NATIONAL INSTITUTES OF HEALTH

Review of INTRAMURAL RESEARCH 1966

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
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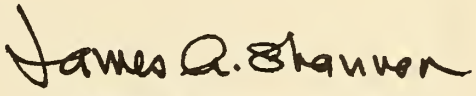
FOREWORD

As the title implies this volume summarizes work done in the NIH laboratories and clinics and in the offices closely connected to these. It does not cover the research supported throughout the United States and in some other nations by grants from the various Institutes. Nor does it include research done on contract for the NIH unless this was a direct extension of an intramural program.

This story covers the year from July 1965 through June 1966. The philosophy behind the Review remains the same as in previous years. These are the words of the staff who followed very general guidelines in writing their reports. They have received only minimal editing. Thus we have tried to preserve for the reader as direct a view as possible into our diverse and extremely active scientific community as its members pursue their investigations under the missions of the several Institutes and Divisions. Together these missions unite as the common purpose of the NIH, which strives to extend fundamental knowledge about the health and diseases of mankind.



JAMES A. SHANNON, M.D.

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NATIONAL CANCER INSTITUTE

GENERAL LABORATORIES AND CLINICS

Introduction

The reorganization of the National Cancer Institute during fiscal year 1966, dividing the Institute activities into 4 program areas, incorporates within the General Laboratories and Clinics (GL&C) program most of the Institute's intramural research, although the former Laboratory of Viral Oncology is now within the Etiology program area, and the Radiation Branch and Medicine Branch are now within the Chemotherapy program area. Currently, the GL&C consists of 4 Laboratories (Biochemistry, Biology, Physiology, and Pathology), 6 Clinical Branches (Surgery, Metabolism, Immunology, Endocrinology, Dermatology, and Pathologic Anatomy), and one section reporting directly to the Office of the Scientific Director (Macromolecular Biology Section), the latter having been within the Laboratry of Viral Oncology prior to reorganization.

Although the major targeted research activities in tumor virology, radiation biology, and chemotherapy are now pursued within the other program areas, there remains substantial representation of these activities within the GL&C, particularly tumor virology and radiation biology. There is comparatively little representation of chemotherapy *per se*. However, the clinical investigations of chemotherapy are a direct operational activity of the Medicine Branch pursued in the Clinical Center, which geographically provides for easy collaborative and cooperative clinical investigations between the clinical branches of the GL&C and the Medicine Branch of the Chemotherapy area.

The organizational identity of the more targeted programs of the Institute in turn identifies the research program of the GL&C as now consisting of the conduct of less targeted basic research but oriented toward developing knowledge leading to the final understanding of the nature of cancer, its causes, its treatment and its prevention. Superimposed upon this are identifiable characteristics of the research program which have evolved over the years from direction exerted by the scientists within the GL&C and the particular program interests generated within the Institute. Some identifying characteristics include a high degree of emphasis and strength in biochemistry, virology, carcinogenesis and immunology. Beyond these areas of emphasis there is, however, a wide range of interests represented within the GL&C which includes significant research activity in electron micrographic anatomy, *in vitro* and *in vivo* tissue culture studies, endocrinology, mathematical biology, genetics, cellular kinetics and anticarcinogenesis. It should be noted there has been little emphasis to date on developmental biology, membrane biology, mathematical and theoretical biology, lipid chemistry and connective tissue chemistry.

The staff of the GL&C now consists of approximately 500 persons, of which approximately 200 are professional level staff.

The following is an abbreviated account of selective research activities within the constituent laboratories and branches of the GL&C during fiscal year 1966. More detailed description can be found in the individual reports of each area.

The Laboratories

The MACROMOLECULAR BIOLOGY SECTION, within the Laboratory of Viral Oncology prior to the NCI reorganization during the past year,

is now a separate research unit not organizationally attached to a laboratory or branch.

Past and present research interests of the Section have involved the interaction of large molecules, including polysaccharides, proteins and nucleic acid polymers. The research course projected has some additional orientation to the process of malignant transformation of cells and tissue recognition phenomenon occurring during the process of differentiation, where the techniques and knowledge of the Section seem to be applicable.

During the past year the active enzyme, B-galactosidase, of *E. coli* has been shown to be a tetramer, with a molecular weight of 540,000 and a sedimentation coefficient of 16s. The inactive unit is found to be a dimer, molecular weight of 220,000 and sedimentation coeffiicient of 10s. Denaturation of the enzyme with 5–6 M urea has yielded a monomer unit with a sedimentation coefficient of 2s.

The base sequence of DNA of normal rat liver and of rat hepatoma has not been found to possess significant differences, on the basis of optical properties identified in "Melting" profiles.

Intact RNA was isolated from the Rauscher mouse leukemia virus and has been characterized as a single-stranded molecule, with a molecular weight of $13x10^6$. The isolation of the molecule should permit studies to determine whether the isolated RNA possesses complete genetic information to effect malignant transformation in the absence of the intact virus particle.

The electron microscope facility has been reactivated, following several months idleness, and will permit certain membrane studies considered to be important in understanding transformation, cell recognition during contact inhibition, morphogenesis, etc.

In the LABORATORY OF BIOCHEMISTRY research is highly oriented toward identifying biochemical changes associated with the cancer process. Most of the work, however, is applicable to areas and problems other than cancer.

Metabolism of tumors, particularly in regard to glycolytic characteristics, received focused attention by several investigators. Recent work utilizing tumors in *in vivo* isolation has shown that tumors under such conditions utilize oxygen and glucose in proportionate amounts, that such tumors do not selectively utilize glucose in the absence of oxygen. An additional observation made on this isolated tumor system is that tumors metablizing high levels of both oxygen and glucose do not grow faster than when lower levels are utilized, but indeed may grow less rapidly. The destructive effects of hyperthermia on tumors in the intact animal seem to be greater than on normal tissue; this disparity is due in part to impairment of the tumor blood flow which secondarily may account for decreased glucose and oyxgen supply.

Production of transplantable heptomas, providing research material for some two dozen collaborative laboratories in the United States and abroad, is now accomplished through a contract with a commercial laboratory (Melpar). Considerable study of these tumors, however, continues at NCI and NIH. It has been found that many tumors, similar to normal liver, are slow growing, have fewer tissue-specific antigens, and have a reduced capacity to incorporate thymidine into DNA. Some of the free polyribosomes will bind to membranes from normal liver but polyribosomes, originally bound to membranes of normal rat liver, do not bind to membranes from hepatomas.

The chemical carcinogens used to produce hepatomas have also been used to induce bladder tumors. The induction of such tumors requires low dietary vitamin B6. Therefore, an "anti-carcinogenic" effect of vitamin B6 may be identified and in this respect is similar to the anti-carcinogenic effect of vitamin A identified in studies performed in the Pathologic Anatomy Branch.

Controls of enzyme functions have been studied in rat mammary tumors, normal livers and hepatomas. Primary mammary tumors from lactating hosts have been found to have a negligible capacity to produce lactose although it is known that mammary tumors possess the anzymes involved in lactose synthesis. This may be an example of the loss of ability of neoplastic cells to respond to influence controlling functions of normal cells.

Clones of hepatoma cell lines, containing either high catalase activity or low catalase activity, have been isolated from liver metastases when mixtures of tumor cell lines have been injected into the spleens of animals.

A positive feedback mechanism has been identified in normal rat liver which involves the enzyme phosphoryl choline-cytidyl transferase, a step in the synthesis of lecithin. It has been found that the enzyme is activated by degraded phospholipid, and inhibited by a polynucleotide.

Studies on the site of synthesis of protein within the cell, and the intracellular distribution of protein after synthesis, have shown that approximately half of the protein in plasma cell tumors is synthesized by the micromosome fraction and mitochondria, and another half synthesized by the free polyribosomes. The protein synthesized by the microsomal fraction and mitochondria tends to remain within these structures, whereas the greater portion of the protein synthesized by the free polyribosomes is distributed as soluble protein within the cytoplasm. The structural ribosomal proteins of plasma cell tumors of BALB/c mice have been found to consist of approximately 35 discrete protein bands electrophoretically. These bands have been found to be identical for 9 different tumors, even though each tumor secretes a different myeloma protein. The ribosomal protein pattern of the tumor is the same as that of the normal liver of these mice. However, the ribosomal protein patterns of mouse liver, rat liver, and rabbit reticulocytes are distinctly different from each other.

Cellular DNA has been found to reside both in the nucleus and in mitochondria. Although a relatively small portion of cellular DNA is found in mitochondria, recent studies have shown that in the resting liver cell there is a tenfold greater synthesis of mitochondrial DNA compared to nuclear NDA, and that mitochondrial DNA has a rapid turnover rate with a half life of seven days. Synthesis of nuclear DNA is accelerated after partial hepatectomy whereas mitochondrial DNA synthesis is unaffected.

Studies of enzymes degrading DNA have shown the existence of a group of DNases which consist of a mixture of at least 2 endonucleases and 1 phosphodiesterase. The latter enzyme hydrolyzes not only DNA but also RNA.

Work carried out in collaboration with the Weissman Institute has revealed that deoxynucleotides, greater than or equal to 7 in chain length, have produced reactivation of the immune response in thymectomized, X-ray irradiated adult mice. Other work on the biochemistry of immune mechanisms has been pursued within the Laboratory of Biochemistry in which polylysine models have been examined for antigenic capability, that is, ability to provoke delayed and immediate skin sensitivity. Results from these studies suggest that development of delayed responses requires a more biochemically complex antigen than that required for the development of immediate hypersensitivity, at least as measured in guinea pigs.

A concerted attempt to develop means for isolating specific cell types is being directed both intramurally and in contract collaboration with outside facilities. A technique currently under exploration is one employing polyurethane foam containing antibody against specific cell types to be passed through the foam. To date, red blood cells have been concentrated within this system by absorption to specific anti-RBC gamma globulin.

The research activities of the LABORATORY OF BIOLOGY, although generally broad in scope, include distinct emphasis on work in immunology, the behavior of cells under *in vitro* environments, genetics, and carcinogenesis. Research in the field of immunology, particularly immunologic aspects of tumorigenesis and tumor recognition, is approached via the plasma cell system and the thymus. A thymic humoral factor has been identified as being responsbile for initiating and maintaining homeostatis of the lymphoid cell population controlling immunologic competence. A new observation is that spleen cells from C57BL mice with thymus intact produce "runt disease" when injected into strain A mice because of the graft versus host reaction; but if the spleen cells are from a thy-

mectomized C57BL they lack the immunologic competence to induce this reaction. Syngeneic thymic grafts in thymectomized C57BL mice restore this competence of the spleen cells.

An inbred strain of mice is being closely observed because these animals develop, by the ages of 12 to 24 months, a reticulum cell neoplasm closely resembling Hodgkin's Disease as seen in man. This disease is fatal within a 1–2 month period after its development. The animals carrying the lesions are found to produce abnormal immunoglobulins.

The plasma cell tumor is used in intensive studies on the differentiation of the plasma cell and its synthesis of specific immunoglobulins. The functional relationships between gene structure and immunoglobulins produced indicates that only one gene is operative in producing the heavy chain of the immunoglobulin molecule and one gene operative in producing the light chain; all other possible operative genes being repressed during that time. Identification of immunoglobulins of mice is done by means of chain specific antisera prepared in inbred strains.

The genetic patterns of tumor development are being studied by biochemical means, the initial work identifying urinary proteins of the mouse by various chemical detection methods. These studies have indicated that some urinary proteins are under genetic control. The effect of specific genes on the occurrence of tumors in mice continues to be studied along classical lines established in the Laboratory of Biology. The development of mammary plaques, a pre-malignant lesion, has been found to be dependent not only on the genotype of the mouse but also on the strain of mammary tumor viruses carried by the animal.

Much of the current research on the behavior of cells in *in vitro* conditions have been directed toward the malignant transformation process and influences within the tissue cultural environment which modify this change. It has been observed that malignant transformation of cells occurs more rapidly in the presence of tumor virus (e.g. polyoma). It is, however, felt that neoplastic transformation may occur in cells free of tumor viruses. Additional work on tissue culture systems is heavily focused on the effects of changes in the culture medium on the behavior of cells in general, their growth responses and metabolic characteristics.

The biologic identities of hepatomas induced by chemical carcinogens (e.g. carbon tetrachloride and fluorenamine compounds) are being studied both by light mircroscopic and electron microscopic techniques. The identification of ultrastructural characteristics of various tumor cells, as well as cells in tissue culture, is only in its early stages of development in this laboratory.

The LABORATORY OF PATHOLOGY and the PATHOLOGIC ANATOMY BRANCH, although organizationally distinct units, maintain an intimate functional relationship. It is, however, recognized that the primary responsibility of the Pathologic Anatomy Branch is to provide diagnostic pathologic service to the several Institutes, as well as to pursue research related to human disease. On the other hand, the Laboratory of Pathology is involved primarily with experimental cancer research.

The research activities of the Pathology units collectively may be grouped into three major categories, namely cancer in man, cancer in animals and viral carcinogenesis. A further breakdown reveals a range of research interests and includes ongoing research in the following: biochemistry, carcinogenesis (chemical, parasitic, hormonal, viral), viral oncology, immunology, comparative oncology and geographic pathology, in addition to usual studies in human pathology.

Recent biochemical work on the mechanisms of interferon action have shown that the synthesis of interferon is dependent upon protein synthesis and is inhibited by such chemicals as puromycin or p-fluorophenylalanine. Interferon in turn blocks synthesis of viral DNA and RNA.

A number of studies are concerned with carcinogenesis in man and animals. Several projects are pursued to determine the carcinogenicity of a large number of chemical agents on one or more organ systems in small laboratory animals and in sub-human primates. The latter animals were used in carcinogenicity studies as long as 17 years ago when 3 MCA was

instilled in the stomach wall of a group of monkeys; no neoplastic lesions have been detected to date in these animals which are still under observation. Currently infant monkeys are receiving, either in the diet or sub-q, several carcinogenic chemicals including methylcholantrene, benzpyrene, aso-dyes, fluorenamine compounds, urethane, aflotoxin, and diethyl nitroso amine. The last named chemical has induced 13 hepatomas and the tumor has been propagated to date via intracerebral transplantation in monkeys. Another monkey has developed chronic myelocytic leukemia in response to MIH, a chemotherapeutic drug currently in use in man. The phenomenon of vitamin anti-carcinogenesis, mentioned earlier, has been identified with vitamin A which has been shown to prevent cervical cancer and stomach cancer in hamsters given dimethylbenzanthracene.

An important relationship between the state of cellular differentiation and susceptibility to carcinogenic influences has been illustrated by the finding that a certain stage of development of mouse salivary gland is necessary before it is susceptible to the tumorigenic influences of polyoma virus. The presence of mesenchyme in turn is required for this differentiation to occur.

A number of studies continue to explore the relationships between several viruses and cancer or cancer-like diseases, and reference is made to the review of this work contained in the Summary Statement of Pathology.

A significant observation in the functional response of lymphoid cells in tissue culture has been made on cells, isolated from a patient with lymphoma and maintained under *in vitro* conditions. These cells have been found to synthesize immunoglobulins. It has not been ascertained whether these are produced in response to a specific antigen, viral or otherwise.

The research interests of the scientific staff of the LABORATORY OF PHYSIOLOGY constitute a somewhat wide spectrum which would include the following subjects: metabolic aspects of cancer, UVL carcinogenesis, thyroid biology and physiology, biochemistry (protein, nucleic acids), radiation biology, mathematical biology and programming, pharmacobiology and immunology.

Recent, but unpublished, work indicates the short ultraviolet wave lengths (2537Å) may be carcinogenic. The usual carcinogenic wave length at the earth's surface is found at 2969Å, the erythema producing wave length, and no VL of 2537Å normally reaches the earth's surface. Whereas the shorter wave lengths may be an additive carcinogenic factor, it should be recalled that earlier work in the Laboratory of Physiology has shown that longer (visible) light is protective against UV carcinogenesis.

Studies of the biologic behavior of the thyroid gland have revealed that the resorption of colloid occurs by a seeming combination of mechanical and enzymatic events in which colloid is taken into the peripheral cells of the follicles via pseudopodic entrapment by the cells; actual resorption of the colloid seems to occur intracellularly, presumably by enzymatic digestion. Recent studies in this unique organ have dealt with the kinetics through which the follicles establish and maintain a concentration of iodide above that of the blood.

Inhibition of RNA sysnthesis in Sarcoma 37 cells by actinomycin has been shown to result in diminished synthesis of proteins, including histones, and sterols. The inhibitory effect is reversed by glucose and other glycolyzable substrates. Another study of intracellular control mechanisms has involved the inhibition of hemoglobulin synthesis by both iron and heme. It is felt that this inhibition occurs via effects on polyribosome assembly.

Mechanisms of recovery from radiation damage are being studied from several directions. In addition to the phenomenon of "spontaneous" recovery it has been found that certain compounds (e.g. colchicine, endotoxin) protect cells against radiation damage when given after or before exposure. Isolated macromolecules in a dry state are found to be protected against radiolysis by metallic ions. Studies of the possible mechanisms by which a cell repairs radiation damage, in which DNA synthesis is controlled or inhibited, have indicated that DNA synthesis is not necessary for

repair to occur, whereas RNA synthesis may be required.

Recovery from radiation affects, and protection against effects, are further explored in studies on radiation inhibition of natural antibody synthesis and recovery thereof, and in studies involving the tolerance or rejection of allogeneic bone marrow grafts and graft versus host reactions. The immunologic behavior of allogeneic bone marrow in animals has been found to be greatly modified by various manipulations of the bone marrow cells prior to innoculation. It may be wondered whether the antigenic identity of the cells is in any way changed by the manipulations.

The Clinical Branches

The clinical and laboratory research program of the DERMATOLOGY BRANCH has continued along lines developed in recent years with the addition of two new laboratory activities, namely research in cell biology and electron microscopy. The major groups of the research interests are: (1) Mycosis fungoides lymphoma, (2) Epidermal growth and differentiation, (3) Biology of lymphoreticular cells, and (4) Melanogenesis.

The clinical research program conducted during the past year within this Branch has almost entirely involved the immunologic reactivity of patients with lymphona, particularly in a comparison of patients with mycosis fungoides with Hodgkin's Disease and other disease conditions having alterations in immune responsiveness. Mycosis fungoides remains as a distinct lymphoma insofar as patients with this disorder retain intact immunologic capabilities.

An incidental finding has been made in patients with mycosis fungoides topically treated with HN_2. Most patients receiving such treatment have developed cutaneous sensitivity to this drug. However, the topical sensitivity either disappears or is markedly diminished following intravenous administration of HN_2.

Reorganization within the past year resulted in the transfer of portions of the ENDOCRINOLOGY BRANCH to the NICHHD with the result that the Endocrinology Branch, NCI, was reduced to approximately half its previous size in both physical facilities and personnel. The research program of the Endocrinology Branch has been reoriented toward studies of androgen and estrogen synthesis and metabolism. Specific laboratory projects, and the recruitment of scientific personnel to implement the Branch program, have been initiated.

The Endocrinology Branch has further extended its interests in the problem of breast cancer. It has initiated explorations into possible means to enhance the development of knowledge that will lead to a more precise definition of the disease and its therapeutic control.

With the recognition that eventual therapeutic control of breast cancer will require identification of a number of biologic determinants operative in the genesis and course of the tumor, the Branch places heavy emphasis on studies of hormonal patterns that may be associated with breast cancer, the isolation of prolactin and its quantitative chemical assay, and biologic assay of prolactin as measured by its effects on the mouse mammary gland. The latter at this time is planned to be initiated by collaborative involvement of other research units within and outside of NCI.

The IMMUNOLOGY BRANCH functions as the focal organizational unit within the GL&C entirely concerned with immunologic aspects of the cancer process. In addition to its own intensive research activities, it has provided laboratory facilities and guidance for the development of immunologic research in other branches and for further training of scientific personnel in immunologic techniques.

Evaluation of human sera for immunoglobulin constituency has been initiated through a contract with a commercial laboratory (Melpar). Through this means it is planned that a large number of sera, representing a wide range of disease states, will be so evaluated. The results will be reviewed for possibilities of identifying immunodiagnostic criteria.

Lymphoid cells from a human lymphoma have been established in continuous culture. In collaboration with scientists in the Pathologic Anatomy Branch, these cells have been found to produce immunoglobulins and constitute

the first possible model to study the production of immunoglobulins by lymphoid cells in *in vitro* conditions, with the goal of producing specific human antibody.

Further research activities of this Branch are identified in the special review entitled "Evaluation of Immunologic Research in GL&C," a separate item in this report.

The METABOLISM BRANCH primarily conducts research relating either secondarily to the cancer process or to matters frequently directly consequent to cancer, e.g., anemia of cancer. Research currently active includes the following: nucleic acid metabolism, amino acid transport, metabolism of albumin and globulin, porphyrin metabolism, erythrophoresis, calcium metabolism and hemaglobulin synthesis. Reference is made to the review given these studies in the summary report of the Branch.

Selected attention is drawn to the findings during the past year from studies on porphyrin metabolism. Delta-aminolevulinic acid synthetase (ALA synthetase) has been partially purified from mitochondria. The kinetics of induction of hepatic ALA synthetase have been studied along with the effects of inhibitors of protein synthesis (puromycin, actinomycin-D, 5-fluorouracil, p-fluorophenylalanine, etc.). Glucose blocks induction of this enzyme as effectively as actinomycin-D. It has been shown that heme, the end product of the pathway which ALA synthetase controls, will prevent induction of ALA synthetase when injected intravenously at certain times before induction is initiated. This is the first direct evidence of heme as a repressor of ALA synthetase in mammalian tissues and supplements the indirect evidence from the tryptophane pyrrolase work reported last year. When heme is injected intravenously, the level of hepatic ALA synthetase oscillates markedly for periods of 4 to 5 days after a single dose. The oscillations can be greatly amplified by administration of inducer 5 hours before sacrifice. This probably is the first demonstrated example of significant oscillations of the level of an enzyme. The only other example is reduced nicotinamide adenine dinucleotide oscillations in *in vitro* yeast extracts of glycolyzing systems. The oscillations of ALA synthetase in mammalian liver explain Watson's observations on variations in bilirubin excretion following heme administration and raise the question of the role of ALA synthetase in biologic clock mechanisms. The effects of diet on preventing the chemical abnormalities of acute porphrin previously demonstrated in the experimental animal have been extended to patients. A decreased caloric intake can initiate attacks of the disease and high carbohydrate diets can end these attacks. A small fraction of women whose attacks of acture porphyria recurred cyclically with their menstrual periods have been maintained free of disease by use of oral anovulatory hormone preparations.

DNA-RNA hybridization techniques have been used to study the taxonomy of microorganisms, mycoplasma and streptococci. Mycoplasms have been found to represent a highly heterogeneous group of organisms by this technique. The reliability of the hybridization technique for the identification of microorganism types is supported by the fact that the results correlate exceedingly well with those from usual serologic typing of these organisms.

A major responsibility of the SURGERY BRANCH is to provide consultative advice and service to the several Institutes in clinical problems requiring judgment in general, urological, otolaryngological, gynecological or thoracic surgery. In addition to this, a clinical and laboratory investigative program, broad in scope, is actively carried out. Much of the research is directly concerned with development and evaluation of surgical procedures applied to control of cancer in man, and control of complications secondary to cancer. Examples include clinical evaluation of combined ionizing radiation and surgery as a cancer therapeutic modality; and evaluation of pre- and post-operative prophylactic antibiotic administration to control infectious complications of cancer surgery. A study of the latter has shown that chloramphenicol, given pre- and post-operatively over a ten-day period, has markedly diminshed infectious complications to the low frequency of 7% compared to 22–30% in patients receiving no antibiotic therapy.

The Surgery Branch has been provided two laser units with which exploration of the ap-

plicability of this type of radiation to research and treatment of disease in man and animals has been initiated. Pursuit of investigations utilizing these instruments will be in accord with initial results obtained and the likelihood of deriving significant information with their continued use.

Greater and greater emphasis is placed on research on immunologic processes involved in transplantation, cellular recognition, and control of the cancer process. Several studies are in progress. A study of the immunologic relationship between pregnant women, or women with a trophoblastic tumor, and their husbands has been carried out using the technique of lymphocyte transformation in mixed leukocyte cultures. Preliminary results show that lymphocytes from normally pregnant women do not transform when grown with their husband's leukocytes in as high a percentage as they do when grown with cells from an unrelated male. Further studies are being carried out to evaluate serial changes in each trimester of pregnancy and the changes that take place during and after chemotherapy of trophoblastic neoplasms.

A chemically-induced gastric adenocarcinoma has been found to be antigenic eliciting an immunologic response which can be adoptively transferred by isologous lymphoid cells, and which may be abrogated by splenectomy or large doses of tumor cells. Attempts to utilize sensitized lymphoid cells to alter the growth of established tumors have resulted in possible slowing of tumor growth; complete regression has not occurred.

Immunologic Research

As earlier pointed out, while the research interests and activities within the GL&C, NCI, extend over a broad range of subject interests, there are several subjects in which there is an intensity of interest and work. These include biochemistry, carcinogenesis, virology and immunology. Because of increasing evidence of the importance of immunologic factors in the development of some neoplasms, and an awareness of possible applicability of immunologic factors to control the course of neoplasms, a summary assessment of immunologic research within the GL&C seems appropriate.

A significant amount of research directly related to or involving cancer immunology is found in the Laboratories of Biology and Biochemistry; and in the Immunology, Surgery, Pathologic Anatomy, Metabolism and Dermatology Branches.

Immunologic research has been fostered and intensively pursued in the Laboratory of Biology for a number of years. Currently the work within this Laboratory is directed towad eliciting antigenic identities of cancer cells; determining the rcle exerted by thymic and lymphoid cells in directing immunologic capabilities of the organism; and determining molecular specificities of immunoglobulins synthesized by the plasma cell. To date it has been shown that some cancers of animals, particularly viral cancers, have cell-related antigenic characteristics which can be utilized to prevent or modify the early development or course of these tumors. Lymphoid cells of thymic origin have been shown to be highly functional in determining and maintaining immunologic competence of the mouse. A number of immunoglobulins, each molecularly specific in composition, have been identified as products of plasma cell tumors. It is found that each tumor continues to synthesize a specific globulin. The specificity of the stimulus accounting for this is under study. In this experimental system, no antibody function of the immunoglobulins has been discerned as yet.

Some studies of molecular identities of immunoglobulins produced by plasma cell tumors, and factors controlling their synthesis, are pursued within the Laboratory of Biochemistry in collaboration with scientists of the Laboratory of Biology. Additional research within the Laboratory of Biochemistry on the chemical basis of immunity has been concerned with molecular determinants of antigenicity. Results to date, from studies utilizing polylysine models, indicate the induction of delayed hypersensitivity requires an antigen with structure more complex than that required to induce immediate hypersensitivtity.

Research of the Immunology Branch is primarily directed toward studies in man. Scien-

tists within this branch have over the years provided leadership in research which has led to better understanding of the biochemical identities and specificities of immuno-proteins in man. Work in animals has yielded fundamental information on the reactivities of the various components of complement and the applicability of complement fixation tests to determine antigenic identities of chemically-induced tumors. The screening analysis of human sera now underway in the Branch is directed toward identification of immunoprotein patterns which might be diagnostic of certain types of cancer in man. The work initiated collaboratively with scientists of the Pathologic Anatomy Branch has provided the first instance of continual immunoglobulin synthesis by lymphoid cells in tissue culture. Inasmuch as these cells are of human origin, the possibility is now raised of *in vitro* synthesis in other culture systems of specific antibody for human use.

Immunofluorescent studies of possible tumor specific antigens in man and animals have been carried out in the Pathologic Anatomy Branch in recent years. Some of these studies have suggested that specific tumor antigens may exist in man, although a definite conclusion cannot be made at this time. Collaborative work along these lines is being continued with other units in GL&C and with units in the other program areas of NCI.

Immunologic research of the Surgery Branch has to date yielded positive information on the role of the small lymphocyte in maintaining immunologic competence in man. Partial depletion of the lymphocyte population by thoracic duct drainage has resulted in significant diminishment of delayed immune reactivity as measured by skin tests and homografts. Immunologic research capabilities are being actively encouraged within this branch.

Studies of the Dermatology Branch have shown that the patients with mycosis fungoides lymphoma remain immunologically competent throughout the disease, in distinction to patients with Hodgkin's Disease. Immunologic challenges, cutaneous and systemic, of patients with mycosis fungoides have been observed to effect partial or complete remissions of the disease in some patients.

Results of clinical and laboratory work to date suggest there is a likelihood of influencing cancer in man by immunologic means. It is intended that early leads will be tested as quickly as possible in man, but at a pace and in a fashion commensurate with standards appropriate to research in man.

MACROMOLECULAR BIOLOGY SECTION

In the recent reorganization of the NCI, the Macromolecular Biology Section was retained in the intramural research area in the Office of the Scientific Director for General Laboratories and Clinics, NCI. The Macromolecular Biology Section was previously under the Laboratory of Viral Oncology, which has been transferred to Field Studies, Etiology. The decision was made to retain the Macromolecular Biology Section in the general intramural research area since the basic research in the Macromolecular Biology Section pertains to cell growth and differentiation in general, as well as to viral oncogenesis.

During the past few years there has been increasing recognition that the new techniques and findings of molecular biology may lead to significant clarification of cellular differentiation on the macromolecular level. Differential macromolecular synthesis and differential utilization of energy through processes controlled by macromolecules are crucial in the control mechanisms of histo-differentiation. In order to understand the control mechanisms of cancerous transformation, greater understanding must be gained of the processes of differentiation, cell division, and contact inhibition.

The combined disciplines of molecular biology (biophysics, genetics, virology, biochemistry, polymer and physical chemistry) were fused in recent years into a unique research facility at NIH in the Section of Macromolecular Biology. The aim was exploration of the role of macromolecules in cellular control mechanisms. Thus the research emphasized the interaction of macromolecules in the cellular control processes which govern differential synthesis of macromolecules, utilization of energy, and growth of cells.

In the field of macromolecular interactions and protein structure pertaining to control processes in energy utilization, Dr. Mora, with associates, was studying the changes in cytochrome c structure associated with interconversion between the oxidized and reduced states. Certain polycations, prepared by substituting polyglucose with basic residues, and certain sugars, reduce cytochrome c in a unique, slow and controllable fashion, so that both the rate and the extent of reduction of the cytochrome c can be varied. This technique permits observations on the changes in the tertiary structure of this respiratory protein *during* its conversion from oxidized to reduced form, heretofore impossible. Greater understanding of protein conformational changes in cytochrome c allows better understanding of the control processes of oxidative phosphorylation in terminal electron transport. Cytochrome c plays a central role in terminal electron transport in the mitochondria and in energy utilization in all types of cells.

Dr. Shifrin shifted his research interest to the study of cellular control processes in bacteria. In collaboration with investigators in the Laboratory of Chemical Biology, NIAMD, he studied the aggregation of the subunits of β-galactosidase isolated from wild-type *E. coli* and from a mutant which lacks β-galactosidase activity. He found that a tetramer of the subunit is required for the enzymatic function, and a point mutation in the *lac* operon yields a protein which is enzymatically inactive primarily because the site required for aggregation of the subunits of the β-galactosidase to an active tetramer is mutated. In another collaborative work with scientists in the Laboratory of Molecular Biology, NIAMD, Dr. Shifrin studied the mechanisms of action of an amino acid analogue, the α-hydrazino analogue of histidine, which inhibits bacterial growth. The study utilized a mutant of *S. typhimurium* defective in histidine transport system. He found that this metabolic inhibitor is functioning not by virtue of its ability to be incorporated into the protein and thus make a defective enzyme, but by acting as a feedback inhibitor. The site of action was found to be on the acetylornithine transaminase in the arginine biosynthetic pathway. Thus, an important linkage between the bacterial cell wall or membrane where materials are transported, and the control of cellular growth is clearly demonstrated in this study.

Other work pertained to macromolecular differences in higher organisms. In one study, Dr. Smith carefully compared the nucleic acids of rat liver and hepatoma from the point of view of base sequence homology. Refined and novel physical measurements were used. Observation of the changes (upon heating) in the optical properties of the DNA at various wavelengths, and comparison of the so-called "melting" profiles revealed no difference in the DNA from the two tissues. In addition, various techniques were employed in hybridizing denatured single-stranded nucleic acids from the two tissues. Currently attempts are being made to compare liver and hepatoma messenger RNA by various hybridization techniques designed to reveal differences in their base sequences.

The study of differences in macromolecular pathways of normal cells and tumor cells can be conveniently accomplished by the comparison of normal cells and tumor virus-transformed cells in tissue culture. Preliminary purification and characterization of the virus and its nucleic acid are essential.

With the help of a small contract at Melpar, Inc. (which expands the limited tissue culture facilities of the MBS), Drs. Luborsky and Wood studied the incorporation of tritium into DNA in mouse cells infected by the polyoma virus. Under specific conditions, in the presence of FUDR and tritiated thymidine, it was found that only viral DNA was synthesized in mouse embryo cells. In the absence of a very critical concentration of the metabolic inhibitor the major amountt of the newly synthesized DNA is induced cellular DNA. These findings were possible after first developing a fractionating technique so that the cellular DNA was fully separated from the viral DNA on methylated albumin column, and after studying the balance of the synthesis of these two DNA species in the cells under various conditions. Conditions were also found to increase the level of incorporation of the tritium

into the virus DNA. The highly radioactive viral DNA is used currently in refined nucleic acid homology studies, following the pioneer observations of Axelrod, Habel and Bolton. The objective is to see what part of the viral genome is incorporated into the transformed tumor cells in different types of polyoma-induced tumors. Another objective is to see if the viral DNA genome, or a fragment, incorporates perferentially into the transformed cell's mitochondrial DNA or into its nuclear DNA. Further objectives pertain to changes in macromolecular synthetic pathways and to cell surface changes during transformation of cells to cancer cells (see below).

A significant achievement in the Macramolecular Biology Section was the first isolation of an intact RNA from an animal leukemia virus. A 13 million molecular weight single-stranded RNA molecule was isolated from the Rauscher mouse leukemia virus. It was shown that this molecule accounts for the full genome of the virus. To our best knowledge this RNA is the highest molecular weight RNA ever observed. Our method of isolating it in good yield opens the way for molecular biological studies of the RNA-containing viruses. One important finding in this respect is that the RNA is single-stranded. Questions which are now under study include: whether and how transformation or virus induction may occur with a pure and intact single-stranded viral RNA; does this RNA possess the full genetic information for transformation, or is other information necessary?

A particularly important change during transformation of a cell from the normal to the malignant state must be on the cell surface regulating the growth of the cell vis-a-vis the other cells. Acid mucopolysaccharides have been reported to be qualitatively different after virus infection and transformation of several cellular systems. Chemical methods are being developed in the Macromolecular Biology Section for the isolation and characterization of these mucopolysaccharides from various normal cells and from virus producing and virus transformed cells. (Dr. Wood)

The possible changes in surface charge during transformation of cells, and the effect of added polyanions and polycations on cell growth and on contact inhibition of normal and malignant cells are also being studied in tissue culture. (Dr. Schutz) Techniques for measuring DNA synthesis, rate of mitosis, cell overgrowth, etc., is being refined to permit reproducible measurement of the changes induced in cell growth by charged polymers.

Lipid components of normal cells and of cells producing certain viruses, especially the so-called membrane viruses were compared (Dr. Johnson). It was found that more unsaturated and short chain lipids were produced in cells which were infected by the Rauscher, Moloney or Rous viruses, as well as in Rous-transformed cells.

Future research will continue to center around the molecular basis of differentiation. With the help of two additional investigators, research on protein biosynthesis in subcellular systems and electron microscopic investigations on cell surface changes will be integrated into this effort.

LABORATORY OF BIOCHEMISTRY

Cytochemistry Section

Dr. D. Burk and Dr. M. Woods have continued their investigations of the effects and modes of action of anti-cancer agents on the metabolism, growth, and death of cancer cells.

Cancer tissues and cells have been exposed *in vitro* and *in vivo* to chemical or physical (hyperthermy, laser, laser, light) agents, and effects on their metabolism, growth, and death have been studied. *In vitro* exposures of 30–90 minutes to temperatures of no more than 43–44° C sufficed to produce extensive or total killing of animal cancer cells, results similar to those obtained clinically by von Ardenne and Kirsch in Dresden, Germany. The thermal death temperatures and exposure times involved varied somewhat with the cancer cell type, and were lowered by certain drugs, such as steroids and serotonin, and raised by insulin and glucose. *In vivo* exposures to water temperatures of 43–44° C (90% total body immersion of host mouse) indicate that the tumor cells are more readily killed under *in vivo* than *in vitro* conditions, due in part to im-

pairment of the tumor blood flow and subsequent decreased glucose supply and anoxia. Concommitant with loss of viability as measured by transplantation is a greatly decreased respiratory capacity and Pasteur Effect (inhibition of fermentation by oxygen gas), followed later by greatly decreased fermentation capacity. The *in vitro* metabolism and *in vivo* growth of two different tissue culture sarcoma cells lines, both derived years before from the same single cell, have remained relatively constant to each other over the last eight years in tissue culture.

Drs. Burk and Woods are the recipients of the 1965 Gerhard Domagk Prize for Cancer Research which was awarded for their paper showing the connection between glucose fermentation and growth rate in the spectrum of Morris hepatomas.

Dr. W. H. Evans in his biochemical studies of normal and leukemic leukocytes has joined with Dr. M. Rechcigl, Jr. in an examination of the peroxidative metabolism of leukocytes. Little is known about the relative roles of catalase and peroxidase although elevated catalase activity of leukocytes has been reported in chronic myelogenous leukemia.

The present studies indicate that myeloperoxidase in the intact cell is active in the presence of sufficient aminotriazole to inhibit catalase. With cells disrupted by homogenization, however, both enzymes are inhibited. New data indicate that the inhibition of myeloperoxidase by amiotriazole depends on the presence of a peroxide-generating system whereas catalase can be inhibited by amiotriazole but under conditions where a much lower peroxide concentration exists. Inhibition by aminotriazole of both myeloperoxidase and catalase also occurs when leukocytes are caused by phagocytize polystyrene particles, another condition leading to increased hydrogen peroxide formation in intact cells. These observations suggest that catalase and myeloperoxidase are located in different compartments within the cell. Purified myeloperoxidase has been shown to oxidize formate-C^{14} to $C^{14}O_2$ in the presence of H_2O_2. Myeloperoxidase may account for the major part of formate-C^{14} oxidation in leukocytes since catalase activity can account for only about 30% of the peroxidative metabolism of leukocytes as measured by formate-C^{14} oxidation.

Nutrition and Carcinogenesis Section

Transplantable hepatomas, originally derived by Dr. H. P. Morris from primary hepatomas induced by a variety of aromatic amines, provide a spectrum of tumors which are being used to examine the relationship between biochemical and biological characteristics and degree of malignancy. A total of 24 cooperating laboratories in the United States and abroad are involved in this continuing study. The growth rate of these transplantable tumors differs many fold and, morphologically, they vary from poorly or undifferentiated to highly differentiated neoplasms.

Many tumors with biochemical characteristics similar to those of normal liver are slow growing, have reduced tissue-specific antigens, are not completely devoid of the slow-moving, h_2 electrophoretic class of proteins, and have a reduced capacity to incorporate thymidine into DNA. A high correlation has been established in cell free systems between growth rates of these hepatomas and the activities of the replicative and terminal DNA nucleotidyl transferases, the faster growing tumors having the higher activities. Degradative enzymes and kinases in such cell free systems were not rate-limiting. The very slow growing hepatomas appear to have more organized endoplasmic reticulum and fewer free ribosomes. Some of the free polyribosomes will bind to membranes from normal liver but polyribosomes, originally bound to membranes of normal rat liver, do not bind to membranes from hepatomas.

It has been amply demonstrated that this spectrum of hepatomas provide a series with graded biochemical alterations, with some biochemical parameters increasing with growth rate, others decreasing, while still others remain unchanged. Biochemical characterization seems to be much more specific than morphological characterization since, despite the large number of tumors with different growth rates but similar morphology that have been devel-

oped, hepatomas with identical biochemical patterns have not been noted.

Glutamic-oxalacetic transaminase (GOT) activity in these transplantable rat hepatomas has been shown by Drs. T. T. Otani and H. P. Morris to consist of two isozymes. Studies of the distribution of these isozymes, their Michaelis constants, and the effect of hormones have been made in the hepatoma and the liver of tumor-bearing animals. The host liver GOT content and isozyme distribution was not affected by adrenalectomy, hydrocortisone treatment, a combination of both nor by the presence of tumor. With the 5123B hepatoma, however, while hydrocortisone treatment or adenalectomy alone showed no effect, there was a shift in the isozyme distribution of the tumor in females, but not in males, when hydrocortisone was administered to an adenalectomized animal.

Dr. Morris has also been studying the role of tryptophan and indole in the induction of bladder tumors in rats ingesting N-2-fluorenylacetamide (2-FAA) and the effects of the level of 2-FAA on the induction of tumors in jaundiced rats. Although all details of these experiments have not been completed, as reported last year, it appears that bladder tumors can be produced in Fischer strain rats without the addition of tryptophan. Jaundiced rats ingesting 0.025% 2-FAA develop as many liver tumors as do nonjaundiced animals of the same genetic background. Lower levels of 2-FAA are now being studied to determine whether the early results were due to an overwhelming exposure to the carcinogen. Animal experiments have been completed and histologic studies are in progress.

A series of "unnatural" β-hydroxyamino acids and their derivatives have been synthesized by Dr. T. T. Otani for use as growth inhibitors and racemic mixtures of disasteromers were tested. Certain of these were shown to possess moderate growth inhibiting activity in microbial and solid tumor tests systems. Since only one out of the four diasteromers is presumed to be active, increased inhibitory activity should result from the use of the pure resolved isomer. Milligram quantities of each of the four isomers of β-hydroxynorleucine and their N-chloroacetyl derivatives have been prepared and remain to be tested.

Tumor-Host Relations Section

The study of the *in vivo* environment of the neoplastic cell by Dr. P. Gullino has been directed toward an examination of the energy requirements of growing tumor tissue. By use of the "tissue isolated" tumor preparation into which has been implanted a chamber for harvesting interstitial fluid, blood flow, glucose, and lactic acid levels, and O_2 and CO_2 exchange levels can be measured continuously in interstitial fluid and in efferent and afferent blood.

In three solid tumors, chosen because of differences in structure and biological behavior, it was found that about 50% of the available blood glucose was removed from the blood and utilized in different degree by each tumor. The limiting factor in oxygen utilization seems to be the supply since, within the limits of the 50% removal ratio, the three tumors used all the O_2 that was received. It was not possible to saturate the ability of the neoplastic tissue to use O_2. Conversely, neoplastic tissues apparently were unable to remove a larger proportion of O_2 from the blood when less is supplied. A decrease of arterial O_2 from 20 to 5 volumes percent resulted in a decrease of O_2 used. Furthermore, during acute *in vivo* lack of O_2, the tumors failed to respond with increased glucose utilization of lactate production. Glucose utilization and lactate production were directly related to O_2 consumption; no "sparing" effect (Pasteur effect) of O_2 on glucose utilization could be demonstrated *in vivo*.

In normoglycemic animals a sharp gradient of glucose concentration was found between plasma and interstitial fluid (from 150 mg/100 ml plasma to 0–8 mg/100 ml interstitial fluid). The tumors utilized all the glucose which passed through the capillary wall. The gradient was lost in hyperglycemia and restored by normoglycemia. Several hours were needed for the two phenomena to occur, suggesting that a glucose transfer system is operating across the capillary wall of tumors. Hyperglycemia (350 mg/100 ml plasma) lasting several days increased glucose utilization to a maximum level

of about 30% above normal. Higher blood glucose levels did not induce greater glucose utilization. Tumors utilizing glucose at the highest possible level did not grow any faster; indeed, growth was poor in many of them. During normoglycemia the 3 tumors transformed about 35% of the glucose utilized into lactate. During hyperglycemia more glucose was utilized and more lactate was eliminated by the 35% proportion was maintained. However, in tumors starved of glucose by severe hypoglycemia (30 mg/100 ml plasma), the amount of lactate eliminated increased sharply in proportion to the amount of glucose utilized. Moreover, in hyperglycemia produced immediately after glucose starvation, the elimination of lactate in tumors was decreased to negligible amounts; some even utilized lactate from the afferent blood. These observations imply the existence, *in vivo*, of several compartments in the neoplastic cell and that only in some of them does glycolysis occur with a special priority.

In an attempt to investigate the relationship between differentiation and the growth of neoplastic cell populations, Dr. P. Gullino and Dr. S. Roskes (NICHHD) have begun to employ primary mammary tumors in rats. Preliminary results have shown that the majority of primary tumors from lactating hosts have a negligible capacity to produce lactose although it is known that mammary tumors possess the enzymes involved in lactose synthesis and an appreciable level of activity can be demonstrated *in vitro*.

It is Dr. E. Shelton's purpose to examine the normal and malignant transformation of cells. The free cells found in the peritoneal fluid of the mouse represent the "wandering cells" of the tissue spaces of the body (i.e., the extra-vascular wandering cells). These cells function by protecting the mouse from adverse effects of foreign materials or debris resulting from metabolism or death of host cells. In performing this function, the cells undergo changes in number and metabolism. However, little is known about the normal development of this cell population which must serve as the basis for a comparison of malignant cell behavior.

A study of the population dynamics of normal free peritoneal cells has shown that the total number of peritoneal cells in the mouse rises from birth to a stable plateau at 4 months of age. The macrophage population reaches a stable plateau of between 2–3 x 10^6 cells at 30 days. The continued increase in total cells is due to the continued increase in total lymphocytes. It is suggested that the increase in number of lymphocytes may be related to maturation of the immune system in the mouse. Major emphasis will be placed upon elucidating the fine structure of the peritoneal cells with reference to the age of the animal. Attention will then be turned to the study of the alterations in fine structure which are attendant upon growth in the diffusion chamber.

As an example of another differentiating cell system, whole thymuses from new-born mice were grown in diffusion chambers implanted in thymectomized or intact, syngeneic or allogeneic hosts of various ages. Whole mount preparations were made of the thymus tissue after it had grown for from 9–86 days. Thymic lymphocytes, granulocytes, macrophages and fibroblasts migrated rapidly from the explant, the latter forming a sheet that covered the entire chamber. Viable lymphocytes were present in chambers removed from the host after 86 days. The striking feature of the cultures was the differentiation of the epithelium into sheets, clusters, and cords of cells reminiscent of glandular epithelium. Whorls of cells resembling Hassall's bodies were occasionally seen. These epithelial cells were distinguished by the presence of large lipid-filled vacuoles and neutral mucopolysaccharide in the cytoplasm, suggesting that the epithelial cells were engaged in secretory activity.

In collaboration with Drs. E. L. Kuff and W. C. Hymer, Dr. Sheton has examined the fine structure of the free and bound ribosomes of mammalian cells, principally the RPC 20 plasma cell tumor of the mouse. Utilizing negative and positive staining, the structure of these organelles has been visualized by electron microscopy in greater detail than hitherto possible. The individual mammalian ribosome appears as a dual structure consisting of a sphere approximately 220 Å in diameter

(probably corresponding to a 50S ribosomal subunit) to which is attached a smaller structure of variable morphology (probably corresponding to a 30S ribosomal subunit). The large 50S subunit is extremely stable but the morphology of the smaller subunit is variable. It has been seen as a flattened sphere, a cleft sphere, two small closely apposed spheres and various other shapes. The small subunit seems to be structurally unstable and instability may be related to the capacity of the smaller subunit to "accept" messenger RNA. Ribosomes are attached to membranes of the endoplasmic reticulum (ER) by the stable, 50S subunit. The unstable 30S subunit stands up and away from the membrane. The base of the 50S subunit appears to form an integral part of the ER membrane.

Polyribosomes are formed by the joining together of individual ribosomes into rosettes or linear arrays. The patterns formed by the rosettes and linear polyribosomes as they settle out of suspension onto the membrane of the microscope grid have provided clues as to the way they are joined together. The evidence is very strong that the units of the polyribosome are joined together at the smaller subunit and that the polyribosome strand is flexible. Positive stains of the polyribosomes provide evidence that the material holding the ribosomes together is a more substantial substance than the single strand of messenger RNA that has been postulated previously.

Dr. M. Rechcigl, Jr. has embarked on studies concerned with the selection and differentiation of cell populations of normal and tumor tissues. Understanding the interrelationships between cells requires systems with minimum heterogeneity of cell types. At present it is not known whether tumor preparations are mixed populations of tumor cells and whether variation in such populations influence results and interpretations by virtue of their varied biochemical characteristics.

It had previously been shown (NCI–388, 1965) that the injection of pieces of tumor into the spleen of a compatible host gives rise to numerous metastases in the liver of the host. This technique has now been successfully used for separation of different cell lines. When pieces of tumor from HC-hepatomas (high catalase line) were injected into the spleen of OM/N rats, all liver metastases found showed high catalase activity (i.e., around 200 units). Pieces from the low line (LC hepatoma) had liver metastases with low catalase values (i.e., around 40 units). Freshly prepared mixtures from HC- and LC-tumor lines injected into spleen produced liver nodules possessing either low or high enzymatic activity. No intermediate values were seen. Upon reinoculation into spleen, the nodules gave rise to liver metastases of either high or low activity, comparable to the activity of the initial inoculated tissue. Similar results were obtained in studies with mixtures of unrelated tumors and grown in different inbred or hybrid strains. Small pieces of a given liver nodule obtained from F_1 hybrid experiments, transplanted subcutaneously into both original parent strains, would develop tumors *only in one* of the strains. Moreover, the developed tumors possessed typical biochemical characteristics of the original tumors grown in these strains. All the evidence obtained so far implicates cloning as the selective process.

On the other hand, it was established by Dr. Kuff that the small tumor nodules observed on the mesentary of mice early after intraperitoneal transplantation of ascites plasma cell tumors (see NCI–366, 1965) do not *exclusively* represent clones derived from individual tumor cells. Transplantation of individual nodules, while feasible, does not presently appear to be a useful cloning technique for these tumors.

Current studies by Dr. E. L. Kuff and collaborators are based upon the hypothesis that important regulatory mechanisms involved in cell reduplication and differentiation may have their basis not only in the molecular structure of the polyribosomal protein-synthetic units but also in the relationship of these units to the membranes of the endoplasmic reticulum. Transplantable plasma-cell tumors, combining cellular proliferation with active synthesis of secretory protein, are particularly suitable systems in which to study the functional activity of intracellular components.

Cytoplasmic extracts of the RPC–20 plasma cell tumor, fractionated by sucrose density gradient centrifugation yield 4 major fractions: (*a*) a "membrane" fraction containing both microsomes with associated ribosomes and mitochondria, (*b*) membrane-free polyribosomes; (*c*) free monomeric ribosomes, and (*d*) soluble fraction. The particulate fractions have been examined by electron microscopy by Dr. Emma Shelton to verify their cytological composition. The kinetics of labelling obtained from *in vivo* incorporation studies with C^{14}-leucine during incorporation times between 1.33 and 11 minutes suggested that protein synthesized on the free polyribosomes was rapidly transferred *in vivo* to the soluble fraction of the cell, while protein synthesized by the microsomes and mitochondria remained localized within these elements. In the RPC-20 tumor, the free polyribosome fraction and the combined microsome-mitochondria fraction accounted for approximately equal proportions of the total cytoplasmic protein synthesis *in vivo*.

These results indicated the existence of two biosynthetic compartments distinguishable in terms of the immediate distribution of their protein products. Accordingly, Drs. M. Wolf and L. Kedes have undertaken to determine whether a corresponding phenomenon could be observed during *in vitro* protein synthesis by the isolated fractions, and whether direct experimental evidence could be obtained that the two compartments are concerned with the synthesis of a qualitatively different spectra of proteins. Tumors were pulse-labelled *in vivo* and the fractions isolated from them were incubated *in vitro* in the presence of soluble fracton, energy, and a complete C^{12}-amino acid mixture. Under these conditions, 30–40% of the radioactive (nascent) protein of the free polyribosomes was transferred to the soluble fraction, the release phenomenon being time and energy dependent. In contrast, only 10% of the labelled protein of the membrane fraction (microsome plus mitochondria) appeared in the soluble fraction, and this transfer appeared to be non-enzymatic since it occurred equally well in the cold and the absence of energy. Control experiments have established that both fractions do, in fact, incorporate amino acid into protein under the *in vitro* conditions employed for the study of nascent protein release. The results are thus entirely in accord with those obtained in the *in vivo* experiments.

The free polyribosome fraction of the plasma cell tumor has been studied in some detail by electron microscopy by Dr. Emma Shelton. The size distribution is generally larger than that reported for the polyribosomes of other types of tissue, with many polyribosomes composed of 15–20 monomeric ribosmal units and with no evidence that the polyribosomes contain any organized membrane structure. Similarly, chemical analysis has failed to reveal either phospholipid or neutral lipid. Since these polyribosomes are active in completion and release of nascent peptide chains, their protein synthetic activity may be independent of lipid components, in contradistinction to results reported with other cell types such as yeast and regenerating liver. Deoxycholate, a detergent in widespread use for isolation of polyribosomes from a variety of tissues, has a marked effect upon the free polyribosomes of the tumor, removing approximately one third of their total protein, without, however, removing the nascent protein. At the same time, the polyribosomes become more sensitive to ribonuclease action and less efficient in carrying out *in vitro* amino acid incorporation

An analysis of the structural proteins of various mammalian ribosomes by Dr. L. Kedes followed procedures established elsewhere but with plasma cell tumor ribosomes as the initial objects of study. The complex acrylamide gel electrophoretic pattern of the ribosomal proteins (up to 35 discrete bands) was identical in the case of 9 different tumors (each secreting a different myeloma protein) growing in BALB/c mice. The tumor ribosomal protein pattern was, in turn, the same as that given by the ribosomal proteins of normal BALB/c liver. However, the ribosomal proteins of mouse liver, rat liver, and rabbit reticulocytes could all be differentiated from one another on the basis of their disc electrophoresis patterns. Comparison of the ribosomal pro-

teins from inbred mouse strains has been initiated.

Efforts are being made by Dr. M. Wolf to test the biological activity of plasma cell tumor RNA upon intact cells in which endogenous messenger RNA synthesis had been blocked by treatment with actinomycin. Although initial experiments seemed to indicate some activity (as judged by restoration of protein synthesis in the treated cells), reproducible results could not be obtained. The major problem appears to be in finding suitable non-toxic substances which, upon complexing with the added RNA, will both protect it from enzymatic degradation and facilitate its entrance into the target cells.

Investigation by Mr. N. E. Roberts of the non-secretory subline of the RPC–20 plasma tumor has continued. It now appears (from immunological analysis of tissue fluids that were fortunately frozen during the time that conversion occurred) that the loss of the capacity to produce specific secretory protein took place quite abruptly, possibly over a single transplant generation. Attempts to reproduce the phenomenon by continued rapid transplantation of the producer line have been unsuccessful. Induction of the non-secretory subline by a viral infection of the cells has not been excluded.

Dr. M. Rechcigl, Jr. has continued his investigation of the physiological role of catalase and related enzymes and the mechanisms of their regulation in normal and neoplastic tissues.

In collaboration with Drs. H. A. Hoffman and W. E. Heston, studies on the biochemical genetics of catalase have confirmed earlier findings that the level of liver catalase activity in the mouse is controlled by a single pair of genes with low dominant to high. *Ce* has been suggested for the gene symbol. Present data indicate that the gene does not act *directly* in the synthesis of the enzyme. The presence of the gene reduces catalase actitivy to one-half the normal amount rather than eliminating it completely and low activity is dominant to high in contrast to genetic control over direct synthesis of enzymes. Furthermore, the effect is apparently limited to the liver, implying that the gene must be providing a regulator of the amount of liver catalase activity. This is in sharp contrast with acatalasemia in the Japanese, where the gene is apparently involved directly in the synthesis of catalase, for homozygous recessive individuals show hardly any catalase in all examined tissues. On the basis of preliminary data obtained by determining the rates and the kinetics of catalase synthesis and destruction *in vivo*, the "regulator gene" action causing the observed differences in the liver catalase activity in the investigated mouse strains is a result of the decreased rate of synthesis of the enzyme rather than the increased rate of its breakdown.

Experiments have been initiated to develop a histochemical technique which would permit a quantitative or semi-quantitative assessment of the relative amount of catalase present in individual cells. The general approach was to immobilize isolated cells in a thin agar sheet, lyse the cells in order to release the contents around the cells, and then specifically identify regions of high catalase by the ability of the enzyme to destroy peroxide, which serves as an oxidizing agent in the iodine reaction with starch. An assay capable of identifying catalase-containing cells as visible plaques has been developed but no quantitative information has yet been obtained.

Preliminary studies using disc electrophoresis indicate that a variety of tissues may contain several isozymes of catalase. The possibility of having broken down the catalase protein into subunits cannot be overlooked though it seems rather unlikely. When the liver homogenate supernatants were heated with 3-amino-1,2,4-triazole, a drug known to destroy catalase, the electrophoretic pattern showed that one of the "catalases" was inactivated while the second catalase was still active.

Nucleic Acids Section

The objectves of Dr. W. Schneider's research are to isolate and identify the deoxyribose-containing compounds present in normal and cancer tissues, to determine the metabolic sequences in which they are involved, and to study their relationship to DNA synthesis.

Previous studies on normal liver and the Novikoff hepatoma showed that the levels of dCDP-choline and dCDP-ethanolamine were 6 to 7 times as great in the tumor as in the liver. Studies on liver after partial hepatectomy now have shown that these compounds also increase in amount as early as 12 hours after the operation. A single determination on a pooled sample of hepatoma 5123, a minimal deviation tumor, showed that these compounds were also increased several-fold in this tissue. The fact that the concentration of these compounds increases in the liver shortly after partial hepatectomy, and is high in the minimal deviation hepatoma 5123 as well as in the highly malignant Novikoff hepatoma, provide further evidence for the importance of these compounds in growth, although an explanation for their increased concentration in growing tissues remains obscure. The enxymatic synthesis of labeled phosphoryl choline, phoscholine, dCDP-ethanolamine, and OCDP-ethanolamine has been completed and the results published.

Previous work demonstrated that the phosphoryl choline-cytidyl transferase of rat liver was activated by degraded phospholipids. This suggested to Drs. W. G. Fiscus and W. Schneider that a positive feedback mechanism was operative in lecithin biosynthesis and led to a study of the transferases. Experiments showed that a change in size of the enzyme occurred in the presence of activating phospholipid and indicated that an association of subunits was involved in the activation process. In addition, the activity of the transferase, in the presence of increasing concentrations of CTP, showed a sigmoidal curve in the absence of activator. In the presence of activator the activity did not show this "lag" phase at low concentrations of CPT. Other experiments have shown that an inhibitor of the transferase is present in rat liver particulate fractions and can be released into solution by boiling the particulate suspension. This inhibitory factor appears to be bucleotide in nature. If labeled CDP-choline is incubated with this fraction and a liver homogenate, the cytosine moiety of the CDP-choline is incorporated into a material which is also inhibitory to the transferase and which may be a polynucleotide.

These results suggest that CDP-choline is involved not only in phospholipid formation but may also be involved in the formation of polynucleotides. Of interest from this standpoint is the observation that dCDP-choline, but not CTP, CDP, or CMP, dilute the incorporation of CDP-choline into the polynucleotide.

Furthermore, the present studies indicate that the transferase belongs to the group of allosteric enzymes since its activity depended upon the presence of phospholipid activator, its conformation changed during activation possibly due to an association of subunits, its substrate concentration curve was sigmoidal in the absence of activator, and it was inhibited by a naturally occurring material which appears to be a polynucleotide.

The work on DNA in isolated liver mitochondria, begun last year by Dr. W. Schneider, was continued and some of the results have been published. A method was developed for isolating DNA from both nuclei and mitochondria in highly purified form. Tests of these DNA samples by buoyant density determinations in CsCl and by melting point determinations confirmed the results reported last year by direct base analyses and show that the guanine-cytosine content of the mitochondrial DNA was 2 to 3 percent lower than that of the nuclear DNA. In addition, these studies also showed that the mitochondrial DNA was of large size with a molecular weight of 8 to 9 million. The mitochondrial DNA also appeared to be more homogeneous than the nuclear DNA, as judged by the sharpness of the bands obtained in the CsCl centrifugations and the temperature range required to melt the nucleic acid samples. Another difference between the nuclear and mitochondrial DNA was in the rate at which labeled thymidine and deoxycytidine were incorporated *in vivo*. Both of the precursors were found to be incorporated to about 10 times as great an extent into liver mitochondrial DNA as into nuclear DNA in adult rats. More recent experiments have shown that although the incorporation into nuclear DNA was increased enormously 24 hours after partial hepatectomy, the incor-

poration into mitochondrial DNA was unaffected. A study of the retention of label in the mitochondrial DNA showed that the specific activity of the mitochondrial DNA fell off rapidly, with a half life of about 7 days.

Preliminary experiments have also been carried out on the livers of rats fed the potent carcinogen, 3′-methyl-N,N-dimethylaminoazobenzene (3′-MeDAB) or the non-carcinogen, 2-methyl-N,N-dimethylaminoazobenzene (2-MeDAB) for 14 and 28 days. The expected increase in liver mitochondria in the rats fed the 2-MeDAB and the expected decrease in liver mitochondria from rats fed the 3′-MeDAB were observed but the rate of incorporation of thymidine into the mitochondrial DNA did not seem to differ in these rats and in controls. The most significant finding of these experiments was a 2-fold increase in DNA in the 3′-MeDAB liver mitochondria. It seems likely that the study of much shorter periods of feeding these dyes will be necessary to determine whether the mitochondrial DNA is affected by either dye.

These studies on mitochondrial DNA provide a new point of departure for experiments in cancer research. Since experiments performed here, as well as elsewhere, have shown that tumors contain fewer mitochondria and have decreased levels of mitochondrial enzymes and that these changes in mitochondria occur in the liver in the earliest stages of chemical carcinogenesis, it seems logical to ask whether the point of attack of the carcinogen is at the mitochondrial DNA. Experiments on this point are in progress.

During the past few months Dr. S. Lerner and Dr. W. Schneider have begun to examine by hybridization techniques the degree of complementarity that may exist between DNA and RNA isolated from rat liver mitochondria. Heretofore, efforts have been directed mainly toward the isolation and clean separation of DNA and RNA. If, however, mitochondrial RNA is found to hybridize with the DNA, this would suggest a transfer of information from the DNA, perhaps analogous to the RNA-mediated expression of information in protein synthesis, and would provide impetus for the investigation of possible autonomy for mitochondrial biosynthesis which endogenous DNA might afford.

Partial purification of TMP kinase free of TMP phosphatase activity was achieved by Dr. R. K. Kielley with successive steps involving pH 4.8 precipitation, $(NH_4)_2SO_4$ fractionation of soluble protein, and treatment with DEAE-Sephadex.

TMP-phosphatase activity was totally absent in the final product. The specific activity of the purified kinase is the highest reported from mammalian tissue, but the purified enzyme is still contaminated with some ATPase. TdR kinase, diphosphokinase and myokinase activity, as determined by spectrophotometric, coupled enzyme assays. Other purification procedures with Sephadex gel filtration and CM-Sephadex have been investigated and shown to be feasible. Removal of the protective substrate (2×10^{-5} m TMP) from the enzyme by passage through Sephadex G–25 led to inactivation, the degree depending on the time required for passage, suggesting a dissociable complex of the enzyme and protector. The kinetics of the time course of inactivation indicated a latent period lasting several minutes during which time an intermediate may be formed. It has been shown that TMP kinase, an enzyme which plays a key role in DNA synthesis, has a sulfhydryl requirement for stability, a requirement not detected in crude extracts of the enzyme. Pre-incubation experiments indicated that glutathione conferred increased stability to enzyme already protected with TMP.

It is noteworthy that stability in the presence of ATP occurred only when GSH was present. A tentative explanation might be that the actual stabilizing nucleotide bound to the enzyme is the reaction product itself, TDP, rather than the substrate, TMP, which appears to be less effective. The GSH effect may serve to increase the binding strength of the enzyme for TDP to such an extent that the "bound TDP" is unavailable for further phosphorylation even though diphosphokinase and ATP are present to convert TDP to TTP.

The nature and extent of the biochemical reactions by which nucleic acid precursors are metabolized in cultured nonmalignant and neo-

plastic cells are being studied by Dr. J. Rotherham, particularly with respect to their possible significance in nucleic acid synthesis and neoplastic transformation.

It has been demonstrated that a strain of C3H mouse embryo cells (Tissue Culture Section, Laboratory of Biology) grown in chemically-defined medium has a nutritional requirement for thymidine. The thymidine can be replaced by doexycytidine or 5-methyldeoxycytidine but not by uridine, deoxyuridine or cytidine. Thus, it appears that the deoxynucleosides which were utilized have a significant metabolic function for this cell strain and that biochemical reactions by which the latter three nucleosides can be converted to the former were not taking place. An explanation for these findings is being sought from tracer experiments in which the cells are exposed to C^{14}-labeled nucleosides in the medium. From isolation of the nucleoside products found in the medium and in the DNA or RNA of the cells and from a determination of the approximate extent of conversion of precursors to product it can be concluded that there is a rapid deamination of deoxycytidine and/or phosphorylation followed by deamination and dephosphorylation—and that the deoxycytidine can also be phosphorylated and utilized for synthesis of DNA-deoxycytidine and/or thymidine.

As part of a continuing study of the function and relationships of the structure and sequence of nucleic acids, the nucleotide distribution in nucleic acids, the development of new fractionation procedures, and the preparation and characterization of nucleotide sequences are under investigation by Drs. G. W. Rushizky and H. A. Sober. Various protein fractionation techniques are utilized for the isolation, from sources such as micro-organisms, of nucleases (both of RNases and DNses) with hydrolytic activities directed toward specific nucleotide linkages. Since DNases, in particular, are quite unstable, new techniques have to be developed. A group of DNases have been characterized and found to consist of a mixture of at least 2 endonucleases and 1 phosphodiesterase. The latter enzyme hydrolyzes not only DNA but also RNA. Separation of the 2 endonucleases by standard protein fractionation techniques has not met with success since loss of the major portion of the enzymatic activity resulted. These DNases are obtained from the mold Aspergillus oryzae, which is also the source of the very useful enzymes, RNases T^1 and T^2. Other previously described RNases have been prepared from *B. subtilis*, *B. cereus*, and various molds, and the possible presence of new DNases has been investigated. A RNase-free phosphatase from *E. coli* C90 has been prepared.

There is always a need for new methods for the separation, and characterization of oligomers of mixed base ratios ranging in size from 10–100. To date, a supply of such large oligomers is one of the limiting factors in designing such procedures. As a source of large oligonucleotides, MS2 phage RNA (chain length of 3,000 nucleotides) was purified in good yield. Octa- and decanucleotides from complete RNase T_1 digests of MS2 RNA were fractionated according to their (Ap+Cp): Up content by mapping or column chromatography at pH 2.7 in 7 *M* urea. Penta-, hexa-, and heptamer mixtures of identical Up (and Gp) composition were separated according to Ap:Cp ratios by the same procedures but at pH 4.3. Such oligomers have been examined for hyperchromy and optical rotatory dispersion. Even larger oligomers have been obtained by digestion of of MS2 RNA after it had been absorbed on DEAE-cellulose, yielding groups of oligomers of chain length 14 to 400 with 51 to 84% recovery of the starting material. Oligomers derived in this manner by Micrococcal nuclease digestion had base ratios very similar to those of the original MS2 RNA. On the other hand, oligonmers with Cp:Gp ratios of 2:1 were obtained when *B. subtilis* RNase was used as the enzyme (the ratio is approximately 1:1 in MS2 RNA). Preliminary results indicate that this procedure also is applicable to the isolation of large oligomers from DNA.

These procedures have been used to prepare pure oligonucleotides of known chain length and nucleotide sequence and has permitted a study of their chemical, physical and biological properties. Oligomers so prepared have been used in studies of nuclease specificity, neigh-

bor-neighbor interactions in single oligonucleotide strands, and as test materials for computational analysis of nucleic acid spectra. Deoxynucleotides, greater than or equal to 7 in chain length, have produced reactivation of the immune response in thymectomized, X-ray irradiated adult mice in exploratory experiments with Dr. M. Feldman (Weizmann Institute of Science). Attempts to duplicate the result in *in vitro* spleen cultures have so far been unsuccessful.

Protein Chemistry Section

The polylysine-polynucleotide system was chosen by Dr. H. A. Sober as a simple model of nucleoprotein interaction. This interaction is known to affect the biological function of the nucleic acid. A study of the nature of the binding forces, stoichiometry, and specificity of this interaction should shed some light on the mechanism by which a basic protein "recognizes" a portion of the nucleic acid structure and affects its properties.

Polylysines react on a molar basis with RNA to form insoluble complexes at low salt concentrations and neutral pH. Soluble complexes are formed, however, at low polylysine-to-RNA ratios. The soluble ccmplexes are susceptible to hydrolytic degradation by specific and nonspecific ribonucleases with release of soluble nucleotides. Digestion continues until the formation of a precipitate with a nucleotide-to-lysine ratio of one. After dissociation of the precipitated complex and separation of its components, the "protected" nucleotide chain is again susceptible to cleavage by the original enzyme. The protected nucleotide sequence is shorter than that of the original RNA. Thus, treatment with polylysine, n = 100, results in a protected RNA sequence of n = 99; the use of oligolysine (n = 13) results in a RNA segment of n = 16. Oligolysines smaller than the heptamer do not form enzyme-resistant complexes.

Present evidence indicates that the nucleotide composition of the protected sequence may be markedly different from that of the parent molecule and that overlapping nucleotide sequences may be obtained by this procedure. While no base specificity has been found with heated or "denatured" RNA, with "native" or unheated RNA, the protected RNA segments contain an *altered* proportion of gyanylic (G) and cytidylic (C) acid residues as much as 76% (G+C).

Measurements by Dr. S. Latt have shown that the binding of oligolysine to MS2 RNA, Poly (I+C), and Poly (A+U) was reversible as long as the polynucleotide-oligolysine complex remained soluble. Preliminary data with the single homopolymers show precipitation even at very low free oligolysine concentrations. Binding of oligolysine to polyribonucleotide decreases markedly with increasing ionic stength, and is virtually nonexistent in 1.5 *M* NaCl, indicating that the major if not sole binding force is electrostatic. Binding strength increases markedly with oligolysine chain length. An equation for reversible binding of a linear oligomer to a much larger linear polymer was derived and experimental binding curves obtained were of the form predicted.

Another approach to nucleoprotein interaction is a new project by Dr. O. W. McBride wherein the role of nucleic acid-bound proteins in the regulation of the transcription and translation of genetic information will be examined. Studies have been initiated recently to determine the most suitable procedures for isolating soluble chromosomal nucleoproteins from rat liver in a physical condition closely resembling the native state of these nuclear components. A variety of mcdifications of two basically different procedures are being examined. These methods involve either the direct isolation and purification of the chromosomal apparatus from aqueous solutions or isolation and extensive purification of whole nuclei prior to isolation of soluble nucleoproteins. The criteria which have been established for a satisfactory procedure are the isolation of a soluble nucleoprotein preparation without degradation or with minimal, controlled, and reproducible degradative methods and the absence of dissociation, association or exchange of chromosomal components with either cytoplasmic or nuclear non-chromosomal molecules. The possible exchange between soluble histones or non-histone proteins as well as be-

tween soluble ribonucleic acids and nucleoproteins will be examined under the specific ionic conditions of the isolation procedure with isotopically-labeled material. Rat liver nucleoproteins will be fractionated by a combination of several independent methods utilizing differences in mass, buoyant density, electrostatic charge, and surface characteristics of these particles. The fractions will be examined for differences in ribonucleic acids, histones, nonhistone proteins, and lipids associated with these DNA segments. Covalent and non-covalent associations between these various components will be investigated to obtain some insign concerning the particular component or components which may be responsible for a specific association with the nucleic acid.

Attempts to purify the Moloney virus, which is known to produce a generalized lymphocytic neoplasm in mice and rats were made by Dr. C. W. Lees and Dr. H. A. Sober with the collaboration of Dr. J. B. Moloney (Laboratory of Viral Biology). Fractionation of viral activity was attempted by ion-exchange chromatography, Sephadex chromatography, and the aqueous phase liquid-liquid partition system of Albertsson. While apparent purification of several-fold was obtained, reproducibility of the assays was inadequate to confirm these results or to permit the desired estimate of the recovery of activity. These exploratory studies were designed to indicate the range within which fractionation procedures must remain in order to obtain good yields of active virus. Because of the large number of fractions which develop during fractionation, a "rapid" and reasonably quantitative assay procedure must be available. The 4-week bioassay used in these experiments does not satisfy these requirements.

Dr. R. W. Hartley, Jr. with the collaboration of Dr. C. W. Lees has been ivolved in the purification and characterization of an extra cellular ribonuclease of *B. subtilis* by physical and chemical techniques and the development of methods of culture and genetic analysis so that the organism and its enzyme product may be applied to a long range study of enzyme structure, function, and synthesis.

A strain of *B. subtilis* has been derived from strain H that will grow in a synthetic medium and produce ribonuclease on a continuous basis. This was achieved by an evolutionary process in a continuous fermenter. Experience with a small (100–200 ml per stage) two stage continuous fermenter has established the feasibility of producing crude enzyme in a continuous and semi-automatic fashion in such an apparatus. Production at a rate of at least 100 to 200 mg of crude enzyme per day is envisaged with a 14 liter per stage set-up currently available.

Amino acid analysis of the purified enzyme has confirmed the absence of sulfur amino acids except for 0.2 moles per mole of methionine which is probably part of a contaminant. All of the weight and nitrogen of the enzyme was accounted for by the amino acid recovery. Dry wevht, nitrogen and U.V. absorption determinations gave a nitrogen content of 17.7% and an E 1%/1 cm at 280 mμof 20.9 The high extinction is in line with the high tryptophan content. Quantitative N-terminal analysis by dinitrophenylation in two experiments showed 0.7 and 0.9 mole of N-terminal alanine per mole of enzyme and no other N-terminal residues. Alanine alone was also found by the DNS ("dansylation" with dimethylnapthalene-sulfonyl chloride) method. The molecular weight of 10,700 was determined by ultracentrifugal analysis.

The enzyme is absorbed in considerable quantity by glass and other surfaces from water and many buffer systems. This is largely prevented by di- and tri-valent cations, especially iron, by amines such as choline and cetyltrimethylammonium bromide, and, fortunately, by 0.1 M ammonium bicarbonate which will quantitatively release enzyme adsorbed from 0.1 M Tris hydrochloride solution but not enzyme adsorbed from water or washed with water.

The dry enzyme is insensitive to heat (100°). In solution (0.1 *M* NH_4HCO_3) however, 90% of the activity is lost within an hour at 90° with significant loss of activity even at 60°. The protein remains in solution, but moves as a discrete band on disc electrophoresis at pH 4.5 behind that of the active enzyme, suggesting that the loss of activity is due to

hydrolysis of amide groups. The inactive enzyme still reacts with a specific *B. subtilis* intracellular ribonuclease inhibitor, preventing inhibition of added active enzymes. Activity remains constant in 1 *N* HCl at 0° up to 24 hours but is largely lost in one hour at room temperature. In 1 *N* NaOH at 0°, about 30% of the activity remains at 1 hour and very little remains at 4 hours.

Several ribonuclease-negative mutants have been isolated as well as a larger number of protease- and amylase-negative mutants.

Dr. E. A. Peterson and Dr. W. H. Evans have undertaken to develop methods for the fractionation of normal and leukemic blood and bone marrow leukocytes as a basis for the study of the fundamental biochemical changes that underlie the maturation of normal leukocytes and the factors that lead to the arrest of maturation in leukemia.

Several stages of maturation in the erythroid and myeloid series of guinea pig bone marrow cells have been partially resolved by sedimentation in a dilute density gradient at unit gravity in a simple apparatus. Besides the clear separation of erythrocytes from the myeloid cells, a useful separation of blasts, neutrophil myelocytes, and mature neutrophils was achieved, although the overlap of peaks left something to be desired. Lymphocytes emerged with the erythroid cells but were well separated from all other types except blasts. Peak enrichments of given cell types ranged from 3- to 15-fold. The time required for such resolution varied from 5 to 20 hours at 4°. However, the myeloid cells were 92 percent viable after 20 hours, as determined by the trypan blue method. Erythrocyte precursors were only 41 percent viable after this period and 67 percent viable after 5 hours. Although migration was almost linear with time, useful resolution did not increase in proportion to the time allowed for sedimentation under the same conditions, indicating an overlapping heterogeneity of the cell classifications with respect to sedimentation rate. The medium employed was a Krebs-Ringer phosphate solution from which calcium and magnesium were omitted and to which 0.01 percent polyacrylate was added. These changes were necessary to prevent reaggregation of the cells after they were dispersed. The presence of polyacrylate did not affect the viability count.

Studies of the behavior of guinea pig bone marrow cells on passage through slowly rotating horizontal columns of open cell polyurethane foam have been continued. Enzyme treatment of the cells in order to prevent nonspecific retardation of a considerable portion of the myeloid cells in columns of foam having 100 pores per inch was mentioned in last year's report. A similar effect has now been achieved by dispersing the cells in a medium containing polyacrylate and treating the foam with the same substance. Whether it is bound to the cell or merely cleans it (of, e.g., nucleoprotein) is now known at present, but polyacrylate *is* bound to the foam. Similarly, these foams have been shown to bind polyethyleneimine and tetraethylene-pentamine. Moreover, foams treated with these polyamines strongly adsorb guinea pig bone marrow cells, including the red cells which pass unadsorbed through untreated foams under the same conditions. Thus, the foams can be converted readily into ion exchanges. Presumably, the polyacrylic acid chain is bound when one or more of its many carboxyl groups engages in an acid-exchange reaction with the polyester moiety of the particular polyurethane foams used in this work. The polyamine chains are similarly bound when one or more of the amino groups cleaves the polyester to form an amide bond. Theoretically, many substances can be attached to the foam in this way, providing the possibility of readily tailoring the nature of the surface for a wide variety of purposes. Among others successfully bound were proteins such as histones, protamine, gamma-globulin, and albumin.

The ability to bind gamma-globulin to the foam columns suggested the feasibility of utilizing immunological affinity for the separation of cells, provided the bound gamma-globulin retained its activity. In collaboration with Dr. Michael Mage (NIDR) a study of such a possibility was undertaken, using the circulating red cells of the guinea pig as a model antigen. These experiments were carried out at room temperature (25°) in order to pro-

mote a reasonably rapid immunological reaction. It was found that the circulating red cells were bound to untreated foam, presumably through the sialic acid on their surfaces, but they were readily dislodged by streams of liquid, by very low-power sonication, or by squeezing the foam. Treatment of the foam with *normal* rabbit gamma-globulin prevented this binding of red cells, apparently by covering the reactive sites. In contrast, foams treated with *specific* rabbit gamma-globulin (anti-guinea pig RBC) at the same protein concentration adsorbed the red cells entering the column and became bright red. Again, the bound red cells could be completely removed from the foam by the low shear applied by the moving liquid when the foam was squeezed. It is not known, as yet, whether the release of the cells involved dissociation of the antigen-antibody complex or cleavage of some other linkage, but the cleaned foam could be used repeately to bind red cells. Similarly, the foam protected with normal gamma-globulin retained its protection through repeated uses. The specificity of this interaction has not as yet been completely established, but sheep cells were not bound by the anti-guinea pig RBC foam. On the other hand, an anomalous low-intensity interaction between rabbit RBC's and normal rabbit gamma-globulin and a strong interaction between rabbit RBC's and normal bovine gamma-globulin have been observed.

The discovery that the polyester-based polyurethane open-cell foams can readily be given a wide range of ion-exchange and immunological properties opens the way for the development of procedures for cell separation based on such principles. Studies of the nature of cell surfaces should also be aided, and applications in basic immunological investigations are foreseen, particularly in view of the potential ability of this type of system to detect and measure monovalent as well as multivalent antibodies.

Studies on the basis of heterogeneity of antibody formed to single, well-defined antigens have been performed by Dr. S. Schlossman (Beth Israel Hospital, Boston, Mass.) and Dr. H. A. Sober. A homologous series of compounds was used because the chemical definition both in position of the hapten and in peptide chain length provide obvious advantages over less well-defined materials in studies dealing with the chemical basis of the immune response.

Immunogenicity was observed in Hartley and strain 2 guinea pigs with α, *N*-DNP-hepta-, octa, and nona-L-lysine, whereas smaller α, *N*-DNP-L-lysines were not immunogenic. The L configuration and the presence of a hapten were also required for immunogenicity in this system. The same antigen, e.g., α*N*-DNP-octa-L-lysine, induced the formation of both delayed and immediate sensitivity. Using chemically defined α-DNP$(Lys)_n$ BuAm peptides, related peptides, and hapten-substituted proteins, *only* the immediate skin response (Arthus) could be elicited with hapten-substituted tetra-, penta-, or haxmers, whereas *both* immediate and delayed skin responses could be provoked by the octamer or nonamer. The hapten is an integral part of the determinant for both immediate and delayed skin reactivity, since poly-L-lysine was unable to elicit either reaction in sensitized animals. Immediate-type cross reactions occurred whenever the sensitizing and test antigen shared a common haptenic determinant. In contrast to this, in this system, delayed-type cross reactions occurred only when the test antigen and the sensitizing antigen contained *both* a large oligo-L-lysine carrier as well as the same haptenic determinant.

These observations imply that the mediation of the delayed response requires a larger determinant than is necessary to elicit the immediate response. High affinity antibody as the mediator of the delayed response is not considered a satisfactory explanation of the observed phenomena. However, the fact that the ability to elicit the delayed response parallels the immunogenic capacity of these pepties suggests that the delayed response may require the continued biosynthesis of antibody and may be analogous to a local *in vivo* secondary response.

The properties of poly-α-amino acids, model substances closely analogous to proteins, are being studied by Dr. H. A. Sober and M. C. Otey with the collaboration of Drs. A. Berger,

A. Yaron and E. Katchalski (Weizmann Institute of Science, Rehovoth, Israel). Oligolysyl peptides, in particular, are useful model compounds for the investigation of the interaction and functions of the basic proteins. These histone analogs, now available in chain lengths ranging from 2 to 25 residues long have been used in a number of studies described above, namely: interaction with nucleic acids and polynucleotides to form nuclease-resistant complexes, where the length of RNA segment is directly related to the length of oligolysine used; after mono- and dihapten addition, provide a chemically-defined series ranging from no- to full antigenicity which has been used to define the antigenic determinants of delayed and immediate skin sensitivity; optical rotatory dispersion studies with Dr. G. Fasman (Brandeis University), examining the effect of chain length on protein conformation; and the variation in chemical properties, such as the apparent pK of the ε-amino group, the color yield with ninhydrin, and adsorption to polyanionic substances as the oligopeptide is extended.

It has long been apparent that metals present in blood and tissues function in association with proteins, and whenever it has been possible to examine this association, areas of major biochemical importance have been uncovered. By collaboration between the Laboratory of Biochemistry, NCI, and the Biophysics Laboratory (Peter Bent Brigham Hospital, Harvard Medical School, Boston, Mass.), a combination of spectrographic analyses and protein fractionation has been developed which makes it possible to isolate and study the association of metals and proteins in much greater detail.

Several new zinc proteins have been recognized in serum by Dr. S. R. Himmelhoch using these procedures. In view of the abnormality of serum zinc concentration reported in Laennec's cirrhosis and in leukemia, the existence of these three zinc proteins is of special interest. In Laennec's cirrhosis at least, preliminary studies indicate that serum zinc alteration in this disease is a reflection of changes in the relative amounts of the firmly bound zinc moieties. Studies are in progress to complete the isolation of these proteins and discern their functional role.

Earlier studies, using limited methods of protein fractionation had shown that a protein could be prepared from human or horse renal cortex which contained about 30 residues percent cysteine, and 5% cadmium and 2% zinc by weight. By application of the techniques developed from the above-mentioned collaboration, Drs. S. R. Himmelhoch and N. H. R. Kagi and B. L. Valley (Harvard Medical School) have been able to show that this protein, metallothionein, is but one member of a family of at least four similar proteins of related amino acid composition, that these proteins occur in the supernatant fraction of renal cortical cells, and that they constitute 3% of the total protein in this source. Methods for preparation of 1 gram quantities of each of these components from both equine and human sources with quantitative recoveries have been developed. Two additional cadmium binding moieties, one of larger and the other of smaller molecular weight than the "metallothioneins" have been identified. Together these proteins account for 98% or more of renal cadmium content. Other metals of great interest in humanpathology, including lead and mercury, have been found in association with some of these moieties. The quantitative amino acid composition of these proteins has been determined and demonstrates distinct differences in detail, but a common high cysteine content.

The metallothioneins, a family of peculiar cadmium- and zinc-containing proteins, represent an important portion of horse and human renal cortical cellular protein. Their unique structure, with every third amino acid possessing a reactive sulfhydryl group, suggests a role in renal transport or detoxification processes. Their unusual metal content and small molecular weight provide a unique opportunity to study metal protein interaction in a definitive manner. Its role in human disease is under study.

Fractionation of human serum on DEAE-cellulose had originally suggested that magnesium might be associated with proteins of gamma-globulin mobility. However, further

studies by Dr. N. A. Cummings point to a loose binding between the metal and proteins, i.e., the existence of a magnesium-protein complex rather than a metalloprotein *per se*. By combining analytic and preparative ultracentrifugation, in collaboration with Dr. E. L. Kuff about 70% of the serum magnesium is found to be free, with the remaining 30% in a weak magnesium-albumin association. The information obtained indicates that magnesium in serum exists in a loosely-bound protein form, capable of being transported and easily released by mild changes in salt concentration or pH.

Cryoglobulins isolated by cold precipitation from sera have been further purified by gel filtration by Dr. N. A. Cummings and have been examined by physico-chemical methods in order to study the phenomenon of cryo-precipitation. The solubility characteristics of 7S-cryoglobulins are similar to those of normal IgG globulins except for temperature effects. Cryoglobulin solubility is a linear function of temperature. Although hydrogen-ion titrations failed to reveal significant differences in surface charges at different temperatures, measurements of viscosity, sedimentation, and sedimentation equilibrium indicated a probable unfolding or anisotropic swelling of the molecule with rising temperature. Further studies to elucidate conformational changes will be undertaken, including optical rotatory dispersion.

LABORATORY OF BIOLOGY

During the year the Laboratory of Biology has continued to make significant advances in the basic areas of biologic research as related to cancer. The program has been expanded particularly in the areas of protein chemistry, electron microscopy, experimental pathology, and cytogenetics.

Progress is being made in bringing in new personnel to strengthen certain promising areas of research. We have also lost a few members. Dr. Morris K. Barrett retired in July and Dr. Margaret K. Barrett resigned in August. Dr. Charles Nicoll resigned in January to return to the University of California in Berkeley, where he accepted a position in the Department of Physiology.

Dr. William T. Hall, an electron microscopist, joined the Laboratory as a biologist in September and now has his electron microscope and laboratory set up and has an active research program underway. Dr. Ettore Appella, a protein chemist, got his A.C.S. Fellowship renewed and joined the staff in the Carcinogenesis Section in September. He has been very actively engaged on the amino acid sequences of the myeoloma immunoglobulins that Dr. Potter has been studying. Request for his appointment next September as a Visiting Associate has been submitted. Dr. Ram Parshad, a cytogeneticist, joined the Tissue Culture Section in November as an International Fellow. He is making significant contribution in studying the chromosomes of their cells in culture. Three new Research Associates joined the Laboratory in July, Dr. Hewes Agnew who is working with Dr. Law; Dr. Frederic Mushinski who is working with Dr. Potter; and Dr. Gerald Mackler who is working with Dr. Evans.

Dr. John T. Mitchell, a developmental biologist, is expected to join the staff of the Tissue Culture Section in May as a Staff Fellow, and appointment of Dr. Raymond Gantt, a biochemist, also as a Staff Fellow in Tissue Culture has been approved for September. Dr. M. Ben-David from Hebrew University has been appointed to the staff of the Laboratory as a Visiting Scientist to report July 1st. He is an endocrinologist who is replacing Dr. Nicoll. His principal interest is prolactin and we hope to investigate the role of this hormone in mammary tumorigenesis. Two new Reserach Associates, Dr. Ruffner and Dr. Burstein, are joining the Laboratory July 1, replacing Dr. Granner and Dr. Lyon. This will make a total of 72 on the Staff of the Laboratory.

No attempt is made to mention all findings in this summary, but examples are chosen to illustrate the scope of the program of the Laboratory under the following headings.

The Thymus in Immunology

Dr. Law is continuing the characterization of the thymic humoral factor that is responsible for initiating and maintaining lymphoid

cell homeostasis and immunologic competence. This has been demonstrated in his studies on effect of neonatal thymectomy. One new observation is that spleen cells from C57BL mice produce "runt disease" when injected into strain A mice because of the graft versus host reaction, but if the spleen cells are from a thymectomized C57BL they lack the immunologic competence to induce the graft versus host reaction. Syngenic thymic grafts in thymectomized C57BL mice restore this competence of the spleen cells, but dissociated thymic grafts do not. The relation of this area of research to viral carcinogenesis is summarized in the next section.

Tumor Viruses

The observation that neonatal thymectomy can increase oncogenesis by certain viruses has been extended by Law, Ting, Agnew, and collaborators. Strain A mice that have never been shown to develop tumors of the salivary glands when infected with polyoma virus do show such neoplasms as well as those of bone, mammary, hair follicle, and subcutaneous tissue when the infection with virus is preceded by neonatal thymectomy. Although the Moloney murine sarcomogenic virus induces tumors in mice it does not in newborn intact rats, but when the rats were neonatally thymectomized and then inoculated with the virus 30 to 50 percent of them did develop tumors at the site of inoculation. Neonatal thymectomy did not increase but actually reduced the occurrence of mammary tumors in C3H female mice with the mammary tumor virus but made no difference in the occurrence of MCA induced sarcomas in C3H or C57BL mice. Furthermore, neonatal thymectomy has made no difference in the occurrence of MCA induced lung tumors in suceptible $(C3HfxA)F_1$ hybrid mide or in resistant $(C3HxB)F_1$ hybrids, as shown by their collaborative study with Heston. These observations would indicate that the thymus is responsible for an immunologic mechanism of the homograft type that causes target cells to resist oncogenesis by certain viruses such as polyoma and MSV, but with certain exceptions as the MTV. It is suggested that no virus is involved in MCA induced sarcomas and lung tumors and thus they are not influenced by thymectomy.

Andervont has continued his observations of the activity of the mammary tumor virus MTV from strain RIII mice as compared with that of MTV from C3H mice when transmitted normally through the milk. The RIII MTV is a relatively weak virus that occasionally disappears or becomes inactive in RIII mice. When transmitted to agent free C3H mice the RIII MTV may also disappear, and when retained does not increase in activity. The highly active C3H virus has never been observed to disappear in C3H mice, but when introduced into RIII mice it can either remain active or disappear from them.

Ultrastructure Cytology

Dr. William Hall has his modern electron microscope laboratory set up and his research program underway. In studying the new strain DD with its premalignant, ormone responsive, plaques he has found that the malignant mammary tumors contain an abundance of both A and B mammary tumor virus particles, and the plaques also contain both A and B particles although not nearly so abundantly. In studying some of Dr. Evans' C3H mouse embryo cells in chemically defined media, he has observed many virus or virus-like particles which are as yet unclassified. Hepatomas in $(C3HxY)$ F_1 mice have not exhibited virus particles. They have, however, exhibited significant anomalies of the microbodies of the cells which represent the uricase component. Cells of spontaneous and induced pulmonary tumors in our mice have failed to show any infectious viruses with the E.M.

Plasma Cell Tumors

The plasma cell tumor is used extensively in the Laboratory by Potter and his group in studies on cell differentiation. Doctors Potter, Appella, and Mushinski have made considerable progress in characterizing the gene-gene product relationships for the heavy chain locus in the mouse. In the mouse there have now been defined 7 immunoglobulin structural genes including 5 heavy chain genes: M, A,

F, G, and H, and 2 light chain genes. A single plasma cell tumor utilizes only one heavy chain gene and one light chain gene, the others being permanently "turned off". Structural variations resembling those in the light chains have been found in heavy chains. Iso-antisera have been produced that are specific for individual myeloma proteins and these provide valuable tools in the search for antibody molecules that might resemble myeoloma proteins. Close linkage with no recombinants has been demonstrated between two heavy chain gene types. In the study of amino acid sequences the amino and carboxyl terminal sequences of five kappa light chains have been obtained. Structural analysis of the Fc fragments derived from mouse gamma and eta chains has shown the presence of twelve normal tryptic peptides indicating these genes are probably duplication products. This work has relevance to the understanding of the mechanism of the differentiation process and the mechanism of antibody formation.

Mushinski is delving further into the protein synthesis in these tumors using radioactive tracer techniques. He is investigating transfer RNA as the mediator of controlled amino acid ambiquities in these plasma cell tumors.

McIntire has been studying the plasma cell tumors and related reticulum cell tumors with more comparison to the multiple myeloma of man. In studying the macroglobulins of one tumor he observed the incorporation of a completely different type of light chain into the immunoglobulin of the mouse, while formerly only one light chain had been shown. This is in line with human beings where two types of light chains have also been recognized. He has been studying renal disease in relation to the Bence Jones proteins produced by these tumors. Of 123 tested for Bence Jones proteins, 56 or 45 percent were positive, which is higher than the incidence cited for human beings. The kidneys associated with these tumors show cast formations in the convoluted tubules. Some reticulum cell neoplasms of the mouse other than plasma cell tumors produce these paraproteins and some of these are under study.

McIntire has studied plasma cell carcinogenesis in germ free mice with a minimum of antigenic stimulation, an undeveloped lymphatic system, few plasma cells, and low level of immunoglobulins, and has observed in these mice a delay in plasma cell tumor development. Most of the tumors that arise are of other reticulum cell types. In this regard, thymectomy apparently has no influence on plasma cell carcinogenesis.

Biochemical Genetics

Hoffman has been studying the urinary proteins of the mouse by immunochemical methods, agar gel double diffusion, and immunoelectrophoresis. Thirty-five inbred strains and substrains have been classified according to two different urinary protein types, Up–1^a and Up–1^b. In crosses between two prototype strains for type 1a (strain SWR) and type 1b (strain C57BL/6) the F_1 shows a combination of the two types and the F_2 segregates in a typical 1:2:1 ratio of the two parental types and the F_1 type. This indicates that these two urinary proteins are under the genetic control of a pair of co-dominant alleles at a single locus. Segregation ratios in first and second backcross generations have confirmed this type of inheritance. By using several spearation techniques the 1a and the 1b protein complexes have been isolated and purified.

Hoffman has built up a linkage-testing stock of mice with 25 marker genes on 15 of the 20 chromosomes. With this stock he hopes to locate on specific chromosomes these genes for biochemical traits including low liver catalase, Ce, immunoglobulin-1, Ig–1, urinary protein, Up–1, and also a sparse coat texture gene, Ca^{De}.

Hoffman has also been identifying serum proteins of the rat. He has found that some of the rat serum proteins cross react with those of the mouse, whereas, others do not.

All of this is directed towards building up a background of knowledge of biochemical genetics in the mouse that can later be related to the genetics of cancer in the mouse at the biochemical level.

Genetics of Tumors

Heston and collaborators have continued this study of effect of specific genes of the mouse on occurrence of tumors. The allelic genes lethal yellow, A^y, and viable yellow, A^{vy}, have received greatest emphasis. Both increase occurrence of a number of tumors including mammary tumors, hepatomas, and lung tumors.

It has been shown that the lethal yellow gene increases occurrence of mammary tumors independently of the mammary tumor virus, and studies of reciprocal transplantation of ovaries and of hypophyses show that its effect is not manifested through control of hormonal stimulation from either of these organs. It, therefore, appears that the gene is controlling the response of the mammary gland cell.

In a study of hepatomas in reciprocal hybrids between strain C3Hf and strain YBR with the A^y gene it was observed that the A^y gene, sex, and the strain of mother all could influence the occurrence of hepatomas, causing the incidence to vary from zero in one group to 100 percent in one with intermediate incidences, depending upon the combination of these factors in the intermediate groups. However, this was all closely correlated with the effect of these factors on normal growth.

Progression of mammary tumors in mice is being studied through premalignant, hormone responsive, mammary plaques that occur in the unusual strain DD. The most significant observation on these plaques during the year is that whether or not they occur is dependent not only on the genotype of the mouse, but also on the strain of mammary tumor virus she carries.

Hepatic Carcinogenesis

Dr. Reuber has described the histology of 20 hepatomas of the rat, including those of Dr. Morris that have been used extensively in studies of enzyme activity. There were classified as (1) highly differentiated heptocellular carcinoma;)2) well-differentiated hepatocellular carcinoma; (3) poorly differentiated or undifferentiated carcinoma; and (4) cholangiohepatomas. He has classified an unusual neoplasm of the liver that has been observed in (C3HxY)F_1 mice as of bile duct origin.

In histogenic studies of the livers of hamsters given N–2–fluorenylacetamide and N–2–fluorenyldiacetamide he has observed that cholangiofibrosis and cirrhosis preceded the development of well differentiated cholangiocarcinomas in both males and females. The lesions could be followed from (1) cholangiofibrosis to (2) cholangiofibrosis with atypical cells to (3) small cholangiocarcinomas to (4) large cholangiocarcinomas with metastases to portoheptic lymph nodes.

Dr. Reuber has described the development of carcinomas of the liver in rats following the administration of carbon tetrachloride. Although carbon tetrachloride has long been known to produce hepatomas in the mouse, it had not previously been observed to produce them in the rat. The older rats were more susceptible to the induction of these tumors than were the younger rats, in contrast with hepatoma induction with fluorenamine compounds where the younger rats are more susceptible. Older male rats also developed a higher incidence and more severe cirrhosis of the liver with administration of carbon tetrachloride than did the younger males. In the females the 12 and 24 week old animals had more severe cirrhosis than the 4 and 52 week old groups.

The Malignant Transformation in Vitro

Much of the effort of the Tissue Culture Section is directed toward the study of the malignant transformation in cells of various kinds *in vitro*, when and under what conditions it occurs, and what are the changes in the cell that can be associated with the transformation.

One of the most interesting observations in this section is that of Evans and Andresen, later confirmed by Sanford, that the spontaneous neoplastic conversion of C3Hf embryo cells *in vitro* is delayed with foetal calf serum as compared with horse serum. Chromosomes in neoplastic cells show structural rearrangements, but Sanford and Parshad have observed that the foetal calf serum appears to

have a stabilizing influence on the chromosomes.

A number of changes in metabolic characteristics have been observed in the cells *in vitro*. Whether these are causally related to the malignant transformation has not been ascertained. In some cases the changes may be merely owing to the cells growing *in vitro*.

In one neoplastic strain Evans and coworkers have observed a change in metabolism of nucleic acid precursors. Some enzymes appear to be lacking or inactive. In another strain a change in lactate dehydrogenase behavior was noted that suggested a change in genetic control rather than selection for cell type.

In a line of cells on chemically defined media studied by Westfall, lactate and malate dehydrogenase activities persisted at a high level but arginase and alkaline phosphatase showed changes in activity. In a line of liver cells arginase activity remained high, whereas there was a drop in production of urea, suggesting loss of critical liver function. The C3H mouse liver cells had twice the arginase activity as human skin cells in culture but both had high storage of glycogen.

Sanford has observed in one clone of cells a great increase in glycolytic activity and at the same time an increase in tumor producing capacity. Transformed lines showed very striking histochemical variation in comparison with non-neoplastic lines. It is hoped to overcome this variation by cloning of the neoplastic lines. In a study of a new strain of teratoma cells in culture that were diluted and inoculated into mice or culture it was found that even at the highest dilution the cells retained their differentiating capacity but the extent of differentiation varied inversely with the rate of tumor growth *in vitro*. With Dr. Rapp, Sanford has been studying Forssman antigen in hamster cells *in vitro*. Forssman antigen persisted in the cells for 12 months *in vitro* and since has been disappearing, although as yet the cells apparently have not become malignant.

Sanford has demonstrated neoplastic transformation in hamster cells *in vitro* following treatment with polyoma virus. Conversion was not rapid and occurred at least one or two transplant generations following virus treatment. It appeared that transplantation antigens may be induced by the virus in cells already neoplastic. Morphologic alterations were noted but were not specific to virus infection. In looking for evidence of virus in her cell lines of both high- and low-tumor producing capacity Sanford has found that some lines are positive for leukemia antigen. Dalton has found C particles in them but assays for biologic activity of the particles have always been negative. However, the neoplastic transformation may occur in cells free of the leukemia virus.

Methodology in Tissue Culture

Possibly the greatest advance in this area in recent years has been the development of the mass stirred fluid culture system with continuous nutrient fluid renewal by Andresen, Bryant, and Evans. This system has been perfected and now has been used for stain L cells in chemically defined media. Conceivably the cells could be maintained in this system indefinitely and examination of various aspects of growth could routinely be made through the malignant transformation. The system should also be of great value in the production of tissues for virus production. Commercial organizations will be very much interested in this system.

Although the agitated fluid suspension culture system developed in the Tissue Culture Section has been used for some time, Bryant and Evans continue to perfect it particularly in respect to growing cells in chemically defined media, and also to adapt and establish new lines of cells of various types including some mouse leukemia and mast cells, and a number of human cell lines.

Five new lines of C3H mouse ambryonic fibroblasts have been grown on the chemically defined media NCTC 135, demonstrating that this media is adequate for continuous growth of freshly explanted cultures. This, together with the fact that these cells can be maintained for a long period of time, provides the system suitable for studying these cells through the malignant transformation and enhances

the chances of causally relating observed changes to the transformation.

A number of commercial establishments are now supplying media made according to the NCTC 135 formula and these are being tested for maintenance of cells in the Tissue Culture Section. Thus far none of these commercial media are wholly satisfactory, with the possible exception of one powdered preparation of NCTC 135.

Biochemistry

Dr. Anderson has been summarizing her studies on glutamine antagonists and with Dr. Lyon has been continuing studies of mechanisms of feedback control over "salvage" pathways for pyrimidine nucleotide biosynthesis. Dr. Anderson and her program are transferring to the Laboratory of Biochemistry that is expected to supply a better environment for her research.

LABORATORY OF PATHOLOGY

Introduction

The work in Pathology is separated into two general areas, the Department of Pathologic Anatomy and the Laboratory of Pathology. The staff of Pathologic Anatomy performs the autopsies and examines the biopsies and surgical pathology and exfoliative cytology specimens for the Clinical Center. The annual reports on the number of these are attached.

Another important operation maintained in the Laboratory of Pathology is the Pathological Technology Section. A report (NCI 517) prepared by the head of this section, Mr. Joseph Albrecht, describes the work performed in this section.

The primary aim of the staff of the Laboratory of Pathology is to carry out experimental cancer research. For this a variety of techniques and experimental animals are used. Each senior staff member carries out his own experiments, but two or more members within the Laboratory may collaborate and individual members collaborate with many others in the National Cancer Institute, and in the National Institutes of Health.

There is free exchange of information and assistance between and among all the pathologists. While the Annual Report reflects in general the work of the two groups in pathology, it also shows that there is considerable interchange. Most of the members of the Department of Pathologic Anatomy have under study one or more problems involving cancer or some other disease in man which they carry out either independently or in collaboration with the clinicians. The autopsies and the surgical pathology specimens and biopsies are examined with the greatest of care. They are initially studied by residents and their diagnoses are all reviewed by senior staff members.

A notable achievement by the head of the Surgical Pathology and Postmortem Service of the Department of Pathologic Anatomy, Dr. Louis B. Thomas, is his work on a committee of the College of American Pathologists which has devised a nomenclature and code for pathological lesions. In collaboration with Dr. Arnold Pratt and the Biometrics Section of NIH this Systematized Nomenclature of Pathology is now being used in a computer retrieval system which permits the accurate and speedy retrieval of all autopsy and biopsy records in the National Cancer Institute.

Most of the members of the Department of Pathologic Anatomy also carry out experimental research using animals, tissue culture and other special techniques. The residents in Pathologic Anatomy are particularly ambitious in carrying out research programs.

Experimental Research

The experimental work in the Laboratory of Pathology is not restricted to a single project, or to a group of closely related projects, but each pathologist follows his particular line of interest and training. Because of this diversity the projects in the Laboratory have been separated into 6 rather loose categories for convenience in preparing this summary. These are:

Cancer in Man, and Related Animal Studies:
- Geographic pathology of cancer.
- Exfoliative cytology and cytogenetics.

Possible etiologic factors in human environment.
African lymphoma.

Cancer in Animals:
Induction and pathogenesis.
Modifications in carcinogenesis.
Biologic factors in neoplasia.
Spontaneous tumors.
Transplanted tumors.

Viral Carcinogenesis and Related Problems in Virology:
Polyoma virus.
SV40 and adenovirus.
Leukemogenic viruses.
Virus replication.
Interferon.

Accumulation of Data on Laboratory Animals and Other Species:
Information on laboratory animals.
Phylogenetic aspects of neoplasia and comparative oncology.

Development of New Techniques and Their Application.

Collaborative Research:
Methods.
Examples.

Cancer in Man, and Related Animal Studies

Since nearly all the members of the Professional Staff of the Laboratory of Pathology and Department of Pathologic Anatomy are medically trained, many research activities are correlated with the problem of human cancer. Several projects are directly utilizing human material, and others are testing in animals substances in the human environment that may be carcinogenic.

GEOGRAPHIC PATHOLOGY OF CANCER—Information is accumulating that certain forms of cancer in human population groups occur in a higher incidence than would be expected. Intensive study of such groups, and the recognition of possible etiologic agents which can be tested on experimental animals may identify some environmental factors which could be eliminated or controlled and thereby prevent many cases of human cancer. The following studies have this aim.

Maps of different geographic areas are in preparation to show the relative frequency of different types of cancer. When it is recognized that some type is especially common, a more intensive study of the population group is indicated. (Dunham and Bailar)

The following types of cancer are being investigated in particular geographic area:

Bladder cancer in New Orleans: White males over 60 in this city are frequently affected. All histologic sections have been reviewed by the same pathologists (Stewart and Rabson) to establish uniformity in diagnosis. Questionaires on past history and possible exposure to carcinogens are being analyzed, and a report is now in preparation. (Dunham)

Uterine cancer in New York City, Israel and Washington, D.C.: This type of cancer is notably frequent in Negro women and infrequent in Jewish women. Histologic sections have been examined from patients with cancer, and over 3,500 women interviewed. The data are being coded and analyzed in an effort to detect an environmental factor that may account for the racial difference. (Stewart, Dunham, Thomas, Edgcomb)

A study of cancer in North American Indians has been started. Cancers of the uterine cervix and biliary tract, and basal cell carcinoma of the skin, are frequent in this ethnic group. The histologic diagnoses have been made on 700 specimens in the NIAMD. Correlations with age and sex will be made. A survey of the literature revealed that very little reliable information is now available on the Indians. (Dunham, Laqueur)

Lung cancer in veterans of World War I: The medical history and biopsy and autopsy material from this group are gathered. All histological material has been reviewed by the same pathologist, and a uniform classification of histologic types has been made. Different histologic groups may have different significance and relationship to environmental factors. This survey indicated the unreliability of random histologic diagnoses which have often been accepted uncritically by statisticians or epidemiologists. The results have been coded and a manuscript is in preparation. This study will supply important information

on the influence of smoking on the incidence of lung cancer within this group. (Herrold)

Gastric cancer in Japan: Cancer of the stomach is remarkably frequent in several unrelated human populations, and in Japan, this type of cancer is especially common. Migrant Japanese in Hawaii offer a good group of genetically similar people in a different geographic area for comparison. The histologic sections are being reviewed by the same pathologist (Herrold), and any association with gastric ulcer, polyps or intestinal metaplasia is noted. A correlation will be made with data collected by a team of epidemiologists. An investigation is also made for viruses (Bryan); and electron microscopy is done when possible. Histochemical studies on the human material indicate a change in gastric mucins, and correlated studies of Syrian hamsters support this finding. This is a long term study that will require many years, but a preliminary report will be given at the International Congress in Japan in 1966 (Herrold, Stewart, pathologists from Japan).

Exfoliatine Cytology and Cytogenetic.—A diagnostic service in exfoliative cytology is supplied to the Clinical Center, but in addition to this, a number of research studies are in progress in collaboration with various clinical services. These relate to the presence of neoplastic or other cells on body surfaces or in fluids, and sex chromatin in exfoliated cells (Malmgren). It has been determined:

Number of neoplastic cells in the blood of cancer patients is not closely correlated with metastasis.

Cancer cells are often recovered from wound washings after cancer surgery, but time will be required to determine whether the frequency is correlated with recurrence in the wound site.

Leukemic cells in the spinal fluid provide a reliable indication of central nervous system involvement and in the assessment of therapy. Evaluation of intrathecal treatment can be made by observation of neoplastic cells in spinal fluid.

An improved technique using a milipore filter makes the identification of sex-chromatin in cells from the buccal mucosa more reliable.

A cell line from a primary lymphoma of the ovary in an American woman has been studied using cytogenetic techniques. Ninety percent of cells in the primary tumor carried a marker chromosome which was not seen in the established continuous tissue culture line. This indicated that the marker chromosome was not an inseparable and necessary feature of the malignant cell. (Chu)

A system for coding findings in exfoliative cytology has been prepared, and is now in operation. (Malmgren)

Possible Etiologic Factors in Human Environment.—An important area in cancer research is the identification of environmental factors in a human population that may alter the expected incidence of cancer. Notable progress in the prevention of cancer has come from recognition of the carcinogeni activity of soot, shale-oil, and radioactivity. Once an environmental factor is suspected, tests on animals are necessary to prove that it is carcinogenic and in a complex substance it is important to identify the most potent fraction. A number of experiments with this aim are now in progress in the Laboratory of Pathology.

Absorbates from drinking water from New Orleans, where bladder cancer is high, and Birmingham, where it is low, are concentrated and given to mice in alcohol or chloroform. Lung tumors, a delicate indicator of carinogenicity were equally frequent in control and experimental groups. (Dunham)

Betel quid chewing is associated with oral cancer in many areas of the world. Ingredients of the quid have been tested in the hamster cheek pouch. Calcium hydroxide caused the most damage to the mucosa, and produced inflammatory and hyperplastic lesions and epithelial atypia, but no cancer. Other substances were ineffective. A paper describing these results has been accepted for publication in the British Journal of Cancer. (Dunham)

The increasing number of lung cancers in man is a cause for concern. No exact prototype of human lung cancer is known in animals, but a technique has been devised where a substance for testing can be incorporated in a beeswax pellet, and inserted into the lung

tissue of a rat. When cancer develops it is epidermoid as in human lung cancer. The formation of keratinizing cysts in the rat indicated a potentially cascinogenic substance. This technique will make the investigation of potentially carcinogenic substances for man more accurate. (Stanton)

Attempts to produce bladder cancer in hamsters with schistosoma were unsuccessful, but since the Egyptian and the Gold Coast strain were each used, a comparison and search for histologic differences in the effects can be made. (Thomas)

DMSO, a solvent suggested for therapy in man was tested in mice. No increase in tumors was noted. (Dunham)

The long term effects of enovid (the antifertility pill) are under investigation in mice. It was found that the sterility dose is 4 or more times that required for women. All animals are living after a year. One mouse that received enovid when newborn developed a granular cell myoblastoma of the cervix. Similar tumors were found in mice receiving estrogens when newborn. (Dunn)

Numerous other substances to which human beings are exposed have been tested, or are now being tested. Dihydrosafrol (DHSF) a food coloring proved to be toxic to OM rats on the usual laboratory chow, but when on an 80% corn meal diet the rats lived longer and some atypia of the esophageal mucosa was found. Esophageal cancer is high in Curacao, and natives use many decoctions of plants. Decoctions from 12 plants used by 11 patients with esophageal cancer have been given to rodents but it is too early to expect results. The quantity of zinc and copper in the soil has been correlated with the occurrence of certain human tumors. To test this in animals, squares of zinc, copper or iron were embedded in subcutaneous tissues of rats. Substances from heated fats were given to rats. Experiments begun by Dr. Wilhelm Hueper on the carcinogenicity of heated fats are being continued. The results are incomplete, but there is an indication of a weak carcinogenic action that is enhanced by repeated heating of the fat. (O'Gara)

AFRICAN LYMPHOMA.—The frequency of this form of cancer in children in Africa has aroused much speculation as to possible etiologic factors. A series of experiments have been conducted in the Laboratory of Pathology, and comparisons made between a lymphoma in an African child, and a tumor of similar cytology in an American woman. Each of the tumors has been grown in tissue culture. Cells are similar and resemble transformed lymphocytes. *In vitro* studies of the cell line from the African lymphoma showed 40-77 chromosomes. Interferon was found in supernatant fluid. The cell line from the American woman produced immunoglobulins (Rabson). Herpes-like particles have been seen in both lymphoma lines and attempts to isolate a virus and to clarify the role of the particles in these tumors continue. The tissue culture cells could be infected with the herpes virus, but other viruses so far tried have not proliferated in the cultures. Twenty-one malignant lymphomas of various histologic types obtained from Clinical Center patients failed to show viruses or virus-like particles by electron microscopy. (O'Conor)

Cancer in Animals

Many of the studies of cancer in animals are concerned with (*a*) induction and pathogenesis, or the steps by which cancer develops after exposure to a carcinogen; (*b*) modifications in the activity of carcinogens produced by altered environmental conditions or substances; (*c*) biologic factors of neoplasia such as the behavior of tumors in tissue culture, and transfer by vectors; (*d*) spontaneous and unusual tumors; and (*e*) transplanted tumors.

INTRODUCTION AND PATHOGENESIS.—Development of gastric and skin appendage tumors produced by 2–7–FAA when given to pregnant or lactating mothers, to newborns, and to adult rats is being studied. The distribution of skin appendage tumors produced by this chemical is being plotted on maps of the skin surface and a paper on histogenesis has been prepared. The tumor yield in the offspring is increased if 2–7–FAA is administered during both pregnancy and lactation. Carcinogenic

action is less certain if given during pregnancy or lactation only. (Stewart, Snell)

Monkeys were injected with MCA in the wall of the stomach 15–17 years ago. No cancers have been found, but degenerative diseases such as arthritis have appeared. (Stewart, Snell)

Several studies have been done with the nitrosamines which have been found to be potent carcinogens. Nitrosamine compounds given by a variety of routes in hamsters produced tumors at similar sites, indicating that the distribution, metabolic pathways, and excretion of the carcinogen were the critical factors. Tumors of the olfactory neuroepithelium in Syrian hamsters were of special interest, because in previous reports these may have been mistaken for brain tumors by others. These studies emphasize that carcinogens may reach remote sites, and that when the site of tumor formation is in the lung, the carcinogen did not necessarily reach the lung by inhalation. (Herrold)

Methylnitrosourea (MNU) was given by a number of routes to rats. Tumors histologically resembling neurilemmomas appeared at many different sites. Since use of this substance as a solvent has been proposed, awareness of its carcinogenic potency is important. (Stewart, Snell)

Nitrosamine was given orally in various vehicles to general-purpose mice. Papillomas of the esophagus and forestomach were found in a few. (O'Gara)

Syrian hamsters were given benzopyrene by intratracheal instillation and early changes in the bronchial epithelium were described. No tumors resulted from atmospheric pollutants or tobacco tar. An unanticipated finding from this experiment was the development of cryptococcus neoformans meningitis in one hamster. This disease occurs in man, where it has been presumed that the organism is bloodborne from a focus in the lung. Observations with the hamster suggest that the primary focus may be the nasal cavity and sinuses, from which sites the infection extends to the meninges. (Herrold)

A prototype of human osteogenic sarcoma has been produced in rats by copper chelated N–OHAAF, introduced into the medullary cavity. Following this observation new experiments have been started. An improved technique is used in inbred rats so that any tumors that develop can be transplanted. (Stanton)

Hepatic cancer is being produced successfully in primates after a short induction period. Newborn and infant monkeys were used, and 13 hepatic neoplasms were produced by DENA. Successful intracerebral transplants have been made. This is the first time quick induction of cancer and transplantation has been accomplished in primates. The transplanted tumors have now been used in therapeutic trials. (O'Gara, Kelly)

Chronic myelogenous leukemia developed in one monkey that received MIH, a drug used for treating Hodgkin's disease. Although leukemia has been induced in one monkey by irradiation this is the first time it has been induced in a primate by a chemical. (O'Gara, Kelly)

Modifications in Carcinogenesis.—Vitamin A delayed the appearance of tumors of the forestomach in hamsters given DMBA. No inhibition of skin cancer was noted. (Chu, Malmgren)

Hepatocarcinogenesis in the rat has been altered by dietary modifications: (*a*) Three times as much carcinogen N,N–Dimethyl–*p*–phenylazoaniline is required to induce hepatomas on a complete diet, as it takes to induce cancer in rats on a deficient semi-synthetic diet. (*b*) n a complete Purina laboratory chow diet, the carcinogen N,N–Dimethyl–*p*–(m–tolylazo)aniline induced cysts and cholageofibrosis and a rare hepatoma, but the same dose of carcinogen gave 100% incidence of hepatoma on a deficient semi-synthetic diet. (*c*) An extract of U.V. irradiated fat in conjunction with subcarcinogenic dose of N,N–Dimethyl–*p*–phenylazoaniline had a tendency to induce hepatomas. The extract alone caused considerable liver damage but no neoplasms. (Mulay)

Manipulation of copper and zinc concentration in the rat diet failed to induce neoplastic lesions. Thus it fails to support the contention that a higher incidence of gastric cancer, in

some areas, is related to use of food grown exclusively on soils with copper and zinc imbalance. (Mulay)

Early alterations preceding the development of cancer are being investigated.

Rats fed a hepatocarcinogenic diet showed a rise in adrenal steroid concentration, two weeks after the start of the diet and continued high. The significance of this finding to azo dye carcinogenesis is discussed. (Mulay)

Biologic Factors in Neoplasia.—Cell cultures have been made from a liver tumor in a rhesus monkey (see O'Gara) above. The cells grow slowly and the morphology remains epithelial with some attempt at differentiation, but show no evidence of function. Transplants back to the monkey brain have been successful. (Dawe)

The ability of insect vectors to transmit neoplasms is under investigation. A reticulum cell sarcoma in the hamster has been under observation for several years. It was proved that it could be transmitted by feeding tumor tissue to susceptible hamsters and it has now been shown that it can be transmitted by the bite of the Aedes mosquito. Transmission is by cells. No multiplication of the cell or a virus within the mosquito could be noted. Attempts to transmit Rauscher cells or the virus have failed. Transfer of Moloney virus by the mosquito was successful in 2 of 61 trials. (Banfield)

Spontaneous Tumors.—These are always under study in the Laboratory of Pathology and descriptions and classifications of them are published at intervals. A considerable number of histologic sections of retriculum cell sarcomas have been accumulated by Dr. Margaret Deringer. Since this group of tumors is especially confusing and complex the slides are being reviewed and a publication on the histological features is being prepared. (Dunn)

Transplanted Tumors.—Observations on transplanted tumors and their behavior furnish valuable information on neoplasia. A number of these are carried and studied in the Laboratory of Pathology, and are made available to other investigators. Among many tumors of interest are an adrenal cortical tumor, gastric adenocarcinomas, a neurilemmoma, and a mesothelioma (Stewart and Snell). Others carried in mice are a myoepithelioma with a leukemoid reaction, a kidney tumor, and a granular cell myoblastoma. (Dunn)

Viral Carcinogenesis, and Problems of Immunology and Resistance

The same interest in pathogenesis and the mechanisms of carcinogenesis will be found in the experiments carried out by pathologists with viruses as was noted in experiments with chemical carcinogens. Experiments now in progress are concerned with: Polyoma virus, SV40 and adenovirus 12, leukemogenic viruses, and Interferon.

Polyoma Virus.—Studies on the interaction of mesenchymal and epithelial elements in organ culture, and the effect of the polyoma virus have continued. The specificity of the mesenchyme for the epithelium is critical and some combinations produce no neoplastic change, even in the presence of PV. Continued contact with natural mesenchymal tissue is required for epithelial neoplasia, and neoplasia will not develop until some morphogenesis and differentiation take place. The salivary gland rudiments must reach a certain stage before neoplasia results. Viral oncogenesis is not simply the result of interaction of the viral genome with the cell genome—epigenetic factors also operate and a proliferative stage is required. (Dawe)

The behavior of a polyoma induced tumor in the hamster was compared with a spontaneous and a carcinogen-induced tumor after intravenous injection of dissociated cells. The resulting nodules in the lung after injection of the polyoma tumor were interpreted as inflammatory reactions rather than true neoplasms. (Herrold)

Thymectomy was shown to increase the oncogenic effect of the polyoma virus. Of particular interest was the observation that C57BL mice resistant to polyoma oncogenesis were made susceptible by thymectomy but sensitized spleen cells will inhibit this susceptibility even when given many weeks after the viral infection and thymectomy. A morphologic

study of this effect is now in progress. (Stanton, Law)

Two strains of the polyoma virus with different oncogenic potentials but with a similar capacity for the induction of tumor antigens are available. These have been tested by the tumor rejection assay. Paradoxically, the more oncogenic virus inhibited the ability of the mouse to reject a non-polyoma tumor, possibly because of thymotrophic action. (Friedman)

SV40 AND ADENOVIRUS.—(*a.*) Newborn hamsters were infected with SV40 and adeno 12 simultaneously and the progeny of the two viruses grown in mixed infection. The SV40 had previously been shown to induce tumors with large cells, while the adeno 12 tumor cells were small and hyperchromatic. The tumors that developed early were of a histologic type characteristic of adeno 12; tumors that developed later were of SV40 type. (*b*) A "T" antigen was shown by immunofluorescence to be correlated with stippling seen by electron microscopy in monkey kidney cells with adenovirus 12. (*c*) The hybrid virus adeno 7–SV40 designated E46 produced SV40 type tumors, described as papillary ependyomas when given intracerebrally. When given subcutaneously, it produced "mixed" tumors showing histologic areas like SV40 and other areas like adeno 12. (Rabson)

Electron microscopic studies on mixed viral infections are continuing. Simultaneous replication within the same nucleus of SV40 and herpes simplex virus has been demonstrated in green monkey kidney cells. Hybrid E46 has protein crystalline structures in the nucleus which are not found in the parent adeno 7 or SV40. EM studies on arbovirus infection are being correlated with biochemical studies in chicken fibroblasts infected with Semliki forest virus. (O'Conor)

LEUKEMOGENIC VIRUSES.—Moloney virus infection in rats has disclosed previously inapparent Bartonella organisms in the blood and encephalazoon lesions in the brain in the preleukemic state. This indicates an alteration in the defense mechanism during the preleukemic state, and raises the question whether an atypical response to infection may be found in a preleukemic state in man, and clinicians should be alert to this possibility. (Stanton)

BALB/c mice infected with Rauscher virus and malaria die earlier. Malaria infection also causes an increase in macroglobulin. Changes in the globulins might be of diagnostic aid in malaria and leukemia. (Edgcomb)

BALB/c mice with Rauscher virus developed an extreme erythroblastic reaction but no leukemia. The erythroblastic reaction was inhibited (but not prevented) by feeding prophylthiouracil or by repeated blood transfusions (Dunn, Malmgren). Moloney virus infection was enhanced by DNA and RNA obtained from a commercial source. (Malmgren)

VIRUS REPLICATION.—Replication of an RNA virus is under study. A model cytopathic RNA virus infection with Semliki Forest Virus (arbovirus, group A) is used as a model in order to elucidate the mechanism of replication of RNA viruses including possibly leukemia viruses. Studies have shown that in addition to the single standard RNA present in infectious virus, 2 other replicative forms are present in infected chick cells. One appears to be a double stranded viral forms. These forms are present in 3 distinct cytoplasmic particles. Protein synthesis by virus and the interrelationship between the 3 cytoplasmic particles are being investigated.

INTERFERON.—Mechanisms of interaction of interferon and the possible role in carcinogenesis are under study. The action involves action protein synthesis. Interferon appears to limit production of viral DNA in vaccinia virus. (Friedman)

Accumulation of Data on Laboratory Animals and Other Species

Because the training of pathologists is not restricted to one organ system or type of disease, they are often able to contribute to the general knowledge of the normal and pathologic anatomy of many different animal species. This contribution is often incidental to the main purpose of cancer research, yet it is indispensable to the intelligent use of biologic material, because, unless the normal anatomy and spontaneous pathologic alterations are

recognized, it is impossible to interpret the effects of experimental procedures.

For ten years or more autopsies have been performed on old rats of 6 inbred strains. The incidence of tumors and other lesions has been accurately recorded. This information has become increasingly more complete and valuable and facts derived from this survey are often requested by pathologists within the NIH and from outside. A detailed review of renal lesions in these old rats, with a review of the literature was recently presented at a Conference on Spontaneous Diseases of Rats and Mice sponsored by the Nuffield Foundation in England. (Snell, Stewart)

Mastomys have been introduced as a new laboratory species, and information on the normal anatomy and spontaneous diseases is being accumulated by autopsies performed on 200 animals from the colony maintained at the NCI. These animals lived out the normal lifespan. Tumors were frequent in the glandular stomach, the liver and the thymus. A kidney lesion resembling human glomerulonephritis was frequent. The female has a well developed prostate gland. (Stewart, Snell)

Another species that has been used extensively as a laboratory animal for a decade or more is the hamster, yet comprehensive and reliable information on the normal anatomy and spontaneous disease of this useful rodent is still lacking. References describing the normal anatomy are now being accumulated and information on the normal anatomy and spontaneous diseases in hamsters used as controls in a variety of experiments is being compiled. (Herrold)

Information from personal observations and the literature is being accumulated on the normal and pathologic anatomy of the laboratory mouse. A paper on renal disease in mice and on amyloidosis in mice was given at the recent Conference in England. (Dunn)

Complete autopsies are being performed on both control and experimental monkeys. At the present time information on the normal and pathologic anatomy of monkeys is deficient, scattered, and inaccurate, and reliable knowledge will be supplied only by competent pathologists who perform many autopsies. A form of vascular disease resulting from subcutaneous injections of polycyclic hydrocarbons as been described. (O'Gara)

An International Conference on Lung Tumor in Animals was held in Perugia, Italy, in June 1965. A principal address was delivered on comparison of histologic lung tumor types in fowls, lower animals and man. In preparation for this address, all previous descriptions on lung tumors in these animal species were reviewed, and the histologic sections were examined when available. Many of these came from the Philadelphia Zoo. This critical review revealed that many tumors previously accepted as primary were probably metastatic. Since captive wild animals and domestic are closely associated with man and share his environment, any neoplasms found have special significance. (Stewart)

PHYLOGENETIC ASPECTS OF NEOPLASIA.—An important project has been started on phylogenetic aspects of neoplasia in cooperation with the Smithsonian Institution and marine biological laboratories. The collection and identification of neoplasms in invertebrates has been started, a literature survey now having over 300 entries will be continued and a registry of specimens will be made. Neoplasia in planaria and cockroaches is being investigated under laboratory conditions. The planaria have developed a curious spontaneous lesion with inclusion bodies, but it is doubtfully neoplastic. Soft shell clams are being investigated for neoplastic changes. It is apparent that adequate criteria have not been used previously to determine neoplasia in lower forms. (Dawe)

Studies in comparative oncology include an effort to determine the neoplastic response of fish to chemical carcinogens. Cycasin was administered and severe liver damage produced which the fish survived. Hepatic tumors developed in a restricted interval of time from 12–15 weeks. This fact together with the small size of the liver which permits serial sectioning should make this species a valuable indicator of potential carcinogenicity. (Stanton)

Development of New Techniques and Their Application

Since modern pathology is not static and is not restricted to anatomical dissection and observations with the light microscope, new techniques must be introduced and old ones improved. New electron microscopic techniques are being developed especially.

The electron microscope probe and the scanning electron microscope offer greater precision in morphologic studies. Biological application of these new instruments will first be on an experimental basis. It is now possible to make a determination of the amount of phosphorus in the nucleus. Three dimensional perspective should become possible, and a better knowledge of the cell surface can be obtained. An analysis of tissue *in situ* can be accomplished by these new methods. (Banfield)

A new technique, the Transer, eliminates fixation and embedding of tissues for EM examination, and offers new opportunities for analytical histological investigation. This will be especially valuable in continuing studies on collagenous connective tissue and elastin. (Banfield)

Ferritin conjugated antibody studies are being applied in viral oncology. An effort is being made to improve the specificity of present methods while preserving cell ultrastructure. Findings are being correlated with immunofluorescence techniques. (O'Conor)

Mice have been placed on a complete liquid diet, the commercially available Metrecal, and found to be healthy after 6 months. Development of an appropriate basic liquid food for mice would be desirable in many experiments, and possibly even for routine matintenance. (Dunn)

Collaborative Research

It is recognized that many research projects at the National Cancer Institute require the collaboration of a pathologist, especially in the final evaluation of the effect of an experimental procedure on laboratory animals. The Laboratory of Pathology has always tried to make this assistance available.

METHODS.—The experimental pathologist may take an active part in planning an experiment and in following it through; he may take the responsibility for all autopsies and histologic diagnoses in an experiment; he may review only the histolcgic sections in a given experiment; or may serve as a consultant to review selected material with no responsibility for the entire experiment and its publication. Finally, he may make use of material accumulated by other investigators for independent studies concerning pathologic alterations. It is emphasized that full collaboration of the pathologist at the time the experiment is planned is the most satisfactory arrangement for it insures the best and most economical selection of material for pathologic studies.

The impressive amount of collaborative work carried on by the staff in Pathologic Anatomy with clinicians at the National Institutes of Health is shown in the report submitted by Dr. Louis Thomas (NCI–853). Pathologists also collaborate in experimental work on clinical problems which may not be directly related to cancer, but which could not be accomplished without pathology assistance to physicians of the Clinical Center staff.

In addition to the use of the light microscope and standard autopsy procedures, individual members of the Laboratory of Pathology have become proficient in special techniques such as fluorescent antibody visualization, electron microscopy, tissue culture, autoradiography, exfoliative cytology, special cytology, and histochemistry. These special skills are often made available in collaborative studies.

EXAMPLE:.—It would be tedious to consider all the collaborative work now in progress in the Laboratory of Pathology, and this should be unnecessary since it is covered in reports from other laboratories. However the following are noteworthy:

With other scientists in cancer research: (*a*) Tumor cells were transplanted to day-old hamsters and the subsequent tumors were were treated with clam juice. Macrosis and regression was produced in the small tumors (Chu, C. P. Lee). (*b*) Variations in the estrone cycle were produced by continuous light and by interruption of the pathway to the pineal

glad in rats (Chu, Wurtman). (*c*) Degenerative joint disease of Mastomys has been studied (Snell, Sokoloff). (*d*) The histology of anterior chamber transplants from tissue culture cells made to the eyes of mice has been described (Dunn, Evans).

Research on clinical problems: (*a*) Emphysema is a crippling disease of increasing frequency. A probable factor in its pathogenesis is increased air pressure in airways. This has been produced by experimental procedures in rabbits. The amount of emphysema and tissue damage correlated with the air pressure, but spontaneous healing occurred. (*b*) Myocardial mechanics of the dog's heart were studied during systole. Apparently two groups of differently oriented muscle fibers determine dimensional changes during systole. (*c*) A series of renal biopsies in lupus nephritis was studied in order to determine how uniform and widespread the lesions might be. (*d*) Endometrial changes and ovulation in non-fertile women after treatment with gonadotrophic hormones were studied. Induction of ovulation was produced in some cases. (Powell and others)

Use of special techniques: Fluorescent antibody studies. The following are some now in progress: antigens in patients with mycosis fungoides, antigens in human leukemia cells, monkey kidney cells with adena 12 and SV 40 virus, PV tumor antigen in lytic infection of mouse embryo cells, antigens to adenovirus 7–SV40 in induced neoplasms of the hamster kidney. (Malmgren and others)

Electron microscopy of normal and pathologic tissue. Application to epidemic diarrhea in mice, where virus particle so far have been found only in the lumen. (Banfield and others)

LABORATORY OF PHYSIOLOGY

Cancer Physiology Section

Dr. S. H. Wollman and research associate Dr. J. E. Loewenstein are continuing their efforts in studying the iodide-concentrating mechanism of the thyroid gland. This investigation is concerned with (*a*) the mechanism by which the thyroid gland maintains a concentration of iodide elevated above that in the blood, and (*b*) the mechanism by which the thyroid gland accumulates protein-bound iodide. Kinetic studies on the exchange of radioiodide between individual thyroid follicles and blood have indicated that their kinetic properties are dependent on the size of the follicle. Experimental results support previous theoretical considerations made by Wollman and Dr. G. Andros (former research associate) that the average epithelial cell clears radioiodide from the blood and releases concentrated radioiodide back to the blood to the same extent in follicles of all sizes and that the radioiodide transport properties of follicles depend on their *surface* to *volume* ratio. Kinetics of equilibrium labeling of the protein-bound iodine in individual follicles reveal that at equilibrium the concentration of radioiodine in follicles, and therefore the stable iodine, is independent of follicle size. Follicles at the periphery of the thyroid lobe equilibrate slower than do the central follicles. The rate of equilibrium of central follicles (which parallels the rate of release of organic radioiodine) varies inversely as follicle size.

Dr. Rabinovitz and Dr. Honig and research associates Drs. Waxman and Freedman have have been doing extensive work on protein synthesis and its control in normal and tumor cells. This entails studies on the pathway(s) taken by amino acids in the synthesis of protein and the controls exercised by the cells in regulating these processes. As information is obtained concerning protein synthesis in the normal cell, it is applied to the tumor-bearing animal with the view of selective inhibition of protein synthesis in cancer cells. With Dr. Honig, studies have been made of the requirement of ribonucleic acid synthesis for some respiration dependent biosynthetic pathways in Sarcoma 37 ascites cells. Inhibitors of RNA synthesis in Sarcoma 37 cells, such as actinomycin D, also inhibit the synthesis of proteins and sterols in these cells. They have found that the inhibition of the synthesis of protein and sterols can both be prevented and relieved by glucose or other glycolyzable substrates. The inhibition of protein synthesis has been demonstrated to occur at the step involving aminoacyl-transfer RNA formation. The level and ac-

tivity of the enzyme catalyzing this reaction was identical in cells treated and not treated with actinomycin D. There appeared to be no difference in ATP level or in rate of ATP synthesis in cells with impaired biosynthetic capabilities. They believe that there is present a short-lived RNA which is involved in the availability of oxidative energy for biosynthetic processes. With Dr. Waxman, studies on the control of reticulocyte polyribosome content and hemoglobin synthesis by heme indicated that hemin enhances polyribosome stabilization and formation as well as globin synthesis. This effect of hemin duplicates the results previously reported for iron salts. Cobaltous ion, deuterohemin and zinc protoporphyrin were all capable of producing the enhancing effects on polyribosomes and globin production. Nickel and zinc salts, as well as other cations, were ineffective. Lead salts, which lead to reticulocyte polysome disaggregation, apparently produce this effect by the inhibition of iron incorporation into protoporphyrin. These data suggest that hemin is an intracellular mediator of reticulocyte polyribosome assembly and maintains the functional integrity of the biosynthetic apparatus in these cells and precludes the formation of globin under conditions of iron deficiency. With Drs. Freedman and Honig, studies on the control of nuclear-histone synthesis in chicken reticulocytes indicate that these reticulocytes *in vitro* can synthesize hemoglobin, nuclear ribonucleic acid and nuclear proteins including histones. If one adds actinomycin midazole, inhibition of nuclear ribonucleic acid D or 5, 6-dichloro-1-β-D-ribofuransyl-benzisynthesis occurs promptly. This also led to a rapid decrease in the rate of synthesis of certain nuclear proteins, particularly histones. One histone fraction characterized by its solubility in 5% and insolubility in 20% trichloroacetic acid was particularly sensitive to inhibitors of RNA synthesis. These results suggest that the synthesis of specific histones may be under precise control in the reticulocyte nucleus through the mediation of newly-formed ribonucleic acid.

Dr. Reid continues his studies on the determination of the pattern of excretion of a broad group of metabolites by leukemic patients with special reference to nucleic acid congeners. The techniques involved are both chemical and mathematical. By employing chemical methods, urinary specimens are fractionated into a large group of mixed fractions of nucleic acid congeners. Mathematical techniques, primarily linear algebra, are employed to further resolve these mixed fractions by means of computational analysis of their ultraviolet absorption spectra. Data-processing equipment is used to handle the larger volume of data generated. Continuing with the computational system for spectral file analysis (reported last year), further developments have been made. As many as 16 spectra can now be compared with the total file and those members which correspond at any one of three discrimination levels are obtained. Output by this procedure can be presented analyzed in various ways so as to identify groupings by clinical type and chromatographic characteristics and demonstrate any relationship within subgroups. Application of the computational system to analysis of chromatographic profiles has yielded helpful clarifications. Certain regions of the profiles are clearly common to the leukemia and controls; a few regions are present in the leukemia profiles which appear to have no counterpart in the controls; and certain regions are peculiar and do not correlate in a meaningful way with any others.

Energy Metabolism Section

Dr. Pratt and his associates, Drs. Morrison and Millar, have pursued their investigations of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity. Their goal is to find the mode and extent to which feeding behavior (patterns) influences heat production and, in particular, how the changes in feeding and general behavior pattern of the tumor-bearing rat influence its energy exchange. They are also investigating the relative changes in water and material exchange in tissue compartments of the rat during imposed and induced changes in food intake and the effect on these of tumor induction and growth. They have found that the relative constancy of the size of the activity compartment of total energy expenditure

has been consistently confirmed in normal rats at normal temperatures. Energy expenditure of normal rats made aphagic by lesions in the lateral hypothalamus increases along with a disruption of normal patterns of differentiated activity. In rats in which the aphagia is sustained, the size of the activity compartment of total energy is greatly increased.

Dr. Pratt, in collaboration with Mrs. Toal and Mr. William C. White, is continuing to develop mathematical and computer programming techniques for application in biomedical problems. Ultraviolet spectrophotometric analysis of oligonucleotides has employed this technique previously for the determination of the base composition and evaluation of sample purity of oligonucleotides. This has been extended to determine nucleotide sequences. The technique involves obtaining spectroscopic data before and after enzymatic hydrolysis of mono-, di-, tri- and tetra-nucleotides of known sequence from which precise difference spectra are computed for each compound, utilizing mathematical (linear) programming solution methods. The difference spectra of oligoribonucleotides containing two or more adjacent adenylic acids were found to have a characteristic pattern, the magnitude of which was linearly related to the number of such adenylic acids present. Difference spectra sufficed to identify base composition and sequence for oligoadenylic acids.

Dr. Pratt has been in charge of the computer operations of the Laboratory. The IBM 1620 Data Processing System with 40K digits of memory, 2 disk storage drives and a line printer have been operated with prime time being shared by Dr. Reid, Mr. White and Mrs. Toal, with occasional use by Dr. Draper and Mr. H. King. A major portion of the computer time available was occupied with information storage files and search and retrieval techniques. The organization of the file of NCI research grants was modified to achieve faster loading and more efficient retrieval and output procedures. About one-fourth of the active Cancer grants have been loaded and completion of the file is awaiting the installation of an additional disk storage drive and the remainder of the punched paper tape input records. A storage and retrieval system for the abstracts of pharmacology papers presented at the 1965 FASEB meetings was organized and implemented. Programs were written to organize the file and supply output for several requests from Dr. Shannon. The information file of medical histories and clinical records from the chorio-carcinoma chemotherapy study has been initiated and the conversion and storage of data has been started. The organization of the file is such that a search and retrieval on 121 lines of patient medical history will be available and the identification and retrieval of specific clinical tests on any date for any patient from a list of more than 165 different tests and spanning several years and many admissions can be made.

Physical Biology Section

Dr. Shack continues his studies on the characterization of the nucleic acids and nucleoproteins of normal and malignant tissues in terms of their physical, chemical, metabolic and biological properties. These studies entail the development of additional procedures for isolation, fractionation and characterization of nucleic acids and nucleoproteins. Specific objectives within this framework include the determination of whether (*a*) DNA of malignant cells, including those of viral origin differs from that of normal cells, such as deletion of a normal component, addition of an abnormal component or a possible detectable change of a normal component, and (*b*) does a "metastable" DNA serve as a template for DNA synthesis. He has improved methodology so that submicrogram amounts of DNA can be resolved by electrophoretic methods. These new procedures include (*a*) carrier systems containing unlabelled native and denatured DNA or (*b*) trace amounts of labelled native and denatured DNA as aids in the characterization of various DNA's. Using such procedures, partially renatured DNA was electrophoretically resolved from both the native and denatured forms. The type of product formed during renaturation of DNA depends on the conditions of original denaturation (temperature, salt concentration). Renaturation of DNA, denatured at high salt concentration, gives a product con-

sisting of both denatured and partially renatured DNA, while denaturation at low salt concentration leads to a single species which appears to be a partially renatured aggregate. Using infected tissue cultures as a source material, it has proved possible by such procedures to separate renatured polyoma DNA from the tissue DNA which remains denatured.

Dr. Breitman and research associate Dr. Cannon are studying control mechanisms of purine and pyrimidine ribo- and deoxyribonucleotides. In collaboration with Dr. S. Perry and research associate Dr. R. A. Cooper, studies on pyrimidine metabolism in human leukocytes were conducted. It was observed: (1) The percent DNA-thymine derived from exogenous deoxythymidine increased from 13% to 87% over a range of deoxythymidine concentrations from 0.03μ M to 300μ M. (2) Deoxythymidine caused expansion of the *total* deoxythymidine di- and tri-phosphate pool but did not influence the contribution of the pathway *de novo* to this pool. Hence, the increasing contribution of exogenous deoxythymidine to the formation of DNA-thymidine occurred because of a progressive dilution of deoxythymidine triphesphate synthesized *de novo* with deoxythymidine triphosphate derived from exogenous deoxythymidine. (3) In concentrations above 0.3 μM, deoxythymidine inhibited DNA synthesis but not RNA synthesis. This inhibition was dependent on the continued presence of deoxythymidine in the medium and was reversed by the addition of deoxycytidine. (4) The incorporation of deoxythymidine-^{3}H into deoxythymidine di- and triphosphate pools of intact leukocytes from normal donors and patients with chronic myelogenous leukemia reached a maximum within 5 minutes at 37° C. A steady state was maintained during the subsequent 20 minutes at 37° C. The time to reach maximum incorporation of deoxythymidine-^{3}H into the deoxythymidine monophosphate pool was slower than into the deoxythymidine di- or tri-phosphate pools. In leukemic cells, the rate of equilibrium of deoxythymidine monophosphate with deoxythymidine-^{3}H was related directly to the deoxythymidine concentration and inversely to the temperature. (5) With increasing concentrations of deoxythymidine, expansion of the deoxythymidine monophosphate pool derived from deoxythymidine was greater than expansion of the deoxythymidine di- and tri-phosphate pools. In leukemic leukocytes the deoxythymidine di- and tri-phosphate pools were approximately equal in size and accounted for 65% of the thymine deoxynucleotides in the presence of 1 μM of deoxythymidine but only 10% in the presence of 300 μM deoxythymidine. In normal leukocytes at these deoxythymidine concentrations, the values were 35% and 5% respectively of the total thymine deoxynucleotides. (6) Over the range of deoxythymidine concentrations studied, incorporation of deoxythymidine-^{3}H into thymine deoxynucleoside-monophosphate pool was 2–5 fold greater and incorporation into thymine deoxynucleoside di- and tri-phosphate pool was 6–15 fold greater in chronic myelogenous leukemia than in normal leukocytes. Incorporation of 3 μM of deoxythymidine-^{3}H into DNA was 30-fold greater in chronic myelogenous leukemia than in normal leukocytes. The results indicate that human leukocytes have the enzymatic capability to convert thymine to deoxythymidine. This enzymatic capability is not expressed in the absence of added deoxynucleosides because of rapid catabolism of deoxythymidine and thymine and a limited supply of an available deoxyribose source.

Radiation Biology Section

Dr. Elkind and Dr. Francesco Mauro (Guest Worker) are studying the mechanism of the repair of X-ray damage of cells grown in tissue culture. Changes in protein, DNA and RNA synthesis and the effects on these syntheses of drugs which affect repair of X-ray damage are under investigation. They have developed a model for the fractionated dose response of cultured mammalian cells which they have named a Repair-Progression model. The *repair* part starts promptly after the first dose. This also occurs at lowered temperature and in the absence of oxygen. The *progression* part also starts promptly but is temperature sensitive and can be retarded at 37° C by either inhibitors of protein synthesis, a deficient medium, or the absence of oxygen. Re-

sults obtained with asynchronous as well as synchronous cells (synchronized by exposure to hydroxyurea) support the general features of the model and, hence, a study of the fractionation responses to be divided into the repair and progression parts can be made. In addition, the influence of drugs on changes in repair and/or progression can be studied. DNA synthesis apparently is not required for repair. Inhibition of DNA synthesis can be produced by high concentration of thymidine (1–3 mM). Under these conditions, division stops after G_2 cells have divided, and from gross chemical measurements very little DNA is synthesized (less than 10%) in the ensuing 10 hours. The advantage of thymidine as a DNA inhibitor is that cell viability is not greatly affected. Using high concentrations of thymidine to inhibit DNA synthesis in synchronous and asynchronous cells, they observed that the "coupling" between DNA synthesis and X-ray response is stronger for first dose responses than for second dose responses (i.e., after sublethal damage repair). Thus it appears that even if X-ray response and DNA synthesis are tightly bound before a first dose, irradiation serves to uncouple this relationship.

Dr. Riesz has been continuing work on the effects of ionizing radiation on macromolecules of biological importance: In collaboration with Dr. F. H. White, Jr., of the Heart Institute, the distribution of free radicals in the gamma radiolysis of several dry proteins has been examined. Gamma radiolysis of dry proteins *in vacuo* and subsequent exposure to tritiated hydrogen sulfide, a radical interceptor, leads to the formation of carbon-tritium bonds. It has been shown that the observed tritium distribution determined by amino acid analysis and scintillation counting, corresponds closely to the free radical distribution. Ribonuclease, carboxymethylated reduced ribonuclease, lysozyme, chymotrypsinogen A, insulin and gelatin have been investigated. A comparison of all the proteins irradiated shows that in every instance methionine, proline and lysine have very high specific activities, while valine, isoleucine and phenylalanine contain little tritium. However, each protein has, within this general pattern, its own characteristic radical distribution. The ratio of the highest to lowest specific activity for a given protein ranges from 20 for insulin to about 70 for ribonuclease. Contrary to past interpretations of electron spin resonance spectra, glycine radicals do not play a dominant role as free radical sites on carbon. Dr. Riesz has also studied the mechanism of the protective effect of certain metal ions in the γ-radiolysis of dry metal-ribonuclease complexes. The reduction of cupric ions during the γ-radiolysis of Cu^{2+}-ribonuclease complexes was measured as a function of dose and of Cu^{2+}/ribonuclease ratio and compared with the survival of enzymatic activity. The results of experiments at room and at liquid nitrogen temperatures support the proposal that metal ions protect because of their ability to act as interceptors of subexcitation electrons and/or of hydrogen atoms.

Dr. Smith is pursuing her studies on the kinetics of recovery of hemopoietic cells in irradiated animals. Employing the assay of the Till and McCulloch transplantation method (counting colonies formed in the spleen), it has been possible to study the radio-protective effects of such agents as β-mercaptoethylamine, bacterial endotoxin, colchicine and other antimitotic agents. While colchicine seems to have a beneficial effect similar to that of endotoxin when given after irradiation, it has a moderate to pronounced effect on femoral colony forming units, marrow cellularity, peripheral leukocyte counts and survival. It appears that the initial depressant effect is necessary for the subsequent early recovery observed when colchicine is given 1–3 days before irradiation. Studies on changes in peripheral blood and splenic colony formation using vinblastine are being undertaken with tumors of low radiation sensitivity (lymphosarcoma 1798) and the Bruce lumphoma.

Miss Uphoff is undertaking studies in the field of immunogenetics of tissue transplantation. She is developing new methods of altering the immune response as well as studying the possibility of modifying tissue antigenicity. In addition, a study of cell to cell or cell-antigen interaction in the initiation of the immune response is under investigation. In these investigations radiation is used chiefly as the

immunosuppressant agent. Transplantation of hematopoietic tissue into irradiated mice is used to test donor and host response, since mechanism, sensitivity and specificity of tissue reactions can be demonstrated by appropriate selection of various inbred strains or hybrid combinations. Tolerance is demonstrated either by skin or tumor homograft test systems. Studies on genetic factors influencing irradiation protection by bone marrow have indicated that the early deaths reported in two different strain combinations following mid-lethal X-irradiation and homologous bone marrow inoculation, were found to be unique to these two strain combinations (CBA vs C_{57}BL and BALB/c or C_{57}BL). If the inoculation of homologous bone marrow is delayed or if one pre-immunizes the X-irradiated host, early death patterns are altered. She has demonstrated that a simple gene difference at the H–2 locus between donor and host strains was found to be sufficient to account for the severe "secondary disease" following lethal X-irradiation and bone marrow therapy. Differences at H–1 provide variable reactions, while differences at H–3 produced no secondary disease. Immunologic tolerance to tumor homographs could be induced by pretreatment with amethopterin and antigen (viable cells from appropriate strain). She has also demonstrated a chimera consisting of three genotypes.

Dr. Maxwell is studying the effect of ionizing radiation upon the mammalian cell both *in vivo* and *in vitro* and determining the role of RNA and DNA in radiation damage. He has shown that in the early stages the surviving ability of an irradiated suspension of rat liver ribosomal particles to synthesize (*in vitro*) acid-insoluble protein is approximately a log function of the radiation dose. Doses of Co^{60} γ-rays of the order of 150,000 rads reduce the synthesizing ability by a factor of two. This factor is approximately the same for both endogenous directed synthesis and artificial messenger (polyuridylic acid) directed synthesis. It also appears to be independent of the presence or absence of dissolved oxygen in the irradiated solution.

Dr. Draper has previously reported that natural hemolysin production in rabbits is severely suppressed for about 10–14 days following a single exposure of 500 R whole-body irradiation. A subsequent recovery stage ensues during which there is a renewal of natural antibody production at a relatively rapid rate. Enhanced serum titers are attained between the third and fourth postirradiation weeks. The recovery phase is usually accompanied by a change in the avidity of the antibody but the direction of the change is largely unpredictable. The production of highly avid natural hemolysin in some rabbits precludes the distinction between natural antibody and that induced specifically by immunization with sheep erythrocytes. In contrast to an active immune response, the production of natural hemolysin continues to be radiosensitive even during the rapid production stage during recovery. This may be explained by the natural hemolysin titers representing the sum of a series of short-term, weak, individual primary responses to the normally occurring heterophile antigens in small but constant supply from the rabbit's environment. This series of responses can be interrupted by moderate doses of X-rays. During the recovery stage it is likely that a larger population of cell is producing antibody. Such cells would be expected to be more radioresistant. Irradiation during the recovery phase often results in a delayed depression of titer indicating that the radiosensitive cells are those exposed to antigen after irradiation. The spleen participates actively in natural hemolysin formation. Studies are in progress concerning the production and nature of antibodies produced against different physical forms of the same antigen.

Office of the Chief

Dr. Blum has pursued his studies in hyperplasia induced in mouse epidermis by ultraviolet light. Methods of measuring hyperplasia are quite difficult. Illumination with visible light after irradiation with ultraviolet light increases the hyperplastic response. Hyperplasia occurs in the epidermis of the ventral, unexposed surface of the mouse ear, concurrently with that in the epidermis of the dorsal surface which is directly exposed to ultraviolet light. This suggests that the increase in cell

proliferation is mediated by a diffusible substance, since the ultraviolet light does not penetrate sufficiently to account for this effect. Injections of homogenates prepared from normal and irradiated epidermis cause epidermal hyperplasia when injected into a local vein of the ear. Homogenates from irradiated ears produce the greater hyperplasia, indicating that some specific substance is released by the irradiation.

Dr. White together with Dr. Millar have been studying the role of a growing tumor as a stressing agent in protein metabolism. When pregnant rats are inoculated with the Walker 256 carcinosarcoma subcutaneously, effects observed are dependent on the time the tumor was inoculated. When female rats are inoculated with the tumor 7 days prior to evidence of pregnancy, the fetuses of *all mothers* are either resorbed or born dead. Rats with tumors less than 7 days of age when pregnant, deliver young which are normal in size. However, the inability of the mother to supply adequate milk results in animals which are stunted (about 50%) at weaning. These animals remain stunted for 3 weeks before they appear the same weight as rats born from non-tumor-bearing animals. It appears that the rat with a 3-week tumor (prior to parturition) cannot consume enough food to satisfy the growth of the tumor and supply protein for the formation of milk. In this competition, the tumor obtains what it needs at the expense of the lactating gland. Pregnant rats with 2- or 1-week tumors, deliver normal litters and the offspring are essentially normal at weaning. With Dr. Bates, a study on the nitrogen excretion and oxygen consumption of Fischer rats bearing a mammatropic tumor has been made. Tumors implanted intramuscularly in these rates grow slowly. After 40 days, this tumor begins growing. A tremendous quantity of protein is excreted by these rats and there is an increase in oxygen consumption. Although the total nitrogen increases, urea values remain constant. Food intake of the tumor-bearing rats increases 2–3 fold. Paired feeding experiments are in progress to determine the effect of increased protein intake on protein excretion.

The machine, glass blowing, and electronics shops, headed by Mr. Mencken, Mr. LeBrun, and Mr. Coffey, respectively, continue to make many research problems practical. The ability of these men to study the problems of the Laboratory and to design operational equipment not on the market has resulted in inestimable progress. In addition, their staffs have done an endless amount of emergency repair and modification jobs which not only have saved valuable time but have prevented the failure of many experiments. These unsung heroes have been an important arm in the structure and progress of the Laboratory.

DERMATOLOGY BRANCH

The clinical and laboratory research program of the Dermatology Branch has continued along lines developed in recent years with the addition of two new laboratory activities, namely research in cell biology and electron microscopy. The major groups of the research interests are: (1) Mycosis Fungoides Lymphoma, (2) Epidermal Growth and Differentiation, (3) Biology of Lymphoreticular Cells, and (4) Melanogenesis.

Mycosis Fungoides Lymphoma

Immunologic reactivity of patients with this disease remains normal throughout the course of the lymphoma. Whereas patients with other lymphomas, particularly Hodgkin's disease, have been found to be immunologically hyporesponsive, patients with Hodgkin's disease show no immunologic impairment, particularly demonstrable in studies of skin sensitization to materials such as dinitrochlorobenzene (DNCB) and the ability to reject homografts. Diseases other than lymphoma have been studied for comparative immunologic responsivenesses. These diseases have included leprosy and Sjögren's disease, both of which show marked impairment in capacity to react allergically to DNCB. Patients with lepromatous leprosy have been found to be more anergic than patients with non-lepromatous disease.

Interesting phenomena has been observed in patients with Mycosis Fungoides who have

developed cutaneous allergic sensitivity to topically applied nitrogen mustard HN_2. When such patients are given 1 mg. of HN_2 intravenously, cutaneous sensitivity to HN_2 disappears. This requires further exploration, particularly in regard to other materials since such a desensitization might have useful clinical applications.

Epidermal Growth and Reactivity

The hypothesis that basal cell tumors result from basal cells' inability to keratinize remains valid and is further supported by additional studies. These studies have tested the ability of basal cell tumor cells to participate in healing processes, a process requiring a cell to keratinize. There has been no evidence that basal cell tumor cells have capabilities of re-epithelializing wounded skin. A futher demonstration of the keratinizing defect in basal cell tumor cells has been found when podophyllin is applied to cutaneous tumors. Whereas normal epithelium manifests a high order of keratinization in response to podophyllin manifests a high order of keratinization in response to podophyllin, basal cell tumor cells show no such response.

Attention is called to the successful tissue culture of human skin, an achievement which has been difficult or impossible in the past by many investigators. When split thickness specimens of skin are put into culture, epithelium grows quite readily and remains morphologically normal over periods of several weeks.

The response of basal cell tumors to topically applied 5–fluorouracil (5–FU) has been confirmed. The selective response of lesions, compared to uninvolved skin, seems to be due to the ease of penetration of drug through the damaged epidermis overlying lesions.

Melangonesis

The ability of a soil micro-organism to produce a dark pigment by utilizing coumarin as a substrate seems to be due to the ability of the organism to convert coumarin to tyrosine, thence tyrosine to melanin. The series of synthetic and degradative steps involved in the conversion of coumarin to tyrosine have been studied. Of particular interest is the conversion of O-coumaric acid to melilotic acid, which utilizes an oxidoreductase enzyme requiring FAD as co-factor. The enzyme melilotic hydroxylase, converting melilotic acid to dihydroxyphenyl propionic acid, has been purified to the extent that it migrates homogenously on gel-electrophoresis. It has a molecular weight of about 65,000.

ENDOCRINOLOGY BRANCH

In the fall of 1965, the Endocrinology Branch was reorganized with somewhat less than half of its previous staff, laboratory space, and clinical facilities. This has necessitated some reductions in program. Several areas of the previous research program are being continued under other auspices and this report will summarize those projects only that have remained within the National Cancer Institute.

The appreciation of the complexity of androgen secretion and metabolism has led to the use of better mathematical models and systems for analysis. In conjunction with the development of new methods for the measurement of plasma androstenedione and dehydroepiandrosterone, it has become possible to describe completely the multiple origins and relative importance of each in the maintenance of plasma androgen levels. Such methods have been applied to the study of diseases involving the gonads and to the analysis of the metabolic activity of several steroid-producing cancers.

Further definition of the hormonal milieu, particularly with respect to such cancers as those of the breast and uterus, awaits the development of more sensitive methods for measuring plasma estrogens and their metabolites. We are attempting two entirely different approaches to this important problem.

Our group has maintained an interest in the endocrine effects and therapy of pituitary tumors. The direct assay of pituitary trophic hormones and better methods of assessing neuro-endocrine relationships have resulted in some new concepts of hypothalamic-pituitary controls. It has become clear that gonadotrophin activity is not necessarily the first function of the pituitary to be lost with increas-

ing destruction of the gland. Further, it has been shown that these tumors may cause significant inhibition of pituitary function by interference with hypothalamic regulation rather than encroachment on the gland itself.

Functional tumors of the endocrine glands have continued to interest our group. Pathways of biosynthesis in a luteoma were examined and the importance of the Δ^5-pathway for synthesis and the role of the sulfoconjugates were delineated. The hypoglycemia produced by a functional adrenocortical cancer and the characteristics of a gonadotropin producing carcinoma of the bronchus were explored.

Since endocrinology is concerned with the regulation of tissue growth and function, it is appropriate for the clinical endocrinologist to study regulatory mechanisms at the biochemical level. The initial studies have used a mammalian system, the chick oviduct, because a specific protein, avidin, is produced in response to a specific hormone, progesterone. Conditions for *in vitro* synthesis of avidin have been defined and methods for measuring small amounts of avidin have been devised.

Our group has been strengthened this year by a visiting scientist from Australia. In the coming year, in addition to our normal complement of clinical associates, we shall have one third-year clinical Associate and a visiting scientist from Kyoto. Dr. Chrambach, a protein chemist, has joined us recently and his skills should complement several ongoing programs. We look forward to an expansion of the effort of the Branch during the coming year with increasing emphasis on the exploration of the hormonal milieu in relation to normal and abnormal tissue growth.

IMMUNOLOGY BRANCH

Two significant advances during the year profoundly influenced investigations in the Immunology Branch.

Development of the C'1 Fixation and Transfer test by Drs. Borsos and Rapp in the Immunochemistry Section has played a major role in antibody-cell antigen studies. The C'1 Fixation and Transfer test has been applied in the Immunochemistry Section to theoretical and practical problems. It has made possible quantitative studies of cell antigens and of antibodies reacting with cell antigens, and has facilitated investigation of antibody: cell antigen reactions. One 18S IgM antibody molecule was shown to be sufficient to fix complement and initiate cell lysis, but two 7S IgG antibody molecules must react with relatively closely spaced antigens to achieve the same effect. IgM and IgG, but not IgA, antibodies were shown to fix complement when reacting with human red cells. Differences in human A1 and A2 erythrocyte antigens have been demonstrated and investigations of human leukocyte (?transplantation) antigens have begun. Viral tumor specific antigens were detected in studies of murine tumors induced by viruses. Applications of the C'1 fixation and transfer technic should prove very valuable in studies of transplantation antigens, "tumor" antigens and genetic (and environmental) regulation of cell membrane composition.

Immunoglobulin (gamma globulin) formation *in vitro* in continuous culture of established human cell lines was demonstrated for the first time. IgG (7S immunoglobulin) and IgM (18S macroglobulin) formation has been accomplished. These cultures will be useful for basic biologic and biochemical study, but they may be most useful if antibody activity can be achieved. Specific human antibody synthesis in continuously cultured cells is now a goal of the Immunology Branch.

The research activities of the Immunology Branch extended beyond the areas noted above. 29 publications for the four Senior Investigators are listed in the Annual Project Reports. Studies of cell, especially tumor, antigens have been extended. A program of transplantation studies was initiated. The mechanism of complement activation and cell damage by immune processes continue to be investigated.

Immunodiagnostic facilities for multiple myeloma, macroglobulinemia and immunoglobulin deficiency syndromes were established through a contract with Melpar. Currently about 40 serums are tested each week. Studies of immunoglobulin structure and function have been extended.

The Immunology Branch provides forums for continuous education and exchange of infor-

mation for NCI investigators through two regular weekly seminars—one on Molecular Immunology, and one on Cellular Immunology. Also, tutorial seminars in Immunology and Immunochemistry were conducted by NCI Immunology Branch staff in both Autumn and Spring terms.

METABOLISM BRANCH

Amino Acid Transport

In an attempt to characterize further amino acid transport mechanisms in fetal rat calvaria, the effect of puromycin dihydrochloride, an inhibitor of protein synthesis at the ribosomal level, was studied. Inhibition of protein synthesis, measured as glycine incorporation into T.C.A. precipitable protein was approximately 90% at 10 ug/ml of puromycin hydrochloride. The transport of alpha-amino isobutyric acid (AIB) was inhibited over a range of 10–600 ug/ml following a two-hour incubation in puromycin dihydrochloride. Ouabain and sodium cyanide have been shown previously to inhibit AIB uptake in fetal rat calvaria. If this inhibition is studied after varying incubation times in inhibitor followed by a standard 30 minute labeled amino acid uptake, there is initial inhibition of 35% for cyanide and 15% for ouabain when inhibitor and amino acid are added together. The rate of inhibition versus time slows on prolonged incubation. In contrast, when puromycin is used as inhibitor, there is small if any initial inhibition and the percent inhibition versus time forms a straight line on semi-log paper. A half-life for the transport process can be graphically determined at about 120 minutes. Active transport of lysine, a dibasic amino acid, was not inhibited by any concentration of puromycin dihydrochloride (10 ug/ml-600 ug/ml) and no inhibition could be seen over a 180-minute incubation period. Lysine uptake could be inhibited by cyanide in a manner analogous to that seen for AIB uptake. These findings suggest that puromycin can inhibit active transport of amino acids by inhibiting the synthesis of necessary proteins, and differences in the catabolism of these proteins allow for further characterization of transport systems.

Investigations have been carried out evaluating the relationship between amino acid transport and collagen synthesis. A steady state model has been utilized where fetal rat calvaria are incubated in 0.14mM Proline for three hours and then carrier free Proline-C^{14} is added for varying time intervals. It has been shown that AIB, hydroxy-proline and L-azetidine-2-carboxylic acid decrease the intracellular free amino acid proline pool, protein synthesis and collagen synthesis. In addition, lowering the proline concentration below 0.14mM results in decreased collagen synthesis. Dipeptides such as glycylproline, glycylhydroxy-proline, hydroxyprolylglycine, and prolylglycine do not inhibit amino acid uptake. This suggests that although hormones may regulate the general level of cellular metabolism, a secondary control of protein synthesis by the extracellular amino acid milieu is of importance. At present, efforts are underway to construct a multicompartmental mathematical model for collagen synthesis so that quantitation of effects of varying substances at specific steps in protein synthesis can be carried out.

Calcium Metabolism

A multicompartmental analysis for calcium kinetics has been developed. Metabolism balance data combined with Ca^{47} disappearance from blood, cumulative radioactivity in urine and feces, and surface counts from two sites are processed on an IBM 70904. By a least squares method of curve fitting, pool sizes and turnover rates may be calculated. Parameters which may be interpreted physiologically are bony accretion, bony resorption, urine and fecal turnover rates and gastrointestinal absorption.

During the past year, application of this program has concentrated on the mechanisms of calcium homeostasis. A series of 8 hypoparathyroid patients were studied. Four of these patients were studied twice; once as a control, the second time at a new steady state produced by injections of purified parathyroid hormone. In the hypoparathyroid patients,

there was found a decrease in miscible calcium pool and a decrease in resorption of bone, but bony accretion, urine and fecal turnover rates were not significantly different from normals. After being perturbed by parathyroid hormone, there were increases in bony resorption as well as in urine and fecal turnovers.

Other studies included three normal volunteers on low and high calcium intakes. While the gastrointestinal tract was adequate in controlling the amount absorbed from high Ca^{++} intake, a low (200 mgm/day) calcium diet produced a negative calcium balance with increased bony resorption. The urinary and endogenous fecal turnovers were only slightly decreased. No significant change was seen in bony accretion.

A patient with calcinosis universalis was also studied and found to have a large miscible calcium pool with slow exchange rates between compartments. The urinary and fecal turnovers were also small. Bony accretion and resorption were not significantly different from normal. These changes were correlated with histochemical and crystallographic studies.

The existing computer analysis is for a steady state system; a new computer program for analyzing the nonsteady state is being prepared. With this method, hormone dependent parameters for calcium control may be calculated. The feedback relationship between serum ionized calcium and parathyroid hormone production may be investigated in man.

Porphyrin Metabolism

δ-aminolevulinic acid synthetase (ALA synthetase) has been partially purified from mitochondria. Heme and other metallo-porphyrins have been shown to produce inhibition of this enzyme *in vitro* only at concentrations above $5x10^{-4}M$, casting some doubt on the significance of heme as a functional inhibitor *in vivo*.

The kinetics of induction of hepatic ALA synthetase have been studied alone with the effects of inhibitors of protein synthesis (puromycin, actinomycin D, 5-fluorouracil, p-fluorophenylalanine, etc.). Glucose blocks induction of this enzyme as effectively as actinomycin D. A mathematical model of enzyme synthesis has been developed which allows the calculation of the half life of the specific messenger RNA for a given enzyme. Using this model the half life of ALA synthetase is 67–74′ and that of its messenger RNA is approximately the same, i.e., 40–70′.

It has been shown that heme, the end product of the pathway which ALA synthetase controls, will prevent induction of ALA synthetase when injected intravenously at certain times before induction is initiated. This is the first direct evidence of heme as a repressor of ALA synthetase in mammalian tissues and supplements the indirect evidence from the tryptophane pyrrolase work reported last year.

When heme is injected intravenously, the level of hepatic ALA synthetase oscillates markedly for periods of 4 to 5 days after a single dose. The oscillations can be greatly amplified by administration of inducer 5 hours before sacrifice. This probably is the first demonstrated example of significant oscillations of the level of an enzyme. The only other example is reduced nicotinamide adenine dinucleotide oscillations in *in vitro* yeast extracts of glycolyzing systems. The oscillations of ALA synthetase in mammalian liver explain Watson's observations on variations in bilirubin excretion following heme administration and raise the question of the role of ALA synthetase in biologic clock mechanisms.

The fact that ALA dehydrase (the enzyme following ALA synthetase in the porphyrin pathway) also oscillates after heme administration is evidence of a genetic operon controlling the two enzymes. A mathematical analysis of the kinetic factors controlling the amplitude and periodicity of the oscillations of these enzymes is in progress.

The elevation of serum protein bound iodine in acute porphyria has been found to result from elevation of the serum level of thyroid binding globulin.

Glucose loading tests in patients with acute porphyria have shown frequent abnormalities in the form of a decreased rate of glucose disappearance from the blood and abnormally increased blood pyruvate levels.

A paradoxical response of blood levels of growth hormone has been demonstrated in

seven patients with acute porphyria after glucose loading. The level of growth hormone rises rather than decreasing as seen in normals. The syndrome of inappropriate ADH in acute porphyria has been demonstrated by direct assay of blood and urine for the hormone. These growth hormone and ADH abnormalities occur in most patients and indicate the frequency of abnormal hypothalamic physiology in acute porphyria.

The effects of diet on preventing the chemical abnormalities of acute porphyria previously demonstrated in the experimental animal have been extended to patients. A decreased caloric intake can initiate attacks of the disease and high carbohydrate diets can end these attacks.

A small fraction of women whose attacks of acute porphyria recurred cyclically with their menstrual periods have been maintained free of disease by use of oral anovulatory hormone preparations.

Nucleic Acid

To estimate the amount of virus DNA in cells, radioactive RNA complementary to this DNA was prepared *in vitro*. This RNA was then reacted with DNA prepared from cells infected with or transformed by DNA viruses and the amount of DNA–RNA hybrid formation was measured. DNA preparation from hamster cell lines transformed by SV40 virus had an increased amount of DNA complementary to SV40 RNA, if and only if "T" antigen was demonstrable by immunofluorescence. This is evidence for correlation between "T" antigen and persistence of viral genome. AGMK cells infected with either adeno-7 virus alone, or adeno-7 and SV40 virus produced rather similar amounts of virus DNA, although the titer of infection virus was 100–1000 higher in the infected cells. Thus, the "enhancement" by SV40 occurs at a level beyond DNA synthesis.

The same DNA–RNA hybridization-techniques have been applied to the taxonomy of mycoplasma and streptococci. By this technique, the mycoplasma are a very heterogeneous group of organisms. No relatedness was evident between *M. pneumoniae* and the other mycoplasmas tested. Other human mycoplasma fall into a number of species with only a low level of interspecific relatedness. Within some species, and of *M. hominis* I, there appeared to be much greater heterogeneity between different isolates. Other groups such as *M. pneumoniae* or the "FS" group were not detectably heterogeneous. Similar studies of the streptococci showed excellent correlation between nucleic acid homology and serologic typing. Different subgroups of streptococci were related and serotypes within a species were distinguishable.

Studies of adeno infected KB cells have demonstrated the appearance of an RNA component (VA–RNA) localized in the high speed supernate of the cell cytoplasma but distinct from transfer RNA. This has been partially characterized with regard to molecular properties, metabolic properties, and base sequence in oligonucleotide maps. Somewhat similar RNA components are extractable from ribosomes of uninfected cells. Preliminary studies by oligonucleotide analysis and nucleic acid hybridization techniques indicate these are not identical to VA–RNA.

Metabolism of Plasma Proteins

The known heterogeneity in the chemical nature of the antibody globulin molecules is paralleled by a markedly heterogeneous pattern of distribution synthesis and survival. In subjects without diseases affecting immunoglobulins, the survival half time of IgD was 2.8 days, of IgA 6.1 days, of IgM 5.1 days, and of IgG 24 days. Approximately 45 percent of the IgG and IgA molecules are distributed within the intravascular pool, while 70 to 80 percent of IgD and IgM molecules are within this pool. The catabolic pathway of each of these four major classes of immunoglobulins was shown to be completely independent of the other proteins and determined by the F_c segments of the immunoglobulin molecules, the part that does not contain the antibody-combining site.

The immunoglobulin levels were studied in the sera of 70 patients with thymomas. Nine such patients were shown to have marked reduction of IgG and IgA, with frequent recur-

rent infections. Seven of these nine subjects had a marked reduction in IgM level. The two remaining subjects had extreme elevations of IgM comparable to that seen in the macroglobulinemia of Waldenstrom. These disorders of IgG, IgM and IgA concentration were shown to be predominantly disorders of immunoglobulin synthesis. In all but one of the patients with a reduced IgG concentration the IgG survival was considerably prolonged. In the one remaining subject gastrointestinal protein loss was superimposed on the hypogammaglobulinemia. One patient with Sjogren's syndrome and lymphoma was shown to have a previously undescribed pathophysiological mechanism causing extreme reduction in the serum IgG concentration. This patient had a complex cryoglobulinemia that required the simultaneous presence both in IgM and IgG molecules. The patient's own 19 S macroglobulin was required for cryoprecipitation. The coprecipitant IgG required could be from any human source. Three factors contributed to the low estimate of the IgG levels. First, cryoprecipitation of serum IgG during quantitation of the levels *in vitro* at room temperature resulted in spuriously low estimates for the serum concentration. However, even when these studies were done under conditions that prevent cryoprecipitation, the patient still had an exceedingly low serum concentration of IgG. This was shown to be due to a combination of defective synthesis of IgG, and accelerated catabolism of IgG. The patient had a normal survival of albumin and normal IgM, and thus had an isolated hypercatabolism of one protein, IgG, presumably secondary to its formation of aggregates with the cryomacroglobulin *in vivo*.

Studies on the serum protein metabolism of patients with myotonia dystrophica were continued. Patients with this disorder were shown to have a reduced concentration and total body pool size of IgG secondary to a unique hereditary disorder of the immunoglobulin catabolism and isolated hypercatabolism of IgG with normal IgG synthesis. Both the patients' and normal IgG were catabolized at the accelerated rate in the patients while the patients' IgG was catabolized at a normal rate in normal subjects. All other proteins studied, including ceruloplasmin, albumin, IgA, IgD and IgM, had a normal survival in these subjects.

Studies on the protein metabolism of patients with ataxia telangiectasia were continued. These patients usually had a marked reduction or absence of serum IgA, but had a protein similar to IgA in the saliva. It was shown that the reduction in IgA seen in these patients was secondary to an extreme reduction synthesis of this protein, and in some cases, hypercatabolism of this protein. It was shown that there is normally little or no transport of IgA from the serum into the salivary secretion, but that the very high levels of IgA normally seen in excretory secretions is probably derived from specialized plasma cells in the mucosal and submucosal surfaces of the respiratory and gastrointestinal tract. The IgA is then coupled with a transfer piece in glandular structures and secreted as a first line of defense against infections entering the body. It should be noted that the patients with ataxia telangiectasia have a very high incidence of reticuloendothelial neoplasms.

Subjects with the Wiscott-Aldrich Syndrome, a disorder characterized by eczema, thrombocytopenia, a very high incidence of infections, and sex-linked recessive inheritance, have a markedly reduced level of IgM, a normal or increased level of IgG, and a markedly elevated serum concentration of IgA.

Patients with the syndrome of intestinal lymphangiectasia were shown to have a short survival of all proteins studied, including IgG, IgA, IgM, ceruloplasmin albumin and fibrinogen secondary to loss of these proteins into the gastrointestinal tract. The synthetic rate for each of the immunoglobulins was normal and the patients were able to produce antibodies to Vi and tularemia antigen. Marked lymphocytopenia was noted in these patients presumably due to loss of lymphocytes into the bowel secondary to the disorders of lymphatic channels. The patients with lymphopenia showed marked skin anergy to skin test antigen. Skin grafts from unrelated donors are surviving for over one year and second set grafts from the same donor are also intact at two months.

A pattern of low immunoglobulins and skin anergy was also demonstrated in subjects with other causes of gastrointestinal protein loss associated with disorders of intestinal lymphatics, including Whipple's disease, constrictive pericarditis, and regional enteritis. Subjects with extreme gastrointestinal protein loss, without disorders of gastrointestinal lymphatics, such as subjects with sprue, allergic gastroenteropathy, vascular diseases of the bowel, and ulcerative colitis, did not have lymphocytopenia or anergy. Gastrointestinal protein loss was demonstrated for the first time in subjects with chronic vascular disease of the bowel, with the blind loop syndrome and in a patient with generalized myopathy and congestive heart failure.

Studies on the role of the kidney in the metabolism of fractions of the immunoglobulins were extended. It was shown that the kidney plays a major role in the catabolism of human Bence-Jones proteins of either the lambda or kappa type, of normal human L chains, and of the L chain type of Bence-Jones protein formed secondary to multiple myeloma in mice. A number of other proteins that appear in the urine do not share this catabolic pathway. Specifically, the F_{ab} and F_c fragments of 7S gamma globulins, the Bence-Jones proteins of mice made up of both H chain and L chain, and those that are fragments of the H chain alone were not catabolized by uptake into the proximal convoluted tubule of the kidney. It is suggested that the H chain of immunoglobulin molecule protects the L chain from renal tubular metabolism.

Previously it has been shown that the fraction of the circulating IgG catabolized per day is dependent on the concentration of this protein, in contrast to the situation observed with IgA, IgM, ceruloplasmin and fibrinogen where the serum concentration of the protein does not affect the fractional catabolic rate. With IgG in man the fraction of the intravascular pool catabolized ranges from 2 percent in patients with agammaglobulinemia to 16 percent in patients with multiple myeloma and a high level of IgG. Similar hypercatabolism of IgG has been demonstrated in mice following the infusion of IgG from a number of species or the F_c fragments of the gamma globulin molecule. Similarly, the F_c fragments survival is affected by the concentration of the intact IgG molecule. It was shown that enzyme induction is not required for this hypercatabolism in response to infusion of the protein, since this phenomenon continues when the animals are given high doses of Actinomycin-D or cyclohexamide, materials that inhibit messenger RNA and protein synthesis, respectively. Mathematical analysis of these studies indicates that the best explanation for this phenomenon is the presence of a saturable protection system for IgG molecules. In contrast to the formulation of Brambell these studies indicate that the absolute amount of IgG protected, increases with increasing concentration of IgG, while the fraction of the total number of molecules protected decreases sharply. The reciprocal of the quantity of molecules protected plotted against the reciprocal of the total circulating pool size gives a straight line function. From this graph, the total number of protection sites and the affinity of the molecule for the protection sites can be defined. It is postulated that the same protection system specific for IgG responsible for the concentration effect is also responsible for the facilitated transport of IgG across the newborn gastrointestinal mucosa in animals and across the placenta in man.

Erythropoiesis

The studies of the relationship between metabolic rate and erythropoiesis in the hypothyroid dog have been completed. These studies show that in the dog made hypothyroid with large doses of iodine that there is a normochromic normocytic anemia characterized by a decreased rate of synthesis of red cells that this anemia could be repaired with thyroxin but not with dinitrophenol. It could not be repaired with iron, copper, cobalt, folic acid or vitamin B^{12}. Dinitrophenol raised the metabolic rate to normal or even greater than normal levels in both normal and hypothyroid dogs but neither in the hypothyroid dog nor in the normal dog did this result in a significant effect in erythropoiesis. This is then a demonstration of the uncoupling of erythro-

poiesis and metabolic rate. In addition, the hypothyroid dog develops a marked lipidemia. This is corrected on the administration of thyroxine and dinitrophenol. Thus the alterations in blood lipids that accompany the hypothyroid state can be repaired by elevation of the metabolic rate to the normal by both thyroxine and dinitrophenol.

The initial stages in the development of a multicompartment (nine-pool compartment) model of iron metabolism utilizing the computer facilities of the Office of Mathematical Research of NIAMD have been completed. It is anticipated that this will yield better information regarding the rate of formation of red cells than the simple mathematical models currently being used.

Studies of hemoglobin synthesis by reticulocytes from thalassemic patients demonstrated that overproduction of alpha chains occurs in thalassemia minor as well as thalassemia major. In contrast, there was slight or no detectable overproduction of alpha chains in hemoglobin Lepore trait. There was a reciprocal relationship between the specific activity rates of alpha to beta chain and that of alpha to delta chains, suggesting that, as the chain production was depressed, there was a stimulation of delta chain production.

Bilirubin Metabolism

The bilirubin clearance method has been developed and sufficient data has been acquired. The data shows that the rate of disappearance of bilirubin from the plasma in the sixteen patients studied can be described in terms of two rate components; the first having a half-time in the range of 20–30 minutes and the second a range from 100–200 minutes. In general, the rapid component is associated with the bulk of the removal. A three compartment model has been developed to quantitate the bilirubin production. This has yielded values ranging from 139 to 1530 mg per day. The principal abnormality seen in these two rate components has been in two patients with Gilbert's disease where the second component was somewhat longer but, more particularly, it was a more significant fraction of the total turnover. With this model it is now thought possible to measure bilirubin production.

Other studies have shown that in two patients with a thoracic duct fistula that there was a small transfer to the order of one-half a percent of the bilirubin from plasma through hepatic lymph to the thoracic duct lymph. This did not appear to be a significant pathway anatomically for bilirubin.

In the turkey, an animal with a nucleated red cell, there is a non-red cell source of bile pigment that is significant. The data have not yet been fully analyzed for its quantitative significance.

Other studies of bilirubin physiology have been concerned with the development of an assay for the measurement of hepatic glucuronyl transferase using bilirubin in physiological quantities as a substrate. The male rat shows higher values than the female rat. The assay has been also carried out in six patients with clinical evidence of inability to conjugate bilirubin and the amount of enzyme has shown to be decreased. The significance of this method lies in the hypothesis that bilirubin glucuronyl transferase may be a different enzyme than other glucuronyl transferases. In the past measurements have used other substrate materials to determine the amount of glucuronyl transferase. The data may not be directly extrapolatable to the situation with bilirubin if another substrate is used.

A series of biochemical studies have been undertaken to explain the mechanism of bilirubin toxicity. The principal accomplishments have been a demonstration that unconjugated free bilirubin uncouples oxidative phosphorylation. When unconjugated bilirubin is complexed with albumin this biochemical lesion is repaired in the liver and kidney but not in the brain. This is a possible explanation for the development of neurological lesions in kernicterus.

The metabolism of bilirubin was studied in the kernicteric Gunn rat. This rat has an inherited lack of hepatic glucuronyl transferase. This is manifest by a marked hyperbilirubinemia and in some animals by the neurological signs of kernicterus. On the kernicteric animal, bilirubin-C^{14} could be demonstrated in the

brain after intraperitoneal administration, while in equally jaundiced, but not neurologically involved animals, bilirubin-C^{14} could not be found in the brain. This animal and bilirubin-C^{14} made it possible to study the problems of bilirubin and kernicterus.

SURGERY BRANCH

The Surgery Branch of the National Cancer Institute continues to act in a dual capacity carrying out its own specific clinical and laboratory investigations and providing consultative surgical service to other branches of the National Cancer Institute and the clinical units of each of the other institutes of the National Institutes of Health. Because of the broad scope of investigative clinical studies being carried out by the various institutes, this service has included urological, otolaryngological, and gynecological as well as thoracic and general surgical consultations.

During the previous twelve months, 1,240 surgical consultation requests were received and answered by the Surgery Branch Staff. Four hundred and twenty-nine of these were from the National Cancer Institute, while 811 were from clinical units of other institutes. These numbers were original requests and do not take into account the minimal five-fold increase that would exist if the repeated visits to each patient were recorded. Three hundred and eleven operative procedures were performed as a result of these consultations, 112 were from the medical units of the Cancer Institute and 199 were performed on other institute patients. Forty-two percent of these procedures were major surgical endeavors. Consultants from the community saw 286 patients, 268 of whom were non-malignant otolaryngeal problems. These consultants performed or assisted in only 12 surgical procedures.

A total of 705 surgical operations were performed by the staff; 345 were major procedures and 360 were minor procedures. Surgery Branch admissions resulted in 394 surgical procedures, of which 63% were major operations.

The clinical interests of the Surgery Branch continue to be varied, and this is in part dictated by the type of patient referred. Since disease which can be controlled by standard surgical procedures is being handled in the community, 85% of the admissions were patients with advanced but regionally localized disease not suitable for the usual surgical therapy. Sixty-eight percent of the admissions had received prior radiation, surgery or chemotherapy and were treatment failures.

Malignant disease of the head and neck continues to be a prime interest. In past years head and neck cancer has constituted approximately one third of the Surgery Branch patient care load. However, it has been gradually increasing and during this past year has accounted for 52% of the patient admissions. This increase might be in part due to the increased awareness of the dentist to malignant oral-pharyngeal disease and the apparent increased prevalence in heavy smokers, consumers of alcohol and "snuff dippers". During the past year, 15 patients have been direct referrals from the National Institute of Dental Research. The study of the effectiveness of preoperative radiotherapy for oral, pharyngeal and laryngeal cancer continues. Radiotherapy is given 24 hours preoperatively on a random basis according to the double blind technique. The number of patients is low because of the limitation requiring previously untreated patients. The 1000 roentgens given preoperatively is based upon several of our laboratory studies which showed that metastases could be decreased significantly by this treatment in animal tumor systems by this therapy. Clinical experience to date indicates that 1000 roentgens in a single dose preoperatively is the maximum dosage which can be used and not have significant wound morbidity.

Survival by conventional means of therapy from paranasal sinus cancer is very poor because of frequent tumor extension into the ethmoid, sphenoid and pterygoid areas, resulting in local recurrence in spite of the usual surgical or radiological techniques of treatment. Thirty-one patients have had a combined intracranial transfacial en bloc resection of the paranasal sinus area. Resection includes the cribriform plate and medial orbital walls and usually leaves the orbital contents intact. Operative mortality has been 7%. Follow-up

for from 4 months to 9 years indicates that 19 patients have remained disease free.

The morbidity often associated with extensive head and neck surgery has become less of a problem. The National Institute of Dental Research continues to furnish these patients with meticulous day by day refitting of well constructed prosthetic appliances. Through their cooperative efforts, we have been able to avoid the prolonged and repeated hospitalizations so often associated with plastic reconstruction. In so doing, the operative defect may be carefully followed for local recurrence rather than have it hidden by an overlying skin pedicle. Morbidity has been further decreased by the judicious use of antibiotics, cervical esophagostomy feeding intubation and better placed skin incisions. Even though radiation recurrent lesions constitute 71% of the head and neck material and surgery is in no sense less radical, there has not been a carotid hemorrhage in 28 months.

We have been exploring the feasibility of full dose preoperative radiotherapy followed 6 weeks later with definitive surgery. Patients thus far chosen for this pilot venture have presented large localized, malignant lesions and have been estimated to have an expected 5-year survival of less than 3 to 5%.

Referred patients with carcinoma of the cervix continue to have had previous definitive surgery, supracervical hysterectomy, irradiation and histories of never having had a Papanicolaou smear. Our patients with advanced disease treated with extensive surgery show an operative mortality of less than 10% and an overall five-year survival of 43%. The patients who underwent radical hysterectomy have a 70% survival, anterior pelvic exenteration, 34% and total pelvic exenteration, 24%. With the development of better techniques for patient selection the survival rate following radical surgery continues to improve. Unilateral leg edema, sciatic distribution of pain, bilateral renal involvement, lymphangiography, renal and surgical physiological studies are all clinical and diagnostic aids useful in determining feasibility of surgery. For selected advanced tumors of the pelvic organs, vagina, cervix, uterus bladder and rectum, surgery continues to offer the best chance of cure, but this is not done without, at times, considerable morbidity. Improved techniques of blood volume determination, red cell survival studies, bowel sterilization isotope renography and vital sign monitoring have provided an increased understanding of postoperative physiology and have permitted more knowledgeable postoperative care. All have combined to make radical surgery a practical means of bringing advanced pelvic disease under control.

Experience with over 500 distal extremity lymphangiograms, indicate that this technique of demonstrating lymph nodes is an interesting research tool, but is of little real diagnostic significance in the surgical patient. Its value as seen by this surgical group is first, to draw the surgeon's attention to an area suspicious of lymph node involvement with tumor, and second to allow a more complete lymph node dissection for the surgeon infrequently involved with radical lymph node resection. The false positive and false negative interpretations greatly outnumber the incidences of accurate interpretations. In many patients this may be due to altered lymphatic drainage secondary to previous radiotherapy.

The ileal conduit continues to be the most satisfactory means of urinary diversion following total pelvic exenteration. This technique results in few complications and preserves excellent renal function in nearly all instances. However, attention must be directed toward continual antibacterial prophylaxis in order to prevent infectious complications. Of interest has been 13 patients who have undergone urinary diversion in spite of preoperative and operative evidence of unilateral ureteral occlusion. High transection and urinary diversion into an ileal conduit has resulted in all but one of the kidneys regaining function. In half the patients very satisfactory function returned, in the others improvement was noted but a variable degree of impairment remained.

Radical cancer surgery is by its very nature extensive surgery and carries a relatively high mortality and morbidity. However, our experience with over 100 radical hysterectomies, 50 anterior exenterations, 250 total pelvic exenterations and 4 combined amputative proce-

dures (2 of which are alive over 2 years) suggest that within the proper environment, this procedure should be offered to more patients with advanced pelvic disease. With intensive colostomy and urinary ileostomy instruction and guidance and vaginal reconstruction in selected instances, these patients who have had radical pelvic surgery can be restored to essentially a normal, pleasant and productive life.

An increasing number of extremity sarcomas, most of which are recurrent following previous treatment are being seen by the Surgery Branch. Selected instances of amputation and particularly hemipelvectomy which allows removal of all extremity muscular and tendonous insertions and origins have been performed with no mortality, a high degree of physical rehabilitation and an increased 3-year survival free of disease.

A small, but significant number of patients are referred each year with malignant melanoma. We are reviewing the perfusion studies and the surgical approach practiced in other centers. Our practice of wide local excision and lymph node dissection continues to be the treatment of choice with selected patients being referred for perfusion and surgery. When we have in, 41 patients, a 5-year survival of 16 patients and a 10-year survival of only 6 patients, there is indication for incorporating well protocolled studies into the regime of this disease. With this consideration in mind, the Surgery Branch has developed a chemotherapy protocol in animals with S–91 melanoma. The limiting effects of methotrexate therapy are bone marrow depression and gastro-intestinal toxicity. Primate studies have been conducted to determine whether small amounts of citrovorum factor given by hypogastric arterial infusion will selectively protect the pelvic bone marrow against the effects of intravenously administered methotrexate and yet not generally circulated to interfere with the systemic anti-tumor effects. In addition, attempts to modify the gastro-intestinal tract toxicity by administration of non-abosrbabe intestinal antibiotics have been undertaken. At this time it appears that both of these premises are valid and that with their implementation much larger doses of methotrexate can be safely given.

A long-term survey of infectious complications following cancer surgery shows that the patient who carries a pathogenic organism with him into the operating room, whether it be from the skin, nose, or throat or parcularly within the tumor, runs a significantly increased risk of developing an infectious complication postoperatively. Staphylococca infections predominate, but are more easily controlled and cause less patient morbidity than other organisms. Antibiotic therapy remains routine with all patients undergoing cancer surgery, chloramphenicol being the most satisfactory and frequently used drug. This drug, has been extensively used for 6 years by the Surgery Branch without evidence of hematological complications. Using high dosage for a limited ten day interval which includes the preoperative, operative, and postoperative periods, infectious complications have decreased during the past year to 7% as compared to 10–15% without preoperative coverage and 22–30% without any antibiotic therapy. During 1965 staphylococcal infections accounted for only 36% of our infections and there were no deaths due to this organism. Staphylococcal infection certainly increases morbidity, but it is the pseudomonas, E. coli, candida albicans and proteus organisms which have consistently been associated with the most resistant infections and the septic deaths.

During the past year a fruitful cooperative clinical study has been carried on in association with the Experimental Therapeutics Branch, NHI. Eight patients with pheochromocytoma have undergone surgery following biochemical evaluation. From the care of this group of patients has emerged a program for relatively simple yet quite effective management of a clinical problem previously considered formidable.

Serial measurement of red cell volume, plasma volume and extracellular fluid volume has demonstrated that (1) following extensive surgery there is no evidence for a slowly circulating or non-circulating portion of the red cell volume; (2) post-operatively after extensive surgery there is a disproportionate plas-

ma volume deficit present which has heretofore been unrecognized and is as yet unexplained; (3) post-operatively there is a deficit in the extravascular extracellular fluid volume suggesting the need for additional amounts of fluid at surgery.

This laboratory has not been satisfied with the usual bio-assay methods of detecting anti-diuretic hormone and in the past 18 months has developed an immuno-assay technique capable of measuring one hundred micromicrograms of anti-diuretic hormone per ml. of solution. Continued improvement in the sensitivity and precision of the technique is anticipated and then comprehensive physiological studies will be undertaken.

During the past year, little attention has been directed toward further refinements in techniques of isolating tumor cells from the blood of cancer patients. It remains apparent that if enough blood is sampled from the cancer-bearing patient, tumor cells will eventually be isolated. Surprisingly enough little correlation has been noted between patient survival and the presence or absence of circulating tumor cells. Tumor cell isolation and identification from wound washings and drainages has similarly been unrewarding in most respects. Possibly due to better techniques of filtering and certainly due to better criteria of identification the incidence of tumor cells has decreased to such a low percentage as compared to previous years that validity of the overall study is questioned. A review of wound drainage studies and survival in 170 patients shows no correlation as to the instance of local recurrence. This study has been unrewarding and suggests that an approach to local tumor recurrence through topical agents may not be successful no matter what agent is used.

The cooperative, prospective study of primary therapy of endometrial carcinoma should provide an answer to an old problem. The different types of therapy under study are surgery alone, preoperative radiation and surgery, and surgery and postoperative radiation. The long delay in starting this project continues, but patients will be assigned to therapy at random. Utilizing the Surgery Branch, as the Study Center, data from cooperating institutions will be collected and analyzed. The study is planned to include 400 patients. Ultimate analysis of the data after the completion of the five year follow-up will provide meaningful information concerning the relative effectiveness of the methods of therapy under study.

A technique for transplanting endocrine glands in isologous animal systems has been used to study not only a state of endocrine hyperfunction but also the ability to regulate the animals endocrine activities through hormones produced by these organs. With these methods a physiological model has been used to study the inter-relationships between the parathyroid and thyroid glands in calcium homeostasis. Using hypercalcemic challenged, inbred rats and combining thyro-parathyroidectomy and parathyroid gland transplantation at a remote site, further evidence has accumulated on the physiological role of a calcium lowering hormone. Preliminary evidence of a thyrocalcitonin-releasing factor from the parathyroid gland has also been demonstrated. In a similar experiment the function of the pineal gland and its relation to the menstrual cycle in normal females was shown. Additional studies using sympathetic cervical ganglia have demonstrated the histologic and biochemical survival of these tissues. A model was thus found which facilitates the study of the effects of pharmacological agents such as reserpine and tyramine on the release of norepinephrine. Observations on the effect of the VX_2 carcinoma in rabbits and on its ability to produce hypercalcemia are being made in an attempt to determine if the calcium imbalance is due to an excess of parathyroid hormone which may be produced by this tumor.

Experiments are being carried out to evaluate the comparative abilities of oncogenic DNA viruses to induce neoplastic transformation in newborn hamster endocrine tissues *in vitro*. Transformation of pituitary, pineal thyroid, parathyroid, adrenal, testicle, and ovary tissue in explant cultures to malignant cells by Simina virus 40 (SV40) and LEE 46 strain of adenovirus. These tumors are being evaluated for evidence of endocrine function and studies are being conducted for either a bio-assay or

direct chemical assay of the tumors for specific hormones.

A biological system for studying tumor enhancement with a specific fraction of the isologous immuno-globulins has been established. This consists of producing a potent antiserum in DBA strain of mice by repeated inoculations by EL4 ascites tumor from C57/BL mice. The antiserum is then fractionated by column chromatography and electrophoresis to see which of the specific immuno-globulins is responsible for the enhancement phenomenon of these tumors across the H2 histocompatibility line.

Further studies have been carried out in solid tumor patients subjected to thoracic duct lymph drainage to ascertain whether cellular or humoral factors in human lymph are responsible for suppression of the immune response. This has been done by taking the lymph from patients and subjecting it to sterile centrifugation to separate the lymphocytes from the protein fractions of lymph and then the protein fractions have been returned to the patients. The immune response was studied in these patients by small skin homografts and their ability to respond to a primary antigen. It has been shown that under these conditions the immune response is dependent more on the amount of protein and the depletion of immunoglobins than it is the loss of small lymphocytes in humans.

Through collaborative studies with the Army Missile Command and Eastman Kodak the role of laser energy as an oncolytic tool continues to be intensively investigated. Pulsed energy from both ruby and neodymium sources and continuous wavelength studies from both Argon and CO_2 sources show tumoricidal activity in rodent, dog and primate liver. The use of the continuous wavelength laser as a "light knife" has been investigated. We are pessimistic about its ultimate clinical use either with skin or soft tissue. It is true that relatively bloodless hemihepatectomines can be safely performed with the CW laser and a massive amount of hepatic tissue may be destroyed with the pulsed laser without significant toxic effects to the host, but practicability is doubtful at the present stage of laser "refinement." At 1000 joule, semi-portable laser is being used daily in our laboratory and a 4 module, 1000 joule, neodymium unit will soon be installed. It is fair to say that we continue to look upon laser energy as the next innovation in cancer cure with "suppressed enthusiasm".

Further studies in renal physiology have been performed which seek causes of renal vascular hypertension and better means of diagnosis and surgical management. Further studies of urinary oxygen tension (UpO_2) in dogs have been carried out before and had inversely related UpO_2 to medullary sodium reabsorption under profound osmotic diuresis. Reduction of glomerular filtration rate by clamping the reneal artery has a variable effect on UpO_2 suggesting the medullary blood flow (MBF) has a significant effect on UpO_2 under these conditions. Under the conditions of these studies, however, medullary sodium transport appears to be the determinant of UpO_2. Until an accurate method of measuring MBF is developed, this variable will remain uncontrolled and unknown.

An attempt to validate ^{133}Xenon renal clearances as indicator of medullary blood flow is in progress. The medullary component of renal blood flow has been derived from clearance curves in a consistent manner and agree substantially with data reported by others using autoradiographic techniques. Further study is indicated before accuracy can be determined.

To test the hypothesis that angiotension is inactivated by the liver, splenorenal venous shunt was performed in dogs made hypertensive by clamping the renal artery. None of the animals sustained relief of hypertension after shunting. Clinical trial of this procedure in hypertensive patients is not warranted.

Epinephrine infusion into the renal artery to create temporary ischemia has been performed in one patient and several dogs. Anoxic tissue is known to be less sensitive to radiation effects. Marked deminution of renal blood flow during infusion of minute amounts of epinephrine occurred. We hope to show that there is protection against radiation damage to the body and thus allow larger doses of radiotherapy to be given to abdominal neoplasms.

Clinical studies in renovascular hypertension have included differential studies of kidney function and UpO_2 radioisotope renography and phonocatheter recording of renal artery pulse. Aortogram and differential function remain the most reliable methods of diagnosing surgically curable renovascular disease.

The value of phonocatheter and UpO_2 studies await larger clinical experience. The isotope renogram is useful in following ureteral obstruction and in management of patients following renal artery surgery, but is too nonspecific to be the sole screening test for renal artery stenosis. The renogram has also been helpful in managing cancer patients after pelvic exenteration procedures requiring ileal conduit diversion. It is a more accurate test of renal function than is the excretory urograms and can be used in patients allergic to urographic contrast medium.

Collaborative studies of patients with cystinuria have demonstrated the value of d-penicillamine in preventing recurrence of cystine calculi. Studies of the mechanism of increased cystein excretion by kidney biopsy and *in vitro* amino acid transport and renal arterial-venous amino acid differences suggest that increased turnover of cystine, lysine and arginine within renal parenchyma may be the basic defect in this disease.

Urinary lactic dehydrogenase activity was studied in hamsters during induction of renal adenocarcinomas. In none of the experimental or control animals has total LDH excretion risen above upper limits of normal, even when both kidneys were almost entirely replaced by tumor. This study confirms previous reports from this department that urinary LDH is not a useful screening test for urinary tract neoplasms.

The effect of gonadotropins, testosterone and estradiol on surgically unilateral cryptorchid rats has been studied. Gonadotropins and testosterone in moderately large dosage for 3 weeks did not appear to have had deleterious effects as judged by bilateral testis biopsy six weeks after the cryptorchid testis was returned to the scrotum.

Intestinal perfusion may still be a method that permits repeated dialysis of uremic subjects over a long period. Emphasis is being placed on definition of the variables of the procedure, including serum and perfusate electrolyte concentrations, serum and perfusate osmolality, rates of intestinal exchange and transfer of electrolytes and other crystalloids and such factors as the flow rate and temperature of the perfusate solution.

Demonstration of the physiologic events in surgically de-vascularized and re-vascularized ureters is in progress. Omental sleeves may prevent the high incidence of uretero-vaginal fistulae in women subjected to radical histerectomy for carcinoma of the cervix.

The diagnostic significance of tetracycline fluorescence in early neoplasms accesible to ultraviolet endoscopy is controversial. It may be that tetracycline fluoresces only when chelated to calcium in tissue fluid or within macrophages. This problem is currently being studied in New Zealand white rabbits with VX_2 carcinomas. The state of calcium metabolism is being varied with parathormone and with parathyroidectomy.

Production of experimental and transitional cell carcinomas of the bladder with aniline dye products is well established. The similarity of these papillary carcinomas to tumors of viral etiology is striking, yet, no experimental studies are on record to assess the role of viral "carcinogenesis" in papillary carcinoma of the bladder. Such a study is being conducted on neonatal hamster bladders and in tissue culture employing polyoma, adenovirus 7 and 12, SV–40 and E46–M viruses.

Serum leucine aminopeptidase values are being studied in patients with normal pregnancy, multiple gestations and patients with trophoblastic neoplasms. This is a follow-up of earlier work which suggested that the rise of values of this enzyme which takes place in normal pregnancy may be higher and/or earlier in twin pregnancies. Since it does not rise in patients with hydatidiform mole, further studies on its usefulness in making this differential diagnosis are in order. The report that the increment in leucine aminopeptidase activity in the serum of women during pregnancy is sensitive to inhibition with methionine is to be evaluated.

A study of the immunologic relationship between women with pregnancy or a trophoblastic tumor and their husbands cellular antigens has been carried out using the technique of lymphocyte transformation in mixed leukocyte cultures. Preliminary results show that lymphocytes from normally pregnant women do not transform when grown with their husband's leukocytes in as high a percentage as they do when grown with cells from an unrelated male. This effect is not found in women with trophoblastic tumors. Further studies are being carried out to evaluate serial changes in each trimester of pregnancy and the changes that take place during and after chemotherapy of the trophoblastic neoplasms. The latter are being correlated with the response of the patients to chemotherapy.

In order to evaluate the possibility of damage to the ovum in a resting Graafian follicle by tritiated thymidine given to study bone marrow cellular kinetics, radioautographs of the ovaries of previously treated Rhesus monkeys were made and evaluated for evidence of tritium concentration in the ova or closely adjacent granulosa cells. These studies are in their initial phase.

Immune suppression and its effect on homograft rejection and the growth pattern of transplanted human choriocarcinoma are being studied in hamsters, mice and monkeys. Techniques used in the hamster to obtain this effect are the administration of drugs (Methotrexate, Methotrexate and Citrovorum Factor, and Crotisone), a total body irradiation plus Cortisone and anti-thymocyte serum. In mice the effects of thymectomy alone and thymectomy followed by a course of anti-lymphocyte sera are being evaluated. Studies outlined in monkeys call for evaluating the effects of thymectomy alone, thymectomy plus anti-lymphocyte sera treatment and also anti-thymocyte sera treatment alone.

The evaluation and improvement of diagnostic and operative culdoscopy has continued. The comparison of culdoscopy and X-ray gynecography has resulted in the realization that either technique can provide satisfactory information depending upon the availability of the techniques and the ability of the operators. The development of the intraperineal probe has converted the previously static, diagnostic culdoscopic examination to a dynamic one permitting the operator to palpate and manipulate the visualized structures. The development of a simple and easy technique for positioning makes the procedure significantly shorter and easier on both the patient and the operating room personnel. The technique of ovarian biopsy performed under culdoscopic examination has been developed and perfected and provides a safe technique for obtaining ovarian tissue with entirely acceptable morbidity.

The increased incidence of cystic ovarian enlargement and multiple pregnancy associated with ovulation induction, using human menopausal gonadotropin (HMG or Pergonal) and human chorionic gonadotropin (HCG) suggest overdosage. To test this impression and to select the minimal ovulatory dose gradually increasing amount of HMG has been given to selected anovulatory or oligo-ovulatory patients and the dose response relationship noted. Results indicate a sigmoid dose response relationship with a greater response being associated with larger doses. Minimal ovulatory doses have ranged from 450 to 1725 international units. Grouping of patients by diagnosis has resulted in a marked grouping of the minimal ovulatory doses. It would appear that continuation of the study would supply information which will permit Pergonal dosage to be selected for the individual patient with a markedly lessened chance of unwanted side effects.

Ancillary studies which are underway as a part of the general Pergonal study include the measurement of human chorion gonadotropin in patients with Pergonal induced pregnancies, the early detection of plasma chorionic gonadotropin in the first two weeks of gestation prior to the first missed menstrual period, determination of anti-Pergonal antibody in patients treated with Pergonal and the determination of the effects of Pergonal on plasma testosterone and androstenedione levels.

The effects of pharmocologic doses of estrogen on the reticuloendothelial system clearance rate of ^{131}I tagged aggregated human serum albumin and on the febrile response to endotoxin and etiochloanolone is being studied in

an attempt to elucidate the effects of this hormone on these physiolgic parameters.

Ethiodol is a commonly used lymphangiographic radio-opaque medium. Preliminary carcinogenesis studies in mice suggested that this agent might have activity which would discourage its further use. These animal studies made use of Ethiodol which was injected into three different sites. Subsequent animal investigations compared Ethiodol with the poppy seed oil component of Ethiodol. The completed data on these two oils shows that tumors appeared in mice at irregular intervals in all three injection sites. The response suggests that there may be some carcinogenic activity at the dosage level administered. Data on the pathological findings is now being assembled for statistical review.

In the study of tumor metastases an accurate and reproducible method of identifying small tumor foci is necessary. Frequently the color of normal animal lung parenchyma and small experimental tumor implants is similar and it is difficult, if not impossible, to differentiate grossly between the two. Microscopic identification of tumor implants is often not practical because serial sections are expensive and the review is time-consuming. The accurate counting and sizing of small, lightly pigmented tumor metastases in the lungs of experimental mice have been facilitated by the development of a method whereby the lungs were insufflated with a 15% solution of India ink. After being washed with Feketes solution, which is a bleach preservative, these tiny implants, not detected in the unstained fresh lung, became discretely visible. The accuracy and time-saving features of this method aid in the verification of lung metastases and permit a much wider range of spontaneous and induced animal tumors in studies of experimental tumor metastases.

A comparison study of survival, tumor growth, and metastases with transplanted, induced and spontaneous tumors in mice has shown that metastases observed in the three separate tumor systems were similar, in both amputated and intact tumor-bearing mice even though the growth rates of the tumor systems varied markedly. The similarity in the number of metastases observed is believed to be due to survival which was obtained by individual animal housing. On the basis of this study, it appears that the three tumor systems evaluated might be interchangeably used in metastases studies if recognition is given to their varied growth rates.

The long term program on tumor metastases is in a transition period of orientation to the immunological approach to the understanding of local recurrence and metastases. Studies have been performed to investigate the nature of the immunity imparted by certain chemically-induced tumors upon their hosts. Particular emphasis has been focused on whether chemically induced tumor immunity exists in the autochthonous host, and whether the specific immunity that we see in later transplant generations is due to antigenic alteration of the tumor in serial passages. The cellular localization of this antigen seems to be in the nuclear fraction of the tumor tissue. With weakly antigenic tumors, i.e., S–91 melanoma, prior immunication with cell-free tumor extracts had no significant effect on tumor growth. Enhancement occurred when this extract was given at the time of tumor inoculation.

Previously used methods for the study of tumor specific immunity have been extended to two new and unique tumor systems. A chemcally-induced gastric adenocarcinoma has been found to be immunogenic, demonstrating an immunity which lasts at best, ten months, can be adoptively transferred by isologous lymphoid cells may be abrogated by splenectomy or large doses of tumor cells, and requires viable lymphoid cells for its effectiveness. Attempts to utilize immune lymphoid cells to alter the growth of established tumor have been only partially successful in that growth has been slowed but complete regression has not been achieved.

Similar immunologic phenomena have been demonstrated in another unusual system, a spontaneous fibrosarcoma. Immunotherapeutic experiments with this tumor of recent origin are in progress.

Efforts to establish a number of new lines of visceral tumors in inbred rat strains are

reaching fruition. Freely transplantable yet isologous tumors of visceral organs have not been studied in inbred rats, whose size is more appropriate to the evaluation of experimental operative surgery models than is the mouse.

CHEMOTHERAPY

The recent reorganization of the Chemotherapy Program has had a markedly unifying effect. The program plan (1), developed in last year's review of 1955–1966 experience, was approved by the National Institutes of Health and the National Advisory Cancer Council, and in September 1965 the new organization took form.

The research strategy of the chemotherapy program is built around two objectives—the use of drugs to achieve selective killing of tumor cells and the search for new anti-tumor agents.

Research on selective toxicity of drugs for the tumor cell advanced sharply during the past year. They key to this advance has been the development by Skipper of an animal model in which it is possible to estimate the number of tumor cells killed by drug and the number surviving. Drugs can be studied in the model to discover the optimal schedule for maximum tumor cell kill ("schedule sensitivity"). Bruce and his colleagues have studied the mitotic cycles of normal and of tumor cells in mice, and discovered differences that permit drug administration to kill large numbers of tumor cells with slight effect on normal cells ("cycle sensitivity"). Merkle and colleagues have developed a mathematical model that uses cycle and schedule sensitivity data in predicting curative drug regimens for a variety of human neoplasms. The application of these new concepts to patients depends on ability to measure generation times of tumor cells. Preliminary analysis of those malignant diseases where drugs seemingly are curative, suggests that the biological and mathematical models are predictive. The strategic objective of highest priority at present is the study of selective toxicity of drugs in patients.

Two correlative approaches support this objective. One of these is a broad expansion of pharmacological studies of the known anti-tumor drugs. It is essential to know both the concentration of active drug at the target site and the time required at target for maximum tumor cell kill and minimal host toxicity. These studies are underway in both the Skipper model and in patients. Secondly, selective toxicity can be enhanced by measures that protect the host against the toxic effects of drugs. The most effective of these are platelet replacement and protection against the infections arising from drug induced host depletion. Research and development in these areas and in national resources have been sharply expanded.

The second major objective is the discovery of new chemicals with anti-tumor activity. The method of search, now in its eleventh year at NCI has been thoroughly reviewed and substantial changes invoked. There are over 30 drugs (counting analogues) with substantial effects in clinical cancers. These are active in 100 or more animal tumors. Examination of the screening data of 10 years shows that all but 2 of these drugs are selected by two animal tumors, L1210 leukemia in the mouse and Walker 256 carcinosarcoma implanted intramuscularly in the rat. The conditions of these two animal tests can be adjusted to that few if any false positives will be selected. The primary screen for the search has been redesigned and at present is under a dry run with all known clinically active and a number of inactive drugs. The new screen permits the dropping of a number of less satisfactory animal tumors and will provide both drug schedule information and dose response data. The amounts of drug required are about one-fifth those needed in the old screen so that much of tissue culture screening (used for the frequently inadequate amounts of sample) can be dropped. While the new primary screen ensures that it can pick up signals of anti-tumor activity of a wide variety of synthetic and natural products, exploration must go forward on screens of other types. Until clinical activity is shown for drugs that are inactive in the primary screen, this question cannot be settled. Drugs highly active in other animal tumors but inactive in the primary screen are being sought for introduction into clinical trial.

The extent of the search for new agents has been under active debate. As long as the search is empirical, it would seem wise to allow considerable latitude. Since there are now a number of effective synthetic agents, there is an opportunity to begin a study of structure activity relationships. From this study it may be possible to concentrate synthesis efforts in the most rewarding areas. There have already been sharp reductions in studies of alkylating agents and anti-purines. At the same time, if the difficulties of synthesis can be overcome, more needs to be done with the anti-folics and nucleosides. In the natural product areas there are a number of materials mostly of high molecular weight highly active in the screen. The large size of these agents poses difficult problems of purification and none of these materials has reached clinical trial. Since the known clinically active drugs are of relatively small molecular weight, perhaps these large molecules from plants may open a new era in cancer chemotherapy.

The new research on selective toxicity suggests the working hypothesis that large intermittent pulses of therapy are more damaging to tumor cells than to normal cells. If one examines the best clinical results obtained with drugs, it is noted that these have in large part occurred with pulsed regimens. Remissions approaching cure have been seen in choriocarcinoma, acute lymphocytic leukemia, Burkitt's lymphoma, childhood solid tumors and testicular tumors. The drugs responsible include 2 anti-metabolies (methotrexate and 6 mercaptopurine), an alkylating agent (cyclophosphamide), a bacterial fermentation product (actinomycin D), 2 plant products (vinca alkaloids), and an animal hormone (corticosteroids). Cure is a word to be used with care in connection with disease, since in its strictest sense, it means that a large sample of treated patients subsequently die off at the same rate, appropriately corrected for age and sex, as the general population. While too little time has elapsed since the recognition of selective toxicity of drugs for tumors, to make a judgment about cure, 5 year complete remissions are sufficiently common to generate a strong suspicion about the ultimate conclusion. A number of other tumors are quite sensitive to these and other drugs. The present tactical problem is to apply the concepts of selective toxicity in each instance.

In addition the search for new agents gives evidence of new weapons. Streptonigrin, mithramycin, daunomycin, dibromomannitol and cytosine arabinoside have recently been found clinically active. All of these are strongly positive in the new screen, as are an increasing number of natural products not yet in production for clinical trial.

Acute Leukemia Service

The intensive chemotherapeutic regimen, POMP, has been completed with an excellent complete remission rate (90%) and a low treatment mortality rate (3%). The median remission duration thus far is 16 months. Morbidity was kept at a moderate level by the vigorous use of platelet transfusions and antibiotics. Between 500 to 1000 platelet and leukocyte transfusions are given monthly. There is very little question that the use of platelet tranfusions has been accompanied by a dramatic reduction in the incidence of hemorrhage in thrombocytopenic patients. On the other hand, the effectiveness of leukocyte transfusions (largely cells from donors with chronic myelocytic leukemia) is not as clear. However, their efficacy is for the first time being evaluated in a controlled study. The answer should be available within the next year.

The importance of this very high remission rate in acute leukemia is that it strongly indicates a shift in emphasis in the treatment of this disease. A major effort now needs to be directed toward the eradication of the leukemia leukocytes remaining in the patient in remission. Accordingly more recent protocol studies have been designed with this thought in mind.

Solid Tumor Service

The major disease emphasis in the Solid Tumor Service continues to be the lymphomas and chronic myelocytic leukemia (CML). Based on the successful use of combination therapy in a small group of patients with Hodgkin's Disease (completed last year), a

larger study is now under way using a somewhat different combination. Thus far, 35 patients have been treated and although it is too early for final evaluation, it appears that more complete remissions are possible, particularly in the advanced stages of this disease, with this program than has been achieved before. The Radiation Branch has been extremely active in the treatment with radiation alone of patients with localized Hodgkin's Disease and of those in Stage III–a. The early results are very encouraging and suggest that cure in some of the late stages as well as in the early stages is quite likely.

Along with the programs in therapy, various other aspects of the lymphomas are being investigated. These include the infectious complications, immune mechanisms and the effect of anti-tumor agents on delayed hypersensitivity, antibody production, and lymphocyte transformation. Surprisingly, the studies to date do not support the previous reports that the immune mechanism of untreated patients with Hodgkin's Disease is uniformly depressed. Studies are underway to clarify our knowledge of immunity in anergic individuals. With the availability of large numbers of normal lymphocytes through the use of the NCI cell separator, certain fundamental studies are possible. In addition, the demonstration that thoracic duct lymphocytes can circulate for as long as 9 days raises the possibility that those cells may be of value in anergic patients under certain circumstances.

In addition to the large program in the treatment of lymphomas, the Solid Tumor Service has been vitally interested in the therapy of the blastic transformation of CML. This has been an utterly hopeless phase of the disease in contrast to the earlier phase in which there are a number of agents which can at least offer palliation to the patient. Myleran remains the drug of choice but a new drug, dibrom-mannitol appears promising and is being evaluated. In the blastic phase, the application of the POMP regimen or modifications of it has not been remarkably successful but new agents including cytosine arabinoside and daunomycin are currently under study.

"Life Island"

Additional experience was gained in the use of the patient protection unit, the "Life Island." Patient accrual has been slow even though a second unit was obtained during the year. Ten patients have been treated in these units and several points appear obvious to date: (1) It is possible to maintain a patient for long periods of time (at least 3 months) in a relatively germ-free environment. (2) Under such circumstances and in spite of severe leukopenia, infection is not a major problem even with serious toxicity. (3) Whether drug toxicity is reduced remains to be seen but thus far the impression is that it is somewhat less even though large doses of drugs being employed. (4) Finally, none of the patients experienced significant remissions of any duration in spite of the super-intensive therapy. The use of these units poses difficult problems in nursing and patient care. Accordingly, plans for laminar flow rooms are in the early stages of formulation. Whatever the ultimate role of the germ-free approach will be, there is no question that this technique has important applications in cancer chemotherapy.

Radiation Branch

In addition to its important role in the studies described above, the Radiation Branch has achieved very encouraging results in applying fractionated spaced total body irradiation of patients with chronic lymphocytic leukemia and lymphosarcoma. With this approach, the selective destruction of abnormal cells apparently occurs while normal hematopoietic elements are relatively untouched.

Clinical Branch, Baltimore Public Health Service Hospital

As indicated there were many administrative problems which had to be resolved in the relationship of this Branch to the NCI. However, in spite of this, the Baltimore Branch made a major contribution to experimental chemotherapy effort. It was particularly active in the leukemias, lymphomas and testicular tumors.

Many studies were performed wholly or in part at the Baltimore Branch. It contributed a large number of patients both to the POMP protocol in the treatment of acute leukemia and the combination treatment (vincristine, nitrogen mustard, natulan, and prednisone) of Hodgkin's Disease.

Cell Separation

The joint effort with IBM to develop a machine capable of separating granulocytes and platelets on a continuous flow *in vivo* basis saw some important progress during the year. With the development of a new model and a new centrifuge bowl design, it is now possible to separate 50% of both platelets and lymphocytes passing through the machine. Attention is now being directed toward the concentration of platelets which will permit their clinical use.

A junior investigator who will work full time on the machine joined the program. Current studies are being done only on an *in vivo* basis using dogs primarily. Early results in the separation of granulocytes appear very promising in that yields are being obtained in the region of 35%.

Conclusion

It is apparent from the summary of the activities of the Clinical Trials area that the coming year should be a very exciting one. The organizational changes should permit a more intensive, efficient and intelligent effort in cancer chemotherapy and in the supporting programs. On the laboratory side important studies being carried out in leukocyte biochemistry, leukocyte kinetics, cytogenetics, lymphocyte transformation, immunity, electron microscopy, tissue culture, etc., which are vital to the understanding of the malignant process. Generally, these have been undertaken because there were no similar studies elsewhere in the NIH and because the Clinical Trials area has the appropriate clinical material.

The necessity for the diversity of studies within the Clinical Trials area makes it imperative that additional senior personnel be acquired so that the various programs receive the direction of highly competent and trained individuals. A vigorous clinical program will be maintained. At the same time, in order to provide laboratory support for the clinical activities, at least two individuals with clinical interests as well as competence in such areas as biochemistry and immunology need to be recruited. Space, however, is a very serious problem, particularly in the Medicine Branch and makes it difficult to carry on existing programs, let alone hire new investigators.

With the studies now in progress, the coming year should bring significant advances in the treatment of acute leukemia and in Hodgkin's Disease. There are strong indications that some of these patients will be cured. An important breakthrough appears imminent in the procurement of granulocytes and platelets in large numbers with the use of the NCI cell separator. Contracts with industrial firms will undoubtedly make it possible to provide these blood elements to other institutions on a wide scale.

LABORATORY OF CHEMICAL PHARMACOLOGY

The overall function of the OASDET in the search for chemical methods for the control of human neoplasms is the development of broad pharmacologic knowledge in laboratory animals and in man of antitumor drugs. The degree of need for a comprehensive pharmacology program related to antitumor drugs depends in part upon the philosophy concerning the expected results of large scale screening programs. Extensive pharmacologic studies are not necessary if one subscribes to the notion that if enough compounds are screened long enough and hard enough a drug will be found, full blown, and fully effective which cures cancer. The alternative, but not mutually exclusive notion, is that such a screening program will only provide leads to active antitumor compounds. Quantitative animal antitumor and host toxicity information concerning these compounds, and quantitative clinical toxicity and antitumor data, in conjunction with a thorough knowledge of the factors effecting the antineoplastic activity of such drugs (I

consider all this pharmacology) is necessary to insure the development of chemical methods for the control of human neoplasms.

While we are not involved in the primary screening of compounds, we have responsibility in the field of experimental therapeutics, such as developing more accurate and reproducible systems for testing drugs, and in exploring more efficient means of treating tumor bearing animals by utilizing pharmacological information. In the field of clinical chemotherapy, we are necessarily involved in the initial aspects at least of a new drug trial in terms of the clinical toxicology and pharmacology of a new drug. Information obtained from these clinical studies and studies in experimental animals is then used in the design and evaluation of the therapeutic trial.

In the field of the pharmacology of chemotherapeutic agents, we are responsible for developing information on the factors, biological or chemical, which affect the antitumor activity of such drugs.

The basic pharmacologic investigations include a study of the absorption, tissue and cellular distribution, and excretion of these agents, and their metabolic fate. In addition, the biochemical mechanisms of action of antitumor drugs are investigated. Such data are of major importance in the design of the initial trial and the subsequent therapeutic trial in man. Another major area of research concerns the toxic effects of antitumor drugs. A thorough knowledge of the quantitative details of the deleterious actions of antitumor drugs on normal cells and tissues is important in defining limitations in the therapeutic use of the drug. Of particular importance are quantitative questions of extent or damage, rate of recovery and interval between dosing.

In all these areas basic research is conducted, not only on the pharmacology of the specific drug, but also on the general principles of pharmacology if these principles are not well known and understood. A recurring problem confronting us is that of comparative pharmacology. The development of any new drug from animal screening to clinical trial is an exercise in comparative pharmacology. The antitumor activity in the mouse and rat and the toxicity in mouse, rat and dog are used to attempt to predict activity and toxicity in man. The metabolism of a drug by mouse or rat tumor and normal cells is used as a basis for extrapolating to man. More information is needed comparing the pharmacology of such drugs in experimental animals and man.

The following examples will illustrate current research activities relative to the aforementioned functions. The alkylating agents, for which the prototype is nitrogen mustard, are still important agents in the treatment of cancer. Work this year has emphasized mechanisms of damage and repair of DNA by nitrogen mustard with particular emphasis on confirming the hypothesis that crosslinking in the eight position of the guanine moiety is a primary mechanism of action and further exploring the possibility that nitrogen mustard may also react with the phosphate groups. It was noted that DNA with at least two crosslinks between the chains, was still able to retain its transforming ability. This suggests that either a modest amount of alkylation does not impair the biological functions of this macromolecule, or that repair processes are available to neutralize the damage caused by crosslinking. A technique has been devised in which the alkylated eight carbon in guanine may be specifically liberated as CO_2 under the proper conditions. This may allow a precise measure of the extent of crosslinking and for the development of firmer evidence that crosslinking in this position is critical to the action of mustard.

The mechanism of action of hydroxyurea has yielded interesting and unexpected results. Hydroxyurea is degraded approximately 10–25% *in vivo* to urea. Evidence has now been developed that this degradation is accomplished by a direct reduction through one unit of the oxidative cytochrome chain. This is similar to the reductive drug metabolic pathway present in liver microsomes; however, this reaction occurs in mitrochondria. It is interesting that this reduction of hydroxyurea to urea occurs *in vitro* in the presence of ferric ion. These results suggest that the mechanism of action of hydroxyurea is to act as a biological oxidizing agent. This is consistent with the suggestion that hydroxyurea may damage cells by in-

hibition of ribonucleotide reductase and subsequent nucleic acid synthesis.

Work continues on the overall pharmacology of MIH, the methylhydrazine derivative active in lymphoma and Hodgkin's Disease. In its metabolic degradation, it is clear now that this compound goes through methylhydrazine. This can react with pyridoxal to yield pyridoxal methylhydrazone, a vitamin B_6 antagonist. There is also evidence that MIH administration can, through other metabolic products, exert monoamine oxidase inhibition. There is experimental and clinical evidence of central nervous system activity of MIH which is consistent with monoamine oxidase inhibition. In terms of its metabolism in mice, methane is one of the excretory products. The exact origin of this methane from the MIH molecule is as yet to be determined.

Work continues on the nitrosourea derivatives. One difference between the metabolism of *bis*-β-chloroethylnitrosourea in man and laboratory animals appears to be that a much higher percentage of the BCNU remains in the body in man than in laboratory animals. In laboratory animals approximately 5% of the compound and its metabolites remain after 24 hours; in man, approximately 25% remain after one week. The implications of this in terms of human toxicology are as yet unclear.

One of the interesting new derivatives of nitrosourea synthesized by the Southern Research Institute group is cyclohexylfluorethyl nitrosourea. This is a highly active compound which shows less delayed toxicity in mice than BCNU. Preliminary toxicology studies indicated that in the rat there were unexpected acute deaths after the administration of cyclohexylfluorethyl nitrosourea. It was suggested that these deaths might be related to fluoroacetate toxicity, arising from the degradation of the fluorethyl moiety of the nitrosourea compound. One biochemical consequence of fluoroacetate toxicity is an increase in the citric acid content of a number of organs, particularly liver and kidney. It was shown that after an acutely toxic dose of cyclohexylfluoroethyl nitrosourea, citric acid concentration in rat kidneys rose strikingly. This supports the notion that the fluoroethyl derivatives of nitrosourea might cause unusual acute toxicity related to the metabolism to fluoroacetate.

Work is continued on the folic acid antagonists. In an interesting combination of biochemistry and experimental therapeutics, efforts are now being made, using the high dihydrofolic reductase containing strains of L1210, to assay for number of leukemia cells by assaying for folic reductase. In certain of these strains the very high enzyme activity will allow an excellent comparison of the direct decrease in enzyme activity and the direct kill of the cells to be compared against indirect methods relying on increase in life span or number of survivors. In other studies the ability of kidney and choroid plexus to concentrate methotrexate *in vitro* has been demonstrated.

There are two main areas concerning the toxicology of antitumor drugs. In terms of research toxicology, we are attempting to define optimum dose schedules for a variety of the antitumor drugs which will minimize host toxicity. In attempts to determine an ideal schedule with benefit with optimum relationship between tumor damage and host survival, one approach is to search for that schedule which gives the least amount of host damage in the presence of large amounts of drug. For this we use the "priming dose study" in which a sublethal dose of the drug is given one day to a large group of animals. On subsequent days, portions of this large group of animals are assayed for the LD50 of this agent. With a compound like methotrexate, it can be shown that the LD50 is very low on days 2–4 after the administration of a sublethal dose. Subsequently, on days 7–9, the LD50 is very high and the possibility exists, in fact, that the animals may be able to tolerate a higher dose of the drug. With a compound like cyclophosphamide, there is only a modest decrease in the LD50 on days 2, 3, and 4, and a return to normal on subsequent days without rebound.

Cytosine arabinoside appears to be similar to methotrexate in this respect. It is interesting to note that this rebound previously described for methotrexate, also occurs for cytosine arabinoside. This rebound can be seen

after a single dose or infusion, but not after a course exceeding four or five days. One can visualize the bone marrow reserve which might be available for mobilization after a single dose may be damaged after repeated doses of the drug.

A number of new agents were evaluated for their preclinical toxicology this year. These studies, vital to the development of new drugs, are summarized briefly in the appendix of this report. The increasing importance of quantitative toxicological studies has encouraged us to begin a program attempting to relate host toxicity and experimental tumor damage to plasma concentrations of antitumor drugs. As mentioned above, the development of methodology and the beginning study of the physiological disposition of a variety of important anticancer drugs has begun. A group has been formed including representative of intramural NIH and all of the contractors who are involved in physiological disposition studies. This group will exchange information on techniques and methods for studying physiological disposition and will begin to pull together all the available data on the anticancer drugs and will initiate new areas in which studies should be performed.

The technique of ventricular perfusion in the animal and its use as a research tool have been reported in the last few years, as well as the adaptation of this technique for clinical use in the treatment of intracranial malignancies. This technique has been further developed in this past year. Doses of methotrexate up to approximately ½ to 1 liter of a 1 mgm/milliliter solution of methotrexate have been used in ventricular perfusion. This concentration of methotrexate seems to be the maximum tolerated because occasionally transient convulsive phenomina occur at this concentration. Such concentration, of course, should be very effective in its antitumor effects. We have also studied the toxicology of 8-azaguanine and have begun a cautious clinical trial of this agent in ventricular perfusion studies. 8-azaguanine is of particular interest because brain tumors are thought not to have the enzyme, guanase, that detoxifies this compound, but normal brain and most of the normal body tissues to have guanase and thus the quantities of 8-azaguanine which return to the blood stream after perfusion will be rapidly detoxified.

Three patients with meningeal leukemia who were refractory to intrathecal aminopterin have had complete remissions of their meningeal leukemia after ventriculo lumbar perfusion. The success of this sort of study encouraged us to move on into the area of primary intracranial neoplasms such as gliomas. In considering the chemotherapy of gliomas and other primary intracranial neoplasms, the need for an appropriate screening and test system became apparent. This is not available and therefore we began the process of developing such a system, preferably in an animal large enough to use for pharmacology studies. Currently we are testing the Rous sarcoma virus in newborn puppies. This has been reported by Rabotti to cause ependymomas or gliomas. We are attempting, so far successfully, to maintain the Kelly-O'Gara rhesus monkey hepatic carcinoma in sequential intracranial transplant in young rhesus monkeys. Although it is not a primary but a metastatic type tumor, its usefulness for pharmacologic studies seems clear. As a third alternative, we are exploring the YABA virus tumor inoculated intracranially. Some success was reported in the past by workers at Roswell Park; with more purified and highly potent virus there is reason to hope that this might yield a satisfactory brain tumor model.

ETIOLOGY

The Office of the Associate Scientific Director for Carcinogenesis was created by the reorganization of the Institute to plan and coordinate programmed research in experimental carcinogenesis. The Chemistry Section of the former Carcinogenesis Studies Branch was elevated to branch status (Chemistry Branch) to support a more comprehensive approach to the chemical biology of cancer. The remainder of the former Carcinogenesis Studies Branch (namely the Carcinogen Screening Section, the Bioassay Section, and the Cytogenetics and Cytology Section) have been regrouped into a branch (Biology Branch).

The work of the Chemistry Branch, which emphasizes the fundamental nature of the interaction of carcinogenic agents with living systems in the induction of cancer at the host, tissue, cellular, and molecular levels, has progressed in a most satisfactor manner during the fiscal year. The progress along specific lines of endeavor has been succinctly presented in the summary report of the Branch Chief; no effort will be made to reiterate at this point. It is important, however, to point out that the fundamental nature of the research has influenced the Branch's scientists in their decision to perform their research activities in their NCI laboratory facilities rather than through collaborative relationships supported by contracts. As a consequence, the size of the research program that can be implemented is intimately related to the amount of space that can be made available to the Branch. Unfortunately, during this prelude to the occupancy of the new cancer building, this Branch works in difficult circumstances of crowding and inadequate facilities, about which little can be done; the staff is to be commended highly for its productivity during this fiscal year.

The Biology Branch has continued to maintain collaborative studies, supported by contracts, in addition to its in-house activities. Its program has been aimed principally at the identification and characterization of known and suspected chemical carcinogens, especially those commonly occurring in the human environment; efforts to improve bioassay methods employed in the identification and characterization of chemical carcinogens with the aim of improving the relevance of animal data to the human situation; and the study of effects of known and potential carcinogens at the cytologic and genetic levels. The major findings emanating from the work during this year are stated in the report of the Biology Branch Chief, and will not be reiterated here.

BIOLOGY BRANCH

The Biology Branch, created by the reorganization, is composed of a Carcinogen Screening Section, a Bioassay Section, and a Cytogenetics and Cytology Section. Dr. Falk is the Acting Branch Chief, pending recruitment of another scientist for this position.

The common thread linking the programs of the various sections in the Branch is experimental carcinogenesis. The purpose of the experimental carcinogenesis in the Carcinogen Screening Section is the identification of environmental carcinogens and the characterization of the metabolism and biological action of these carcinogens *in vivo*. The Bioassay Section is concerned with improvement of the methodology of identifying and characterizing chemical and other carcinogens in living systems to the end that screening in animals and *in vitro* may be made more efficient and relevant to the human situation. The cytogenetics and Cytology Section emphasizes the morphologic and biochemical interaction of carcinogens with the host at the cellular level. As will be readily appreciated, these sectional divisions are somewhat arbitrary, the activities of the three Sections truly representing a continuum rather than discreet items.

The Acting Chief of the Branch, in addition to fulfilling his responsibilities as the Associate Scientific Director for Carcinogenesis, has maintained active in-house research programs described in Individual Project Report Nos. NCI–4600 and NCI–4601 concerned with the contribution of pesticides to carcinogenic burden in rats and pesticides synergists, and has been the responsible Project Officer on a spectrum of contract-supported projects (#PH43–64–546, "Study of Air-borne Carcinogens by Means of Photodynamic Bioassay"; #PH43–64–45, "The Co-Carcinogenicity of Aliphatic Hydrocarbons and Organic Disulfides"; #PH43–65–1036, "Carcinogenesis: Toxins in Foodstuffs"; #PH43–64–933, "Studies on Mucous Flow in the Intact Mammal Exposed to Tobacco Smoke"; #PH43–63–1165, "Oxidation Products of Fats"; #PH43–64–1158, "Piperonyl Butoxide as Carcinogen or Co-Carcinogen"; #PH43–64–567, "The Preparation of Products Related to Dibenz (*ah*)Anthracene and Benzo(a)Pyrene"; #PH43–64–938, "Tumor-Promoting Principles of Tobacco and Tobacco Smoke"; #PH43–64–865, "Investigation of Potential Anti-Radiation and anti-Neoplastic Compounds"; #PH43–64–

504, "Biochemical and Morphological Components of Hepatic Carcinogenesis; #PH43–66–508, "Potential Carcinogenicity of Cancer Cheotherapeutic Drugs"; #PH43–65–1000, "Research on Free Radicals and Alkylating Agents in Tobacco Smoke"; #PH43–65–1029, "Carcinogenic Mycotoxins"). In addition, he has had a major share of the responsibility for the contract relationship with the Chicago Medical School (#PH43–65–67), under which a large-scale carcinogen screening program is maintained and research on the fundamentals of cancer induction mechanisms is pursued.

The pesticides carcinogenicity studies being conducted under contract by the Bionetics Research Laboratories, with close supervision by the Biology Branch scientists, has been progressing in a highly satisfactory manner. An exemplary number of valuable contributions to the literature have been made with respect to pesticides chemistry; the long-term carcinogenicity experiments will be completed during the ensuing six months. The information to be obtained from these studies has been long awaited with great interest. The contract-supported research project on the carcinogenicity of oxidized lipids, being conducted at General Foods Corporation, has been augmented to include the determination in animals of carcinogenicity of these lipid products. A new contract-supported research project has been initiated, in collaboration with the CCNSC, at the University of Pittsburgh for the determination of the carcinogenicity of a number of chemo-therapeutic agents employed in clinical medicine. A complementary study of the carcinogenicity of nitrofuran derivatives is being negotiated with the University of Wisconsin. The detailed descriptions of the established projects will be found in the contract narratives of this Branch.

The Acting Branch Chief has been a prime force in the effort to obtain a collaborative relationship with Dr. Norton Nelson's group at New York University, for experimental carcinogenesis involving asbestos products, polyurethane foam products, peroxides, and epoxides, and research into the carcinogenic and co-carcinogenic constituents of tobacco and tobacco smoke.

The Head of the Carcinogen Screening Section, while serving as an Assistant Project Officer on the Chicago Medical School contract, has been responsible for a number of other contract-supported carcinogen screening activities and has maintained his own active in-house research program. These screening activities have resulted in the identification of about fifteen hitherto unrecognized carcinogens. The Section's in-house research has made important contributions to the literature on the effects of androgens and estrogens on the susceptibility of rat liver to chemical carcinogens; has developed a new system for the separation of urinary metabolites of carcinogenic aromatic amines; has described the forms in which carcinogenic amines and their metabolites circulate in the blood and transfer from one tissue compartment to another; has shown some of the effects of pituitary and adrenal hormones on the metabolism of N-hydroxy-N-2-fluorenylacetamide to increase the level of the proximate carcinogen; has shown that some carcinogens combine with existing cellular and molecular targets rather than with the same targets during their periods of synthesis; and has demonstrated the competitive inhibition of binding of carcinogens at the target, using chloramphenicol. The utility of a standardized means of large-scale chemical carcinogen screening and further progress on the synthesis and structural studies of naturally occurring carcinogens, such as aflatoxins, have been accomplished by contract-supported projects. Members of the staff of this Section authored a number of review articles intended for wide circulation to alert chemists to the potential carcinogenic effects of the products of industry.

The Bioassay Section has maintained contract-supported projects for the improvement of the methodology of carcinogen screening, while conducting its own research on the inhibition or anti-tumorigenic action of actinomycin-D. The screening methodology studies have emphasized the utilization of the mammary fat pad technique of DeOme and Faulkin, and the exploitation of the unusual susceptibility and promptness of response to carcinogenic

stimuli by animals in their neonatal period. Efforts are being made to evalute the usefulness of animals born in particularly immature states, such as marsupials. More fundamental studies have demonstrated an anti-tumorigenic effect in skin carcinogenesis with 90μg of actinomycin. In addition, actinomycin (1 to 90μg) inhibited RNA synthesis in skin, whereas initiating amounts of DMBA produced a slight inhibition of RNA synthesis. This inhibition occurred shortly after treatment with DMBA and was transitory. The inhibition of RNA synthesis by actinomycin occurred somewhat later and was of a more lasting nature. The antitumorigenic effect of 90μg of actinomycin was accompanied by an increase in protein synthesis, a change in mitotic index for interfollicular epidermis, and hyperplasia. Skin tumorigenesis was also inhibited by maleic anhydride, N-ethyl maleimide, and iodoacetamide, agents which combine readily with –SH groups. These findings support the hypothesis that DMBA skin tumorigenesis may involve interaction with these –SH groups. Antitumorigenesis is not seen when DMBA is applied 2 to 8 weeks after treatment with maleic anhydride, but is noted when this period is reduced to one week. Treatment with maleic anhydride for ten weeks produced inhibition, while treatment for one week was ineffective. Actinomycin was ineffective in inhibiting lung tumorigenesis even when administered at a high dose, despite a transient inhibition of RNA synthesis. Studies of DNA metabolism have been pursued, and interesting findings reported in the Individual Project Reports for this Section. A fascinating and important observation has been the stronger carcinogenic stimulus by smooth-surfaced plastic implants than by those with rough surfaces. This finding may have some significance in the use of plastic prostheses in human beings.

The transfer of Dr. Klein to Extramural Activities, NCI, will necessitate a recruiting action for the position of Head of the Bioassay Section. Dr. Klein's responsibilities as a Project Officer will be distributed among other members of the Branch.

The Cytogenetics and Cytology Section is studying the quantitative effects of carcinogens, employing models designed primarily to investigate somatic viruses. The systems in use include microbial, cell, and organ cultures and intact animals. Thus, this Section tends to bridge both the biological and chemical approaches to carcinogenesis. A critical review of both the cancer and teratology literature made by the Section has resulted in the concept that the same environmental insult may be responsible for either cancer or malformations or both. In this area, it has been demonstrated that important carcinogens, such as aflatoxins, uracil mustard, and urethan, all produce malformations; the severity of the malformations being dependent upon the dose administered. The extent of the parallelism between carcinogenic and teratogenic activity is being explored by the use of analogues and inhibitors of carcinogens. The effects of carcinogens at the cellular level in terms of drug resistance and neoplastic transformation are under way using mouse and hamster material. Heart tissue, which is rarely the site of primary malignancy, has been shown to undergo spontaneous neoplastic transformation resulting in mesenchymal tumors. In addition, tissue culture cells underwent chromosomal changes consistent with an evolutionary pattern of genetic readjustment. Hamster embryo cells when exposed to carcinogenic polycyclic hydrocarbons underwent a series of morphological changes, including loss of contact inhibition, inhibition of mitosis, and death of many cells. Cells grown in the presence of the carcinogen resulted in permanent cell strains which produce tumors when inoculated into hamsters. Transformed cells were resistant to the exposure of additional carcinogens while control cells exhibited toxicity. Current experiments are aimed at determining the possibility of selection of spontaneously transformed cells by the carcinogen, the acceleration of the carcinogenesis process by the chemical or the actual induction of the change by the carcinogen. Alkylating agents have been studied using specific synthetic messenger ribonucleic acid (RNA) and using tissues of mice treated with uracil mustard. The synthetic messenger was inactivated by alkylating agents by either esterification of chain terminal phosphate or

cross-linking strains of RNA by forming diguanyl derivatives. The administration of a high dose of uracil mustard to Strain A mice caused a marked inhibition of DNA synthesis in lung, liver, and kidney. Inhibition of total RNA synthesis and nuclear RNA synthesis was also observed at relatively high dosages of uracil mustard in the three tissues studied. Additional descriptions of these studies are contained in the Individual Project Reports of this Section and in the report on the Temple University contract (#PH43–66–68) with Dr. Daniel Swern.

CHEMISTRY BRANCH

The program of the Chemistry Branch is designed to clarify the fundamental nature of the interaction of carinogenic agents with living systems in the induction of cancer at the host, tissue, cellular, and molecular levels, and to investigate the interaction of certain environmental agents with functioning biological systems. The biochemical alterations in carcinogenesis and in the resultant tumor tissue are expressed as a number of phenotypic changes which include altered enzyme profile, altered responsiveness to environment, increased growth, loss of contact inhibition, and loss of specialized function. Since the phenotypic changes are heritable, they presumably reflect alterations in gene function, either at the genetic transcription level or at the translational level in the cytoplasm. The Chemistry Branch studies the biochemical nature of the factors controlling gene activity and messenger RNA translation, as well as the chemical nature of the genetic factors characterizing susceptibility to carcinogenesis. Studies are made on the effects of carcinogens of various types, including chemical and viruses on geneaction systems. The studies are performed on both the nature of the chemical and physical interaction of carcinogenic agents with various cellular components, and the effect of their interaction on the functional integrity of cellular control systems.

Studies designed to elucidate the biochemical nature and the time required for the initiation of skin tumorigenesis are in progress. One definitive result has shown that the time required for the initiation of skin tumorigenesis is of short duration, of the order of 1 to 2 days. In addition, the initiation of skin tumorigenesis was found to depend on functioning gene activity and potent carcinogenic agents did not elicit initiation of tumorigenesis when the inhibitor of gene activity, actinomycin D was present.

In related studies, actinomycin D was found to decrease the amount of carcinogen bound to skin DNA by 35% and not to affect the amount of carcinogen bound to total skin protein. In other studies actinomycin D as well as the carcinogen, 7,12-dimethylbenzanthracene (DMBA), were both found to inhibit DNA and RNA synthesis.

Investigations on the interaction of liver carcinogens with cellular macromolecules have demonstrated that three potent liver carcinogens, N-hydroxy-AAF, 3′-methyl-DAB, and aflatoxin B_1, interact with DNA and cause a reduction in the RNA to DNA ratio of purified liver nuclei. In addition, these agents were found to inhibit the incorporation of radioactive precursors into nuclear RNA. Thus these carcinogenic agents are altering gene activity at the level of RNA synthesis.

Investigations on the mechanism of malignant transformation by Rous sarcoma virus (RSV) has shown that the initial stage of the transformation process requires DNA synthesis, and that subsequent stages allowing for the growth of RSV require the continuous participation of cellurlar DNA. These important results have clearly increased our understanding of the nature of malignant transformation by Rous sarcoma virus. As a corollary to these studies techniques have been developed for the growth of Rous sarcoma virus in liquid spinner cultures and in cells suspended in agar. The ability of cells to grow under these conditions requires their prior infection by Rous sarcoma virus. These techniques will permit the large scale production of Rous sarcoma virus.

Investigation of vesicular stomatitis virus infection has shown that it induces in infected cells a new enzyme capable of synthesizing RNA. This enzyme did not appear in cells in-

fected with Rous sarcoma virus or Rous-associated virus.

Methods have been developed for the isolation of macromolecular components of liver chromatin under conditions in which the physiological relationship between these components is minimally disturbed. Using some of these techniques, DNA molecules with a molecular weight of 3 times 10^8 daltons have been isolated from chick embryo. The distribution in DNA size was found to be unimodal, which is unlike the size distributions of metaphase chromosomes in this organism. An accurate determination of DNA size will permit the investigation of the relationship between DNA size and carcinogenesis. In other studies DNA from mouse liver nuclei and mitochondria has been isolated and examined in respect to molecular weight and buoyant density. These studies have found that the minor component of mouse liver DNA, the satellite band, comprises about 10% of the nuclear DNA and is not found in mitochondrial DNA preparations.

The molecular basis for the mutagenic action of hydroxylamine, nitrous acid, formaldehyde, and the carcinogen, N-methyl-N-nitroso-urethan has been investigated. Hydroxylamine acts by altering the coding properties of deoxycytidylic acid from guanylic acid incorporation to adenylic acid incorporation. Formaldehyde causes inactivation of the template properties of polymers, and the carcinogenic N-methyl-N-nitroso-urethan alters several parameters of template activity which are now being studied.

Studies on the removal of messenger RNA-like activity from rat liver microsomes have found that this activity can be removed without disturbing the capability of microsomes to incporporate amino acids in the presence of synthetic messenger RNA. Indeed the removal of endogenous messenger RNA increases the sensitivity of microsomes to added synthetic messenger RNA in respect to both the ability to bind synthetic messenger RNA and the activity of the synthetic messenger RNA in directing amino acid incorporation.

The effects of carcinogens, non-carcinogenic drugs, or environmental chemicals on the induction of specific drug metabolizing enzymes, and the mechanisms of this induction have been under study. The induced enzymes control to a significant extent the duration of drug action; their altered activity may be responsible for often-observed altered drug resistance and susceptibility. In addition, these hazards of drugs and environmental agents are capable of altering normal metabolism. Thus, phenobarbital was found to increase the messenger RNA content of microsomes. It also increased the sensitivity of microsomes, but not ribosomes, to the addition of synthetic messenger RNA. Phenobarbital also increases the endoplasmic reticulum of the cell. Investigations have also been made of the effect of some of these agents on the genetic apparatus, and in particular, on RNA polymerase activity of the nucleus.

Methods, utilizing the zonal centrifuge, are being developed for the preparation and isolation of macromolecules and cellular and subcellular fractions of biological systems. Separations have been made of *E. coli* RNA, *E. coli* ribosomes, rat and mouse liver ribosomes, microsomes, mitochondria, and whole cells. In addition, preliminary work suggests the feasibility of separating cell membranes and nerve endings. These techniques will greatly enhance the ability to isolate large amounts of cellular components needed for biochemical investigations.

An extensive electron microscope study of transformed tissue culture cells is in progress. This study has revealed that the so-called nonproducer cells release morphologically complete but noninfectious viral particles. In addition, certain surface morphology appears altered in the transformed cells. In addition, many of the subcellular materials separated by the zonal centrifuge are being examined by electronc microscopy. The electron microscopic pictures have contributed a semi-quantitative estimate of the number and kinds of riobosomes of varying polymeric units. The distribution of these subcellular particles in the tumor and normal cells is being compared. The electron microscope has also been applied to the study of the epithelial cells of the bronchial tree in animals exposed to air pollutants. The electron microscopy study has provided evidence for a differ-

ent degree of response and rate of recovery in the epithelial cells at different levels of the bronchial tree.

Studies in the Section on Proteins have in the past year included the use of zone electrophoresis in acrylamide gels as an analytical and preparative technique, studies of serum proteins in man and rodents, correlations between serum haptoglobin type and disease, and studies on the preparation and properties of haptoglobin type 1–1 in human serum.

A study of several hundred normal human serum specimens has confirmed the high resolution of "disc" electrophoresis in acrylamide gels. The major polymorphic systems demonstrated by such analyses are the group-specific component, transferrin and haptoglobin. Most variations between individuals could be attributed to these systems. Other variations also exist, but could not be similarly explained.

The high resolving power of such gels has been adapted to preparative purposes with excellent results in the case of hemoglobin and haptoglobin type 1–1. Both these proteins have been isolated in highly purified form, with less than 1% detectable impurities.

The interaction of haptoglobin and hemoglobin has been studied with this purified material. This interaction passes through an intermediate in which one-half molecule of hemoglobin reacts with one molecule of haptoglobin. This complex may add another one-half molecule of hemoglobin to form a fully saturated complex. A detailed analysis of this reaction was possible.

An analysis of haptoglobin types in mice has shown that inbred strains differ with respect to whether they have haptoglobin. AKR and C_3H mice, although normally without serum haptoglobin, are easily induced to produce it. Variations between mouse strains in this connection may be related to ease of "spontaneous" tumor formation.

Further data on haptoglobin types in human keukemia confirm that such patients have an excess of the H_p^1 gene, over normal groups. Similar, but less marked, differences are found between control groups and female patients with carcinoma of the breast and male patients with carcinoma of the lung. A report showing an excess of H_p^1 in patients with juvenile rheumatoid arthritis could not be confirmed. An improved haptoglobin typing method was devised and used in these studies.

VIRAL ONCOLOGY

Activity in the tumor-virus program of NCI was further increased during the past fiscal year. An increase during the preceding year had brought about a split of the Laboratory of Viral Oncology and the creation of an additional laboratory, the Laboratory of Viral Carcinogenesis as well as of the Office of the Associate Scientific Director of Viral Oncology to coordinate tumor-virus research within the two laboratories. With the reorganization of NCI during the past fiscal year, this office and its constituent laboratories were transferred to the Office of the Scientific Director for Etiology, where they were consolidated with the tumor-virus program already in that Office within the branch previously designated as the Virology Research Resources Branch. The new designations of these laboratories and branches and their respective Branch Chiefs, are as follows: (1) Viral Biology Branch, Dr. A. J. Dalton, Chief; (2) Viral Carcinogenesis Branch, Dr. R. E. Stevenson, Chief; and (3) Viral Leukemia and Lymphoma Branch, Dr. F. J. Rauscher, Jr., Chief.

The Viral Carcinogenesis Branch, which superseded the former Virology Research Resources Branch, continued its functions with respect to the development and management of resources essential to tumor-virus research, and assumed additional responsibility for direct program activities in the field of naked DNA tumor viruses. Pending completion of the new NCI building and the availability of laboratory space, the NCI, through the Viral Carcinogenesis Branch, will embrace an intramural research program on these DNA tumor viruses for the first time. Key staff scientists who will participate in this research as well as in other program activities have already been recruited. This Addition of scientific personnel and expertise has made possible the activation under contract of new major activities in support of cancer-virus research, such as: (1) the

viral diagnostic laboratory which will identify known agents and differentiate new agents turned up in the search for human cancer viruses by scientists participating in special programs of NCI; and (2) research on the development and production of simian virus reagents, which will be critical for monitoring for and identification of contaminant simian viruses if a human leukemia or other cancer virus is found which is transmissible in these primate species.

The oncogenic naked DNA viruses, which include 5 of the human adeno-viruses, were discovered by virologists employing the tissue culture techniques of classical virology, and their intensive investigation has been pursued primarily in general virology laboratories. Up until recently the principal activity of the NCI in this area has resided in support of the community of virologists engaged in oncological investigations, through its virology research resources program. Developments during the past two years, and particularly during the last fiscal year have revealed neoantigens in tumor cells of hamsters and other rodents induced by human adeno and other oncogenic naked DNA viruses, although infectious DNA virus is not reproduced in the oncogenic interactions.

At the joint suggestion of Doctors Albert Sabin and Robert Huebner, an *ad hoc* group of leading virologists concerned with this problem was brought together for several discussions during the past year, under the sponsorship of the Viral Carcinogenesis Branch. The question was examined whether the time was ripe for a joint programmed activity in search of neoantigens in human cancers that are characteristic for certain known human viruses, including the adenoviruses. Although the time seems near when such studies might be initiated, it was concluded that refinements in existing techniques must be made before a satisfactory study can be launched. This burgeoning collective effort may presage a future major program activity of NCI.

The Viral Leukemia and Lymphoma Branch and the Viral Biology Branch have continued their singular as well as their joint activities involving the longer known membrane-bound tumor viruses of the types associated with naturally prevalent and transmissible neoplasms of animals. These include: (1) the C type RNA viruses associated with avian and murine leukemia and related neoplasias, and which bud from cytoplasmic membranes; and (2) "herpes-like" particles such as the Lucke frog kidney carcinoma virus which matures in the nucleus, and probably contains DNA. Viruses of both of these types are reproduced during, and remain associated with the overt neoplastic disease which they induce.

In studies directed toward speeding the search for comparable agents in human leukemia and lymphoma, scientists of the Viral Leukemia and Lymphoma Branch have adapted two additional highly sensitive immunological techniques to the detection of murine leukemia virus antigen, and are now investigating applications to the human problem along the same lines as previously reported for the fluorescent antibody technique (i.e., the detection of specific, leukemia associated, antigens and/or antibodies which could be of viral derivation). The additional techniques, which have proven more sensitive than the fluorescent antibody technique in murine systems, are: (*a*) the micro-Ouchterlony (double diffusion in gels) precipitin reaction; and (*b*) specific hemagglutination of antigen-coated tanned erythrocytes. Considerable progress has also been made in the controlled degradation of murine leukemia viral particles into morphological subunits, and purification of the subunits by density gradient centrifugation.

The Viral Biology Branch continues to play a leading role in the search for viral etiological agents associated with human leukemia and lymphoma, using particularly the methods of electron mircroscopy and tissue culture. In collaboration with clinical staff members 141 plasma specimens from 120 separate cases of leukemia were studied during the past year. Of these, about 13% were found to contain virus-like particles resembling the C type virus particles of animal leukemias. This is approximately the same proportion of positive specimens observed in previously reported studies. The results are consistent with those obtained in neoplastic diseases of animals induced with low

doses of RNA tumor viruses or in the naturally occurring animal diseases.

Membrane-bound virus particles of the "herpes" type have been observed by electron microscopy in 3 of 4 additional cell lines of Burkitt lymphoma sent to Dr. Manaker from Nigeria by Dr. Pulvertaft (University of Ibadan). This brings to 9 the number of established cell lines of Burkitt lymphoma that have been investigated in several laboratories for the presence of virus-like particles. The "herpes-like" particle has been observed in 8 of the 9 cell lines. Similar particles were also observed in tissue culture cells from: (1) an induced myeloid leukemia of a monkey (original specimen supplied by Dr. Margaret Kelly of the Chemotherapy area of NCI); and (2) a human patient with chronic myeloid leukemia. The latter observation brings to 5 the number of established cell lines of human leukemia that have shown "herpes-like" particles, out of a total of 12 investigated by participants in the Special Virus-Leukemia Program.

Whether the "herpes-like" agent is a ubiquitous "passenger" virus, or whether it is a serious contender for an etiological role is not yet clear. It cannot be propagated in any cell line thus far tried, other than the explanted malignant cells in which it is found naturally. It shows no antigenic relationship to other members of the herpes group, or any other known virus thus far compared.

In addition to basic research on membrane-bound tumor viruses, and experimental participation in the search for counterpart human agents, members of the Viral Biological Branch are Chairmen of two major Segments and Vice Chairmen of two Segments of the Special Virus Leukemia Program. They also serve as Project Officers and supervisors of developmental research under contract amounting to approximately $5 million.

VIRAL BIOLOGY BRANCH

Electron Microscopy Section

Highlights of the results of collaborative studies on animals in which electron microscopy played an important role include the demonstration that the etiologic agent of Moloney sarcoma is indistinguishable morphologically from the murine leukemia virus; the visualization of the process of replication of avian-type viruses in brain tumors of the dog, induced by Rous sarcoma virus; and the demonstration of the presence of particles in tissue culture cells derived from the buffy coat of a monkey with chronic myelogenous leukemia having the same morphology as those associated with tissue culture isolates from Burkitt lymphoma.

In studies on human material, it has been found that the virus particles associated with Burkitt lymphoma differ from known herpes type viruses by the acquisition of a granular coat at some time during maturation. Also worth of note is the finding of Burkitt type particles in tissue culture cells derived from the buffy coat from a human case of chronic myelogenous leukemia. These findings may still be explained on the basis of passenger virus, but the presence of these particles in 8 out of 9 Burkitt lymphoma isolates and in isolates from 5 human cases of myelogenous leukemia (including the 4 Roswell Park isolates) suggests that something more than simple coincidence is involved.

In the screening program, 19 out of 141 plasmas from leukemic patients were found positive for virus-like particles, an average of something more than 13%. A shift in emphasis is planned for this work with increased concentration on lymph node biopsy material and less on plasma pellets.

Microbiology Section

Work in this Section has included the successful propagation of Burkitt lymphoma isolates, EB–1, 2, and 3 of Epstein and the Jiyoye, Kudé, Ogun, and Ragi lines of Pulvertaft. Modification of the culture media and other experimental interventions were attempted with the aim of increasing virus yield.

In addition the crossing of species barriers has been demonstrated by tumor induction following intracranial inoculation of high titer Rous sarcoma virus into rabbits, guinea pigs, hamsters, cats and dogs. The common forms of tumors have been gliomas and meningiomas,

although the tumors in guinea pigs have been difficult to classify.

An experiment involving the feeding of mosquitoes (Aedes egypti), first on Burkitt tissue culture cells, then on a monkey resulted eventually in a modified peripheral blood picture and enlarged lymph nodes in the monkey. There has been no evidence of the development of the leukemic state, but this experiment should be followed up.

It should be noted that the head of the Section on Microbiology spends fully two-thirds of his working hours on administrative matters connected with the SVLP.

Virus Studies Section

Extracts from the SL–1 isolate from Burkitt lymphoma suspended in 5% dimethyl sulphoxide (DMSO) inoculated intracranially into newborn hamsters induced CNS symptoms, eventually as early as 5 or 6 days after inoculation. Members of the staff at the Pfizer Laboratories, Maywood, N.J., have confirmed these findings for material obtained from the Virus Studies Section but have been unable to confirm them starting with fresh homogenates of SL–1 or EB–2 Burkitt isolates. To date the virus which undoubtedly is present has not been visualized and it is important to determine whether it is of human origin or is a hamster virus activated by the treatment.

Virus Leukemia Section

Studies have continued on the virus induced murine sarcoma. Histopathological analysis indicates that it is a rhabdomyosarcoma with cross striation sometimes apparent in the larger of the two cell types. The large cells when prepared as whole mounts are very similar in appearance to cells present in human rhabdomyosarcoma.

Attempts to pass this virus into cortisone-treated rats have been successful with tumors similar to those induced in mice appearing within 1 to 2 months. Continued passage in rats has resulted in death within 24–36 hours after inoculation. This phenomenon is at present under study.

An analysis of the results of the examination of human leukemic plasma pellets has demonstrated that there is a direct correlation between high white cell counts and plasma pellets positive ofr virus-like particles. No other correlation has been found.

It has been reported previously that an agent present in the plasma cell tumor MPC–2 induces reticulum cell sarcomas. Since these tumors are very similar to Hodgkins sarcoma in man, it would be important to determine if they are consistently induced by a viral agent. Studies under way suggest this as a possibility. In other experiments an agent has been demonstrated to be present in Sarcoma–37 which induces bone lesions in mice. These lesions are similar to the osteopetrosis induced in chickens by viruses of the avian leukosis complex. Evidence to date suggests that the agent from S–37 is the same as or closely related to the Moloney leukemia agent. This in turn suggests the existence of an important and fundamental similarity between the range of tumor types induced by viruses of the avian leukosis complex and viruses of the murine leukemia family. Improved transparent chambers were involved in the development of the information obtained from the last two studies. It should be noted that the head of the Virus Leukemia Section spends fully two-thirds of his working hours on administrative matters concerned with the SALE segment of the SVLP.

VIRAL LEUKEMIA AND LYMPHOMA BRANCH

Introduction

The Viral Leukemia and Lymphoma Branch was organized and officially approved in December of 1965 as a part of the overall reorganization of the National Cancer Institute. The personnel and intellectual nucleus of this Branch were derived almost entirely from the Laboratory of Viral Oncology. Dr. W. Ray Bryan, as Chief of this Laboratory, assembled and guided a group whose outstanding competence and productivity became internationally acknowledged. Organizationally the Branch as proposed will include sections on Comparative Viral Oncology, Immunology, Molecular Virol-

ogy, Ultrastructural Studies, and on Virus and Disease Modification. Laboratory personnel wh are currently functioning as Section Heads are: Dr. Mary Fink, Immunology Section, with 8 support personnel; Dr. Timothy E. O'Connor, Molecular Virology Section, with 5 support personnel; Dr. Robert F. Zeigel, Ultrastructural Studies Section, with 4 support personnel. In addition, the transfer of Dr. Michael A. Chirigos and 4 support personnel from the Chemotherapy area to the Viral Leukemia and ymphoma Branch will be consummated within this fiscal year. Dr. Chirigos will head the Virus and Disease Modification Section. Recruitment actions of key scientists are currently being processed. These and other actions and activities are being conducted in a manner that will take advantage of currently available space and equipment and in a manner that will allow a minimum of delay between the current level of activity and the expanded level which will be possible as soon as the new cancer building (Building 37) and the emergency virus isolation building are completed (approximately January 1968).

The principal activities conducted within the Branch during fiscal year 1966 are discussed briefly according to the following categories: (*a*) research and other activities conducted within the Branch, and (*b*) Special Virus-Leukemia Program.

Research and Other Activities Conducted Within the Branch

Dr. Mary Fink in collaboration with other members of the Branch, Institute, and outside laboratories has continued and extended her studies which are designed to evaluate existing techniques and to devise new methods for the serological and immunological detection and characterization of viruses from animal and human neoplasms. In particular, during the past year, these studies have been concerned with the techniques of immunofluorescence, micro-Ouchterlony (double diffusion in gels) and with specific hemagglutination of antigen coated tanned erythrocytes. In a longitudinal study of the bone marrows from 26 cases of acute human leukemia, 79% of the bone marrow specimens from patients in relapse showed positive immunofluorescence. Only 25 % of the bone marrow specimens from patients in remission showed positive immunofluorescence. In 6 of 8 human leukemia patients, followed through several cycles of relapse-remission, there was no evidence of the presence of immunofluorescent cells during remission. Dr. Fink and co-workers continued to test for immunofluorescence many different materials received from investigators throughout this country and abroad. In regard to the 21 different lines of human leukemia or lymphoma cells now maintained in tissue culture, the immunofluorescent findings parallel the finding of herpes-like virus in these cultures. In collaboration with Drs. Manaker and Dalton, several of these human lymphoma cell lines were examined for the purpose of establishing the optimal time during tissue culture for demonstrating immunofluorescence. These studies showed the fluorescence increases up to a maximum on the fourth or fifth day after fluid change. Virus particles are also more numerous as detected by electron microscopy 4 or 5 days after fluid change. These and other studies designed to control the specificity of the immunofluorescent reaction have been essentially confirmed in the laboratories of Drs. Yohn and Grace of the Roswell Park Memorial Institute and in laboratories of Dr. Zarafonetis at the University of Michign. A microdiffusion precipitation method using agar gels (Ouchterlony) was successfully adapted to the detection of several murine leukemia viruses. With the Rauscher strain, virus could be detected in the plasmas of viremic BALB/c mice as soon as 13 days post infection. Preliminary studies have indicated that the Rauscher and Friend viruses share at least 2 antigens but differ in at least 1 and that the Moloney leukemia virus shares at least 1 antigen with the Rauscher virus. This technique can be used not only to detect murine leukemia viruses but also to quantitate the amount of virus present in various systems and to differentiate the various murine leukemia viruses. A third technique which has been developed and applied to the detection and assay of murine leukemia virueses and their antiserums is that of hemagglutination of antigen coated tanned erythrocytes. This tech-

nique is not only useful as a quantitative measurement of antibody against murine leukemia viruses but also, by blocking the reaction with antigen, the technique becomes an exquisitely sensitive test for the presence and amount of virus in materials of unknown potency. As little as 0.2 micrograms per ml of antigen protein can be detected with this technique.

Each of the 3 above mentioned techniques are being intensively applied to the detection and quantitation of viruses and/or virus antigen presumed to be present in selected materials obtained from human leukemia and lymphoma patients. These *in vitro* monitoring techniques are of paramount importance to the human leukemia problem because it has not yet been possible to develop a sensitive laboratory animal system (including primates) which will support replication of and/or disease induction by candidate human leukemia viruses. It becomes necessary, therefore, to employ *in vitro* serological techniques not only to monitor for the presence of virus in laboratory systems but also to conduct sero-epidemiological surveys for the presence or absence of experience with selected viruses in different human population groups.

Dr. Robert Zeigel and staff continued their numerous collaborative activities with scientists of this Branch as well as those associated with other areas of NIH. In collaboration with Dr. Mary Fink, they obtained electron microscopic evidence for the presence of significant amounts of virus in mice 5 days following infection with a murine leukemia virus (Rauscher strain). This finding is important because it shows that large quantities of virus are present in animals shortly after infection and long before the infection process culminates in frank disease.

Dr. Zeigel's studies have also shown that tissue cultured cells which support active replication of murine leukemia viruses are also capable of phagocytosing virus particles produced and liberated by these same cells. This finding suggests a mechanism for a cyclic reinfection of cells and/or a means for the clearance and potential inactivation of extracellular infectious virus. Studies performed in collaboration with Dr. Gordon Theilen, a visiting investigator of NCI during fiscal year 1966, established the ultrastructure of avian reticuloendotheliosis virus. Although this virus produces a leukosis like disease, it appears to be morphologically different from other viruses of the avian leulosis complex.

During the past year Dr. Zeigel confirmed a extended his findings that type "A" virus particles are associated with all tumors induced in conventional and germfree BALB/c mice with tumor viruses and/or with chemical carcinogens. It has been shown that the endoplasmic reticulum of cells "infected" with type "A" particles is substantially modified. This finding is of potential importance in view of the possibilities that "A" type particles may (*a*) serve as biological precursors for the eventual appearance of leukemogenic "C" type particles, (*b*) interfere with the replication of virus or the induction of disease by "C" type particles, and (*c*) may serve as a helper virus for the replication of and/or diease induction by "C" type particles.

Studies conducted in the laboratory of Dr. Duc Nguyen, a visiting investigator, who joined the VLLB in August of 1965, are aimed at the application, to tumor viruses, of classical detection and bio-assay methods developed and used for studies of the necrotizing or nontumorigenic viruses. In particular, he has attempted to device an *in vitro* method for the bio-assay of murine leukemia viruses based on the production of interferon or the detection of interference of necrotizing viruses by the noncytopathogenic murine leukemia viruses. Studies are well under way in his laboratory to apply the ferritin labeled antibody technique of electron microscopy, used so successfully with influenze virus, for the detection and localization of murine leukemia viruses in infected cells. Dr. Nguyen was successful in establishing a line of embryonic rat kidney cells in tissue culture. These cells were infected with a murine leukemia virus (Rauscher strain) and appear to support the replication of more recoverable murine leukemia virus than any other cell line available for this purpose.

The goal of studies conducted by Dr. Timothy O'Connor as principal investigator is to

provide an integrated background of physical, chemical and immunological data on animal leukemia viruses toward a determination of the possible association of a virus or viruses with human leukemia and lymphoma. The predominant antigenic activity of murine leukemia virus (Rauscher strain) purified by density gradient centrifugation was shown by 3 different immunological techniques to be associated with the virus particle. It was also shown that both the Rauscher and Friend strains of murine leukemia virus after extensive purification on density gradients still showed a significant reaction with antiserum prepared against normal BALB/c mouse tissue antigens. This was not entirely unexpected since collaborative studies with Dr. Guy de Thé showed by electron microscopic techniques that purified murine leukemia viruses were coated with low amounts of host-derived enzymes. Experiments are in progress to determine whether these virus-associated, host-derived antigens arise by adsorption or from true structural integration into the viron.

Treatment of murine leukemia viruses with a combination of ether and the non-ionic detergent, Tween 80, resulted in emulsions which could be separated into ether and aqueous phases. Density gradient centrifugation of the ether extract yielded low density materials which contained considerable amounts of viral-specific antigen. It was not possible to definitely determine the morphological characteristics of these components. Density gradient centrifugation of the aqueous phases yielded materials having a density of 1.24 to 1.27. These materials, therefore, are much more dense than the intact virus and in addition did not react with viral-specific antisera or with antiserums prepared to BALB/c mouse antigens. Electron microscopically, 2 types of "subviral" particles were seen. The most common type was a polygonal structure, the center of which appeared to be occupied with a fibrillar material of low electron density. These particles may represent the naked nucleoids of the mature virus particle. The second type of subviral structure appeared as a rigid circular structure with an electron lucent core and in some instances appeared to have a helical structure composed of morphological subunits. These structures may represent the nucleoids of immature virus particles. These studies are important because they may make it possible to compare the chemical and physical characteristics of intact and degraded murine leukemia viruses to similar characteristics of viruses isolated from human leukemia and lymphoma materials. Dr. O'Connor's studies have made it possible to apply density gradient centrifugation to the routine quality control of virus production. This has been done in his own laboratory and, with his monitoring collaboration, is now being routinely conducted in commercial laboratories engaged in the production of large quantities of murine leukemia viruses under contract to the National Cancer Institute. This test is simple and rapid and provides an excellent estimate of the relative homogeneity and physical titer of different batches of murine leukemia viruses produced in different laboratories at different times.

Members of the Branch served as Project Officers on 9 contracts, the total funding level for which was approximately $3.7 million. The workscopes of these contracts include fundamental and applied studies on animal and human leukemias, research services, and the production of large quantities of highly purified concentrates of infective murine leukemia viruses. One of these contracts deserves special mention because it provides a critical resource and unique technical competence to the research activities of the Branch as well as to the entire Special Virus-Leukemia Program. The objectives of this contract (with Bionetics Research Laboratories, Inc.) are: (*a*) to determine whether newborn, mother-deprived primates of various species are susceptible to the oncogenic and/or leukemogenic effects of known viruses and of candidate viruses recovered directly from man, and (*b*) to test available means and develop new means to enhance the susceptibility of primates to virus replication and/or disease induction. Pursuant to these objectives, the contractor has provided, largely from his own breeding colony, over 700 newborn viable primates suitable for inoculation. These animals have been inoculated with high

priority materials received from over 50 different investigators from 40 different laboratories throughout this country and abroad.

Members of the Branch presented, by invitation, over 40 lectures on their studies and on the studies of others at various meetings and to many different research groups in this country and abroad. Considerable time was devoted to training, advising and collaborating with many different scientists from intramural and extramural laboratories. In particular, Dr. Mary Fink and staff have trained investigators in the theory and use of the immunofluorescent technique reported by Drs. Fink and Malmgren for the detection and monitoring of an antigen which appears to be associated predominantly with the cells of human leukemia and lymphoma patients. Similarly, Dr. Timothy O'Connor has trained visiting investigators in his own laboratory as well as in laboratories throughout this country in the use of density gradient centrifugation and other biochemical and biophysical techniques for the purification and characterization of viruses and of subviral products. During the past year members of the Branch have dispensed liter quantities of different types of murini leukemia viruses, and advise on effective utilization of these viruses to over 120 different investigators.

Drs. O'Connor, Fink, and Rauscher served on several different panels and committees among which were the Field Studies Contract Review Committee, the NCI Primate Study Group, Program Segment Working Groups of the Special Virus-Leukemia Program, and as members of the Civil Service Microbiology and Chemistry Panels.

Special Virus-Leukemia Program

During past year overall management of the Special Virus-Leukemia Program, which was planned and implemented in September of 1964 through a special appropriation, was placed within the Office of the Chief, Viral Leukemia & Lymphoma Branch. The main objectives of this Program are: (1) to determine whether viruses comparable to those now known to be associated with avian and murine leukemia are etiological agents of human leukemia, and (2) to develop an effective vaccine or other means for the prevention and/or control of human leukemia and lymphoma if such etiological agents are found. The main assumption or working hypothesis on which the overall Program is based is that at least one virus is an indispensable element for the induction (directly or indirectly) of at least one kind of human leukemia (including lymphoma) and that the virus persists in the diseased individual. The overall Program was originally planned and modified during and following numerous discussions with key program leaders and research scientists of NCI as well as with university and industrial personnel expert in the general areas of virology, oncology, chemotherapy, etc. From the research standpoint the program is divided into 4 major areas of effort: Human Leukemia Etiology and Prevention, Special Animal Leukemia Ecology Studies, Biohazards Control and Containment, and Human Leukemia Therapy. Operationally the Program has been divided into seven program segments: Developmental Research, Testing and Monitoring, Resources and Logistics, Epidemiology, Special Animal Leukemia Ecology Studies, Human Leukemia Therapy, and Biohazards Control and Containment. These working groups, which with the exception of the Human Leukemia Therapy Segment, are chaired by senior scientists within the Etiology Area who are responsible for the development of research programs in their respective segments within the context of the overall plan. Approximately 70 of 180 projects, which currently make up the program plan, are being conducted by investigators in governmental laboratories and clinical facilities, in universities, in nonprofit laboratories, and in commercial facilities. Selection and implementation of all projects are done on the basis of both scientific excellence and high priority relevance in terms of the integrated program.

NATIONAL HEART INSTITUTE

CARDIOLOGY BRANCH

The fundamental objective of the research program of the Cardiology Branch, NHI is to provide an increased understanding of the derangements in cardiac function which occurs in various forms of heart disease. In the pursuit of this long-range objective it has become evident that the mechanics and energetics of the normal contractile process are poorly understood and that gross clinical and hemodynamic studies on diseased hearts provide only superficial descriptions of disease processes. Accordingly, efforts have been underway for several years to analyze the behavior of the normal and diseased heart as a pump. More recently, these approaches have been broadened and cardiac performance is now also being analyzed from the point of view of the heart as a muscle. As in the past, investigations are simultaneously being carried out on isolated heart muscle, intact canine hearts, diseased canine hearts, as well as on patients with normal and diseased cardiovascular systems.

Mechanics and Energetics of Myocardial Contraction: Studies on Isolated Heart Muscle

The active state of muscle has been defined as a mechanical measure of those processes in the contractile elements which generate force and are responsible for shortening. In a study designed to determine the manner in which the active state in heart muscle is established and dissipated it was found that: (1) in contrast to skeletal muscle, the onset of maximum active state in heart muscle is delayed, developing 100 to 150 msec. after the first evidence of active state; (2) the maximum active state is maintained for about 100 msec. and does not decline until just prior to the development of maximum active tension; and (3) inotropic interventions which alter the contractile state of the muscle, such as changing frequency of contractions, or norepinephrine, accelerate the onset, increase the intensity, and hasten the decline of the active state.

There has long been controversy as to whether stimuli which exert a positive inotropic effect on the myocardium also induce a change in the true compliance of the heart muscle. Since these inotropic interventions are generally associated with substantial increments in the force of contraction, the possibility was considered that the changes in compliance, were they to occur, might be mediated through increases in systolic force per se rather than through a direct effect of the inotropic intervention on the contractile system of the muscle. However, if changes in diastolic compliance of heart muscle are indeed due to alterations in the contractile system, they should be apparent whether a muscle is contracting under isotonic or isometric conditions. On the other hand, should changes in systolic force alone explain these findings, then no change in compliance should be observed under isotonic conditions, but should occur whenever systolic force is increased, regardless of whether or not an inotropic influence is introduced. In a study on isolated papillary muscles it was observed that postextrasystolic potentiation, norepinephrine or calcium administration produced no change in diastolic compliance in the isotonically contracting muscle or in the afterloaded muscle in which increased shortening occurred with no change in force. However, isometric contraction consistently induced a small fall in diastolic tension when the force of contraction rose. It was concluded that inotropic interventions per se do not induce a true change in myocardial compliance. However, diastolic compliance may increase

when the force of contraction rises. These findings provide evidence for a series viscous component in heart muscle which is altered when changes in contractile force occur.

A study has also been initiated to compare the magnitude of stress relaxation that occurs in series viscous elements of isolated cardiac muscle with that occurring in another component which had not been defined previously and which has been termed the "parallel viscous component." This parallel viscous component appears to be several times as extensible as the series viscous component. It also appears that the interplay between effects due to these two viscous elements and their opposite effects on the resting length of the contractile element may serve to explain many of the phenomena associated with homeometric autoregulation of the heart.

The series elastic is one of the fundamental components of heart muscle and its properties must be characterized in order to allow an understanding of the behavior of the contractile elements. Accordingly, the series elasticity of cat papillary muscle was determined by a variety of techniques and revealed an extension of 5 to 10% of muscle length at a developed force of 10 gm. Inotropic interventions did not alter the series elastic component and calculation of contractile element velocity revealed that in auxotonic contractions contractile element velocity exhibits a secondary rise during the transition from the isometric to the isotonic phase. Data were also obtained to support the position that a portion of the series elastic is in series with both the contractile elements and the parallel elastic elements.

Although ultrastructural and biochemical similarities are known to exist in the contractile systems of cardiac and skeletal muscle fundamental differences in their force generating processes have been postulated. This view is based on a ten-fold disparity in force generating capacity between the two types of muscle, which has previously been reported. However, an accurate comparison of the force generating capacity of cardiac and skeletal muscle depends upon establishing a maximum contractile state of cardiac muscle and utilizing electronmicroscopic analyses to correct for its greater proportion of non-contractile elements such as mitochondria, nuclei and interfilamentous spaces. When this is applied to the cat papillary muscle preparation, the maximum contractile force of cardiac muscle achieved during paired electrical stimulation was found to be comparable to that reported for skeletal muscle, suggesting that the force generating mechanisms are basically similar quantitatively.

The mitral valve is generally thought to consist of elastic and fibrous tissue and its motion has been considered entirely passive, the results of changes in ventricular and atrial pressures. However, this view was re-evaluated by assessing the mechanical properties of the mitral valve in vitro. The anterior leaflet of the mitral valve was placed in a myograph. With stimulation, it was found that the valve contracts actively and as with other contractile tissues, the force of contraction was found to be a direct function of length. Electronmicroscopic study of the mitral valve has confirmed the presence of heart muscle underneath the valve. These studies have demonstrated the need for a total re-evaluation of the control of mitral valve motion both in normal and pathological states.

It is well known that stimulation of cardiac sympathetic nerves and the administration of norepinephrine (NE) increase the force of cardiac contraction. Thus, the sympathetic nervous system provides a mechanism for augmenting basal cardiac contractility, but it has not been known whether or not the cardiac stores of NE are necessary to establish normal baseline contractility and to maintain the potential for increasing contractility in response to positive inotropic interventions other than sympathetic stimulation. This question is of particular interest in view of the depletion of myocardial NE stores associated with congestive heart failure. Accordingly, in order to assess the role played by endogenous norepinephrine (NE) stores in the intrinsic contractile state of cardiac muscle, the right ventricular papillary muscles from normal cats and cats with cardiac NE depletion produced by chronic cardiac denervation or reserpine pretreatment

were studied. Length-tension curves, force-velocity relations, the effects of alterations in frequency of contraction and the response to sustained postextrasystolic potentiation were determined. In addition, the electrical excitability and absolute refractory periods were measured. All of these properties were found to be normal in both groups of NE depleted muscles. It is concluded that cardiac stores of NE are not fundamental for maintaining the intrinsic contractile state of the myocardium, its absolute refractory period or electrical excitability. Further, release of endogenous NE from cardiac muscle does not appear to play an essential role in the mediation of the positive inotropic effects of increasing frequency of contraction or of sustained postextrasystolic potentiation.

Major interest has been directed to the effects of sympathetic nervous system activity on cardiac contractility, but far less is known about the action of the parasympathetic nervous system. Acetylcholine (ACh), the parasympathetic neurotransmitter, is said to exert a positive inotropic effect on the mammalian ventricle, an effect which has been attributed to the release of stored NE. In order to test this hypothesis, the effect of ACh on normal cat papillary muscles was compared to the response of muscles from hearts depleted of NE by chronic cardiac denervation and reserpine pretreatment. In the absence of NE the positive response produced by ACh is unchanged from that observed in normal muscles. By examining the response to ACh over a wide concentration range and comparing it to that in isolated atrial tissue in the presence and absence of atropine, the following hypothesis was developed to account for an otherwise bewildering array of apparently contradictory data on parasympathetic control of the heart: There appear to be two types of receptor sites in the heart responsive to acetylcholine—Type I, intimately related to vagal nerve endings and whose stimulation is associated with a negative inotropic effect and Type II, unrelated to the vagus nerve and whose stimulation results in a positive inotropic effect. This study thus emphasizes the need for a reexamination of the role of the parasympathetic nervous system in the control of myocardial contractility.

The direct influence of the thyroid state on cardiac muscle has not been defined. While profound cardiovascular alterations are known to occur with hyper- and hypothyroidism, it is not clear from studies in the intact organism whether these result from fundamental changes in the heart muscle itself or merely reflect the heart's response to the systemic effects produced by these endocrine disorders. The papillary muscles isolated from cats rendered hyperthyroid and hypothyroid provides a preparation in which it is possible to exclude as much as possible the role that neurohumoral and metabolic factors might play in indirectly altering the contractility.

The level of thyroid state was found to have a profound influence on the intrinsic contractile state of isolated papillary muscles. Those parameters related to the speed of contraction, i.e. the V_{max}, the time to peak tension, the rate of tension development, and latency were particularly affected, in a manner indicating that the basic processes controlling the rate of force generation are accelerated by increased levels and slowed by decreased levels of thyroid hormone. These effects were found to be independent of temperature, frequency of contraction, and endogenous NE stores. The level of thyroid hormone was found to have less effect on contractile force than on the speed of its development, but the positive inotropic response to paired electrical stimulation, NE, and strophanthidin are dependent on the thyroid state. Thus, it appears that thyroid hormone may directly affect the contractile state of heart muscle and condition the responsiveness of the heart to other agents that act upon it.

In order to define the chemical energetics of cardiac muscle, the utilization of ATP and creatine phosphate (CP) in the papillary muscle of cat heart was measured. To accomplish this, cat right ventricular papillary muscles were poisoned with iodoacetic acid and nitrogen to prevent further production of ATP and CP. The basal consumption of high energy phosphate was determined in resting muscles with-

out tension by freezing muscles at various times after poisoning and assaying these muscles for CP and ATP. It was found that the utilization of high energy phosphates could be accounted for entirely by the disappearance of CP, indicating that creatine phosphokinase was intact.

The basal rate of CP consumption was 0.71 μmoles/g/min. Increasing resting tension (or muscle length) was found to increase basal CP consumption to a linear fashion over the physiologic range of muscle lengths. In addition, CP utilization was determined in 49 isometrically contracting muscles stimulated to contract 10 to 50 times at the top of their length-tension curves. The utilization of CP by these muscles could be accounted for by a prediction equation with terms for the number of activations and the calculated contractile element work. The mechanochemical efficiency of cardiac muscle, defined as the work done divided by the energy cost of work plus activation, averaged 39% in these studies. This is similar to the efficiency of skeletal muscle previously determined by other investigators.

The in vivo steady state levels of myocardial high energy phosphate stores (adenosine triphosphate-ATP and creatine phosphate-CP) may indicate the relative balance of energy production and utilization in the heart. Techniques developed in this laboratory have made it possible to determine in vivo stores of ATP and CP in rapidly frozen myocardial biopsies. Three groups of cats were studied in addition to normal controls: (1) cats with right ventricular hyertrophy and failure; (2) cats made hyperthyroid; and (3) cats made myxedematous. Right ventricular stores of ATP were not found to be significantly different in these groups. Right ventricular stores of CP were found to be depressed in hyperthyroid and pulmonary artery constricted cats. Right ventricular creatine stores were depressed in all three states. These findings indicate that the contractile state of the heart is not dependent on its high energy phosphate stores. In hyperthyroid cats, the absolute depression of CP stores may be related to a depression of total creatine stores. In pulmonary artery constricted cats, the low CP/total creatine ratio may indicate a relatively higher demand/production ratio for chemical energy.

Application of Myocardial Mechanics to the Intact Heart

In order to increase understanding of the gross architecture and the ultrastructure of the left ventricle during various phases of the cardiac cycle, a technique was developed for the rapid fixation of the canine left ventricle during various phases of the cardiac cycle. A Gregg cannula was placed in the left main coronary artery, and at a selected time during diastole or systole, the fixing agent glutaraldehyde, preceded by a bolus containing potassium chloride and acetylcholine, was injected with a power syringe. Silastic casts of the left ventricles were then prepared, and ventricular volumes measured directly. Portions of the myocardial wall were prepared for electron microscopy, sectioned and analyzed for sarcomere lengths. Sarcomeres of the ventricular wall have averaged 2.10 μ during diastole and 1.84 μ during systole. In the acutely dilated heart, sarcomeres of more than 2.25 μ have been observed with the formation of H zones. Analyses of the gross dimensions of the fixed ventricle and of the silastic cast of its chamber have also been undertaken. Data have been obtained on ventricular wall thickness at the apex and at the waist of the left ventricle during systole and diastole, and on the base to apex and base to outflow tact dimensions.

The relations between tension and the velocity of shortening in the intact left ventricle of the dog were examined in a manner analogous to that employed in isolated muscle, i.e. by serial, reproducible variations in the afterload alone, from a constant end-diastolic volume. Sudden increases or decreases in the aortic pressure during diastole were produced, and ejection rate was measured with an electromagnetic flowmeter; left ventricular wall tension and the shortening velocities of the myocardial fibers and the contractile elements were then calculated. By analyzing isovolume points early in ejection, effects resulting from two

other determinants of shortening velocity, i.e. duration of the active state and the instantaneous muscle length, were minimized. Shifts in the basic force-velocity relation with alterations in V_{max}, obtained by extrapolation, and maximum tension were clearly demonstrated. Norepinephrine and paired electrical stimulation caused a shift in this relation to the right, with increases in velocity at any tension. Similar shifts were also apparent in curves relating tension to velocity, calculated during single isovolumic contractions. It was suggested that determination of these relations expands traditional definitions of ventricular performance, and that estimation of changes in maximum velocity as well as maximum strength relative to muscle length provides direct information concerning alterations in the contractile state of the intact heart.

The contractile state of the intact canine left ventricle was then studied by analyzing the instantaneous relations between force and contractile element velocity (V_{CE}) during the course of single isovolumic beats, and the sensitivity of this relation was compared to that of the ventricular function curve by exerting small inotropic influences. In 7 dogs, norepinephrine always shifted the isovolumic force-velocity (FV) curve, increases occurring both in maximum V_{CE} and maximum tension (P_o); the ventricular function curve was unchanged in 4 of the 7 dogs. Moderate hypothermia (avg. 30.8° C) in 4 dogs increased P_p, with no change or a fall in maximum V_{CE}; the ventricular function curve was shifted upward and to the left in 2 of these dogs and unchanged in 2. In 4 dogs moderate increases in heart rate (avg. 32 beats/min) increased the maximum V_{CE}, with little change in P_o; no shifts in the ventricular function curve occurred. Thus, the isovolumic FV curve was more sensitive than the ventricular function curve in detecting changes in myocardial contractile state, and provided more complete definition of alterations in left ventricular performance by allowing separation of effects due to changes in shortening velocity from those due to alterations in the strength of myocardial contraction.

The effects of increasing heart rate on the relationship between contractile element velocity and myocardial wall tension were determined from single isovolumic contractions in 9 canine right heart bypass preparations. Increases in heart rate ranging from 10 to 70 beats per minute were produced by crushing the sino-atrial node and stimulating the right atrium. An increase in heart rate always produced an increase in maximum calculated contractile element velocity and in other variables reflecting the speed of contraction, such as peak aortic flow rate, the peak first derivative of the left ventricular pressure pulse, peak contractile element power and the time to peak isovolumic tension. Maximum isovolumic tension usually increased. It is concluded that in the intact canine left ventricle, increasing the frequency of contraction always increases the maximum contractile element velocity and maximum isovolumic force of contraction.

The effect on contractile force of changing heart rate had not been determined directly in man, and accordingly a study was undertaken to provide definitive information concerning this relationship. At the time of corrective cardiac surgery a Walton-Brodie strain gauge arch was sutured to the right ventricle of 8 patients. In man, a "velocity staircase" i.e. an increase in the rate of tension development rather than a "force staircase" i.e. an increase in the peak tension development, occurs with changes in heart rate. This mechanism apparently permits the human ventricle to maintain its force of contraction and to preserve the duration of diastolic filling with increases in rate.

It has been suggested that serotonin, known to act on vascular smooth muscle and known to be present in the heart, may play a role in controlling cardiac contractility. The direct effects of serotonin on cardiac muscle have been examined in the isolated cat papillary muscle and in the intact dog heart, utilizing the right heart bypass isovolumic preparation described above. Preliminary results indicate that serotonin produces an increase in isometric tension in the isolated muscle and a shift in the isovolumic force-velocity relationship to the

right, both observations reflecting a positive inotropic effect. The physiologic significance of these findings is currently under investigation.

An attempt has been made to determine whether the technique of sudden occlusion of left ventricular outflow could be applied to the study of force-velocity-length relationships in the intact unanesthetized dog. In addition, efforts have also been directed to deriving, for the first time, normalized values for the force-velocity-length relations in a group of normal intact dogs and to use these as a basis for comparison with the relations obtained in dogs subjected to various chronic interventions.

Several weeks before the experimental procedure a thoracotomy is performed. The pericardium is sutured to the left chest wall to facilitate later percutaneous left ventricular puncture. Isovolumic left ventricular contractions are produced by balloon occlusion of the ascending aorta during diastole. Analysis of left ventricular pressure and the rate of development of pressure in isovolumic beats has been found to give meaningful force-velocity-length relationships in the lightly sedated, closed chest dog. Blood volume loading, by increasing cardiac filling and end-diastolic left ventricular fiber length, shifts the force-velocity curve to the right with an increase in maximum isovolumic tension development but produces no change in maximum velocity of contractile element shortening. Positive inotropic influences (digitalis glycosides, catecholamines, and paired electrical stimulation) produce changes in the force-velocity-length relationships similar to those seen in isolated cardiac muscle. Beta-adrenergic blockade in the intact dog depresses the left ventricular force-velocity curve, both in the presence and absence of digitalis glycosides. In addition, the relationships between left ventricular tension and shortening in ejecting contractions, and maximum tension development in isovolumic beats promises to provide further insight into the mechanics of left ventricular function in the normal intact animal.

As indicated above, it has been shown in studies in the intact dog heart that the relation between instantaneous myocardial wall tension and the velocity of fiber shortening during systole provides a sensitive means of examining the contractile state of the left ventricle (LV). Previously, it has not been possible to quantify this important relation in the human LV. A study was therefore undertaken to determine the course of tension development during systole and the velocity of circumferential shortening of the human LV and to examine the instantaneous tension-velocity relation in patients with and without LV dysfunction.

In 15 patients, LV pressure was measured continuously with a cathetertip transducer, while radiographic contrast material was injected into the left atrium. The true radius (r) of the minor LV circumference (circ.) was then determined at 170 msec. intervals from cineangiograms, and this measurement correlated with instantaneous LV pressure (Pr), the tracing of which was recorded directly on the cine film. The velocity of circumferential shortening (V_{CF}) was calculated as $2_{\pi}dr/dt$. Wall tension in the corresponding slice of muscle was computed as:

$$\frac{Pr \times r}{\text{Wall thickness}}$$

In patients without LV disease, the maximum V_{CF} averaged 2.05 circ./sec.; the corresponding wall tensions ranged from 374 to 384 gm./cm^2. In the patients who exhibited other hemodynamic evidence of LV disease, the maximum VCF's were consistently lower and averaged only 0.95 circ./sec., at levels of wall tension comparable to those observed in patients without evidence of LV dysfunction.

These measurements provide the first description of the tension-velocity relation in the intact human LV. In addition, the normalized measurements of VCF allow comparisons of the contractile state of the LV in patients with and without LV dysfunction. These preliminary results indicate that the contractile state of the LV, as determined from the basic myocardial tension-velocity relation, can be assessed by the technique employed, and clearly serves to distinguish normal from abnormal LV function.

Contractile Properties of the Failing Heart

Studies on Isolated Heart Muscle and Subcellular Fractions

It has long been appreciated that a quantitative definition of the contractile state of cardiac muscle obtained from hypertrophied and failing hearts is needed. The isolated cat papillary muscle preparation allows quantification of intrinsic cardiac contractility per unit of muscle, as well as determination of the responses of heart muscle to inotropic interventions. The contractile properties of normal papillary muscles can be compared with corresponding muscles from abnormal hearts. Accordingly, techniques were developed for the production of right ventricular hypertrophy and right heart failure in cats by graded constriction of the main pulmonary artery.

Doubling of right ventricular weight occurs within several weeks after constriction. When the degree of pulmonary artery constriction is less severe, ventricular hypertrophy occurs with right ventricular systolic hypertension but without evidence of heart failure. In animals in which the pulmonary artery is constricted more severely, hypertrophy is accompanied by heart failure with visceral congestion, ascites, pleural effusion, elevated right ventricular end-diastolic pressure and decreased cardiac output.

The muscles from cats with cardiac hypertrophy and heart failure demonstrated profound depressions of the maximum isotonic velocity of shortening as well as of the maximum isometric tension. It is concluded that congestive heart failure is associated with profound abnormalities in the contractile state per unit of heart muscle, with reduction of both the functional level of contractile state and its ceiling or maximum. These abnormalities do not appear to be dependent on an alteration of the normal relation of sarcomere lengths to length-tension dynamics. Further it is tentatively proposed that ventricular hypertrophy alone, in the absence of demonstrable heart failure, is associated with depression of contractile state per unit of cardiac muscle, although the absolute increase in total muscle mass may be sufficient to maintain cardiac compensation.

It has been the general impression that the hypertrophied ventricle is less distensible than normal. This might be due either to an increase in the total muscle mass or to the decreased compliance of individual fibers. Accordingly the effect of hypertrophy on the resting length-tension relationship of isolated segments of normal and hypertrophied rat ventricles was determined. It was observed that although the hypertrophied ventricles exceeded the normal ventricles by an average of 20% in weight, there was no evidence that the hypertrophied myocardium is less distensible than normal, if proper corrections for differences in weight, length, and cross-sectional area were made.

Collagen comprises approximately 4 percent of the dry weight of heart muscle and may contribute to the diastolic compliance of the ventricle. The few investigations of the connective tissue content in cardiac hypertrophy have not revealed a change in the collagen to muscle ratio in hypertrophied ventricles. However, only the central part of the ventricular wall, after removal of the epicardial and endocardial surfaces, has been studied. Since the surfaces also contribute to the hemodynamic characteristics of the entire ventricular wall it seemed important to determine if any area of the hypertrophied ventricle had altered amounts of collagen. Accordingly, tissue collagen content was determined in normal cat ventricles and in those with right ventricular hypertrophy due to banding of the main pulmonary artery. Each ventricle was split into epicardial and endocardial halves for determination of collagen content and the ultrastructure of these muscles was also examined. The hypertrophied right ventricles had markedly and significantly increased quantities of collagen per unit of muscle, while the left ventricle from the same hearts had normal collagen values. The epicardial half of the right ventricles had the highest collagen content but the content was also increased in the endocardial half. Large bundles of connective tissue were evident in the ultrastructural studies of the right ventricles.

In vitro function of mitochondria from failing hearts has been variously reported as normal and abnormal. A re-evaluation of the question of mitochondrial integrity in heart failure has become necessary because of: (1) the recent development of polarographic techniques offering more accurate data than manometric techniques; and (2) the ability to determine precisely the physiologic function of failing heart muscle. Chronic heart failure was induced in cats by tight constriction of the pulmonary artery and in guinea pigs by tight constriction of the aorta. The presence of heart failure in the cats was identified by: (1) in vivo cardiac catheterization indicating high end-diastolic pressures; (2) pathologic findings such as ascites and massive right ventricular (RV) hypertrophy; (3) depressed mechanical performance of the isolated RV papillary muscle studied in vitro. After isolation of mitochondria from the right ventricle (cats) and left ventricle (guinea pigs), polarographic determinations of oxygen consumption (QO_2), respiratory control ratio (RC), and the ratio of phosphate esterified with ADP to the oxygen consumed (P/O ratio) were carried out. Determinations were performed at 26° C and 37° C with both pyruvate/malate and glutamate substrates. Initial studies have indicated no abnormalities of QO_2, RC or P/O ratio in mitochondria isolated from hearts in experimental heart failure.

The possibility that there may be a bioenergetic defect in the myocardium involving an impairment of oxidative phosphorylation in heart failure in man was examined in myocardial tissue obtained from patients at the time of cardiac operations. Mitochondria were isolated from papillary muscles removed from the left ventricle during replacement of the mitral valve in 11 patients with left ventricular failure. Measurement of oxidative phosphorylation with pyruvate/malate as substrate gave P/O ratios averaging 2.8. Respiratory control ratios, determined both manometrically and polarographically averaged 6.6 with pyruvate/malate and 5.9 with alpha-ketoglutarate. Endogenous ATPase activity averaged 0.14 μmoles P liberated per minute per mg mitochondrial N; it averaged 0.36 with Mg^{++}, 1.79 with 2,4-dinitrophenol, and was completely inhibited by oligomycin. These values are comparable to those reported for normal mitochondria obtained from experimental animals.

Examination of myocardial tissue by electronmicroscopy revealed no apparent abnormality of mitochondrial size or crystal pattern. Creatine phosphate was determined in rapidly frozen ventricular biopsies from 15 patients with heart failure undergoing valve replacement. The mean tissue concentration was 4.0 μmoles per g compared to 4.1μmoles per g in similar biopsies from 5 patients without heart failure. These biochemical studies indicate that electron transport and coupled phosphorylation appear to be normal in mitochondria isolated from failing human hearts and that there is no consistent reduction of the myocardial store of high energy phosphate. It is concluded that the formation of chemical energy is not impaired in the failing heart, and it is suggested that the biochemical abnormality responsible for defective myocardial function involves utilization of energy in the contractile process.

Studies on the Intact Dog Heart

There is general agreement that both the high energy phosphate stores and the mechanical performance of the myocardium are depressed during severe hyposia. To determine whether a casual connection exists between these two phenomena, 18 dogs were respired with 6% O_2, 94% N_2 after sympathetic blockade (hexamethonium and propranolol). Serial left ventricular (LV) biopsies were obtained as myocardial failure occurred, and the concentrations of adenosine triphosphate (ATP) and creatine phosphate (CP) were measured. A transient increase in left ventricular stroke work during initial hypoxia was followed by a progressive rise in left ventricular end-diastolic pressure and fall in left ventricular stroke work. There was no change in mean ATP, even with severe heart failure. With early failure a significant fall in CP occurred in 10 of 18 dogs, and with late failure CP was always depressed. Since ATP, the final energy source for contraction, was unaffected in all dogs and

CP, the secondary energy store, was not depressed when failure first developed in some animals, it is concluded that hypoxic depression of myocardial function is not initiated by a depression of the total myocardial high energy phosphate stores.

The levels of high energy phosphate stores and the status of anaerobic metabolism at the onset of ischemic heart failure induced by restricting flow to the left coronary artery were also studied. Left ventricular biopsies were obtained before ischemia and shortly after the onset of ischemic heart failure for measurement of concentrations of ATP and CP. Samples of blood were drawn from the aorta and coronary sinus for determination of lactate and pyruvate concentrations. At the time of failure, arteriovenous lactate differences decreased from an average of 1.29 to 0.60 mM and "excess lactate" increased by an average of 1.74 mM. There was no change in mean ATP (control, 6.45 vs. failure, 6.49 μmoles/g). Mean CP concentrations fell significantly (12.84 to 8.00 μmoles/g) but concentrations of CP were within 2 SD's of paired control samples in 5 of 19 observations. It is concluded that heart failure may be induced in the presence of relatively minor degrees of anaerobic metabolism, and that the total high energy phosphate stores are not necessarily compromised at this time.

Depressions of cardiac norepinephrine stores have been described in this laboratory in chronic heart failure in man and in experimental heart failure in the dog, cat and guinea pig. Disturbances have been noted in both the uptake and storage of exogenous norepinephrine, although the relative rate of norepinephrine turnover is normal. These findings have suggested that there may be an absolute loss of sympathetic nerve endings in the heart in chronic heart failure. Tyrosine hydroxylase is the rate limiting enzyme in the synthesis of norepinephrine. It was thought, therefore, that the determination of cardiac tyrosine hydroxylase activity might provide insight into the mechanism of the depletion of norepinephrine stores in heart failure. Initial studies of myocardial tissue extracts have demonstrated the possibility of assaying tyrosine hydroxylase activity in canine right ventricle and have suggested that the activity of this enzyme is low in chronic right ventricular failure in dogs.

As outlined in an earlier section of this report, other studies in this laboratory have shown that analysis of the tension-velocity relation throughout a single isovolumic beat provides a sensitive index of the contractile state of the intact left ventricle of the dog. Accordingly, the effects of acute experimental heart failure on tension-velocity relations during isovolumic contractions and auxotonic contractions were studied. Cardiac failure was produced by infusion of large doses of the beta-adrenergic blocking agent pronethalol, or by infusion of barbiturates. The right heart bypass preparation was used to control cardiac output. Blood flow was measured with an electromagnetic blood flow transducer positioned about the aortic root. When ventricular end-diastolic volume was maintained constant, the tension-velocity relation during isovolumic contraction was always shifted markedly downward and to the left during acute failure of the left ventricle, with marked reductions both of maximum measured contractile element velocity, the extrapolation to V_{max}, and the maximum isovolumic tension (P_0). Acute failure also produced a decreased extent of fiber shortening and an increase in the ratio of the tension actually developed (P), to P_o. In some experiments, stroke volume was held constant so that end-diastolic pressure and volume were increased during acute cardiac failure. Although, under these circumstances, maximum measured velocity and V_{max} are depressed, P_o remains unchanged or more often is actually increased during acute failure. Furthermore, it appears that while total contractile element work may not be greatly affected, the ratio of internal contractile element work to total work may be increased substantially.

A marked augmentation of myocardial contractile force regularly accompanies paired electrical stimulation, a technique in which two stimuli are repetitively delivered to the ventricle with such timing that the second stimulus of each pair is introduced at the termination of

the preceding refractory period. Profound positive inotropic effects have been demonstrated in isolated heart muscle, in a variety of animal preparations and in conscious human subjects. However, the potential clinical usefulness of this technique has been sharply restricted by the finding that this augmentation of ventricular contractility is not always translated into an elevation of cardiac output. Accordingly, an investigation was carried out in order to identify circumstances in which the improvement of myocardial contractility produced by paired electrical stimulation would result in an elevation of the cardiac output.

The effects of paired stimulation were studied in intact anesthetized dogs before and after barbiturate induced depression of myocardial performance. In the non-depressed state, paired electrical stimulation exerted a positive inotropic effect but tended to impair circulatory performance, lowering cardiac output and arterial pressure. On the other hand, when applied during barbiturate-induced myocardial depression, paired electrical stimulation elevated cardiac output by an average of 129 percent, increased mean arterial pressure by 34 mm. Hg, and lowered mean right atrial pressure by 2.4 mm. Hg. The effects of paired electrical stimulation were compared to those of acetylstrophanthidin and were found to be quite similar both in the failing and non-failing heart. It was concluded that the effects of paired electrical stimulation are dependent on the circulatory state which exists at the time it is applied.

Clinical Studies

The maximum cardiac output response to intense exercise might be limited either by the pumping ability of the heart, or by extracardiac factors limiting ventricular filling at a time when the ventricles are still capable of augmenting the cardiac output. Previous studies have suggested the latter mechanism, since a relationship between total blood volume and maximum exercise capacity has been demonstrated; the increased exercise capacity produced by training is associated with an augmented blood volume, and an impaired circulatory response to maximal exercise has been produced by decreasing blood volume. However, it is not known if cardiac output during maximal exercise can be elevated by acute expansion of blood volume. Accordingly, six normal men were studied at rest and during maximal treadmill exercise before and after an acute infusion of 1000–1200 ml. of the subject's own blood. Transfusion increased resting central venous pressure from an average of 0.0 to 1.9 mm. Hg and cardiac output from 5.34 to 6.81 L/min. ($p<0.01$). During exercise the augmented blood volume increased central venous pressure from an average of 1.2 to 8.6 mm. Hg, but cardiac output, which averaged 21.60 L/min. in the control studies, was not significantly changed following transfusion (21.62 L/min.); maximum O_2 uptake and the postexercise O_2 debt were also unaltered. These results indicate that since total blood volume and ventricular filling pressure normally do not limit maximum cardiac output and O_2 uptake, cardiac performance during maximum exercise must be limited by the pumping ability of the heart.

Left ventricular function has been analyzed in a variety of patients with and without cardiac dysfunction by assessing the cardiovascular responses to supine muscular exercise on a bicycle ergometer. Left ventricular end-diastolic pressure was measured continuously and cardiac output and oxygen consumption were determined before and during exercise. A normal pattern was established in 7 patients without left ventricular dysfunction and consisted of an exercise factor equal to or greater than 600 ml./100 ml. VO_2, a left ventricular end-diastolic pressure (LVEDP) during exercise of less than 12 mm. Hg, and little change or a decrease in LVEDP, which was accompanied in most instances by an increase in the stroke volume. This response was then compared with that observed in 14 patients studied after stenotic or regurgitant malformations of the aortic valve had been corrected by valve replacement with a Starr-Edwards prosthesis, and with that seen in 31 patients with various other cardiac lesions.

In the majority of patients with mitral stenosis the pattern of left ventricular function during exercise was considered to be normal, and in 5 patients with aortic valve pros-

theses, a normal pattern was also observed. Among the remaining patients, including those with prostheses, aortic stenosis, or left ventricular myocardial disease, two types of abnormal performance of the left ventricle were identified. In some patients, an increase in LVEDP was accompanied by an increase in the stroke volume, a response considered consistent with abnormal left ventricular dynamics in which the ventricle appeared to utilize primarily the Frank-Starling mechanism. In the remaining patients, an increase in LVEDP was accompanied by no change or a fall in the stroke volume, a response considered indicative of depressed left ventricular function. Thus, in the majority of these patients, determination of the LVEDP before and during exercise permitted definition of normal or abnormal left ventricular function. The method described thus appears to supply a practical and useful means of evaluating the functional status of the left ventricle in patients with and without myocardial dysfunction.

Although a considerable body of information is available concerning the circulatory response of patients with heart disease to exercise in the supine position, little is known about the circulatory response of these patients to exercise in the upright posture. Since many of the symptoms of cardiac patients occur during exertion in the upright posture, it was considered that a thorough understanding of the hemodynamic alterations occurring during mild to intense upright exercise would be of physiological and clinical importance. It was also hoped that a more reliable means of evaluating the relative impairment of cardiac function could be obtained. Accordingly, patients with various types of heart disease and normal subjects were studied during mild and intense levels of treadmill exercise. A Cournand catheter was introduced into the pulmonary artery in order to measure pulmonary arterial pressure and to obtain samples of mixed venous O_2 content. Cardiac output was measured by the Fick principle, and oxygen consumption (VO_2), arterial blood pressure, and the electrocardiogram were continuously recorded.

When cardiac output was plotted against VO_2 in the conventional manner, considerable overlap was found between the two groups both in the absolute values of cardiac index and VO_2, as well as in the slope of the line relating these two variables. Since the "exercise factor" describes the slope of the line relating cardiac output to VO_2, these results demonstrated that the "exercise factor" is an insensitive method for assessing the degree of cardiac impairment. On the other hand, if the cardiac index is plotted against the pulmonary artery (PA) O_2 saturation, a clear difference in the response to exercise between normal subjects and patients was evident. At lower levels of exercise (PA saturation $>$ 40%) the cardiac output response of the two groups did not differ greatly. However, at levels of exercise which produced a PA O_2 saturation of 35% or less, the circulatory response of the patients contrasted dramatically with that of normal subjects. It was found that at a PA O_2 saturation of 30%, all normal subjects achieved a cardiac index greater than 7 liters/min, while no patient exceeded a value of 5 liters/min. Thus, the level of the cardiac index at a PA O_2 saturation of 30% provides a new and sensitive index of cardiac function which may prove to be of considerable clinical value.

A comparison of the hemodynamic responses to exercise in the supine and upright positions was undertaken in patients with varying degrees of impaired cardiac function. In patients with insignificant cardiac defects, cardiac output, mixed venous O_2 saturation, and pulmonary arterial pressure were slightly but consistently higher at any given level of VO_2 in the supine position as compared to the upright. In contrast, in the patients with significant cardiac impairment, much higher pulmonary arterial pressures occurred in the supine position than during upright exercise. It also appeared that there was less of a difference between the cardiac output during exercise in the supine and upright positions in the patients as compared to the subjects with insignificant cardiac disease.

Two of the more prominent manifestations of imparied cardiac function are an inability to augment the cardiac output appropriately in response to increased metabolic demands, and a diminished abiilty to excrete Na, with re-

sultant fluid accumulation and edema. Since it has recently been observed that the cardiac response to maximal and submaximal levels of exercise in both normal subjects and patients with various forms of heart disease is reduced by blockade of the beta-adrenergic receptors, it became of interest to determine if this impairment of hemodynamic performance is associated with changes in the patterns of Na excretion.

Sixteen subjects, 13 men and 3 women, ranging in age from 21 to 57 years were studied. Six of the men were normal volunteers; the remainder of the subjects had heart disease of various types. Beta blockade altered the pattern of Na excretion in all subjects, and this impairment of Na excretion could be divided into three grades of severity. The mildest degree of impairment, observed in normal subjects and patients with reduced cardiovascular reserve, consisted of an alteration in the diurnal pattern of Na excretion, the total 24-hour Na excretion remaining unchanged. An intermediate degree of impairment, observed in some patients with heart disease, but not in normal subjects, was manifest by retardation of the rate of increase of Na excretion in response to progressive increases in Na intake, although Na balance was ultimately achieved. The most serious degree of impairment, observed in only one patient with heart failure, consisted of progressive Na retention, resulting in increasing fluid accumulation and edema. Although the results of this investigation suggest that beta-adrenergic blockade will not cause gross Na retention in most cardiac patients, it is stressed that inhibition of cardiac sympathetic nerve activity can occasionally precipitate dangerous cardiac decompensation unassociated with prior Na retention and weight gain. The results of this study further demonstrate the importance, particularly in patients with impaired cardiac function, of the support which the sympathetic nervous system provides to myocardial performance.

Although there has been extensive investigation of the central circulation in patients with heart failure, little information is available concerning possible abnormalities of the peripheral vascular beds in these patients. Furthermore, only a few of the several variables of the regional circulations have been correlated both among themselves and with the variables of the central circulation, even in normal subjects. At rest, forearm blood flow was found to vary directly with cardiac output, forearm resistance varied directly with total systemic resistance, venous tone varied directly with forearm resistance and inversely with cardiac output. Patients with heart failure always had lower values for forearm flow and higher values of forearm resistance and venous tone, providing complete separation of the two groups in the above comparisons. Cold stimulation and leg exercise resulted in an excessive elevation of forearm resistance and venous tone in patients with heart failure as opposed to normal subjects.

It has been established that the elevation of the lower extremities in supine patients with congestive heart failure results in a decrease in forearm blood flow, while in normal subjects an increase in flow occurs. The mechanism of this paradoxical response in congestive heart failure has not been elucidated. In studies in which arteriolar and venous tone of the forearm were determined by plethysmographic means, it was found that leg raising resulted in arteriolar and venous constriction in heart failure while the opposite effect was seen in normal subjects. In addition, it was observed that local nerve block in the forearm abolished the alteration of forearm blood flow in both groups of individuals, indicating that the vasoconstrictor response in heart failure and the vasodilator response in the normal subjects was mediated through a reflex. Since it is known that rapid changes in atrial pressure are important in initiating the abnormal vasoconstrictor response to leg raising in heart failure, it is postulated that a critical increment in right atrial filling pressure reflexly initiates the response and these findings suggest that stretch receptors exist in these low pressure areas of the central circulation.

The reactive hyperemia response, that is, the increase in blood flow immediately after restoration of the circulation after a period during which arterial inflow is occluded, was com-

pared in normal subjects and in a large group of patients with congestive heart failure, utilizing a plethysmographic technique. It was observed that the peak level of reactive hyperemia as markedly reduced in the patients with heart failure compared to the normal subjects. The magnitude of the reduction of the hyperemic response was found to be a function of the duration of the period of circulatory arrest in normal subjects, progressive increases in postischemic blood flow occurring as the duration of ischemia was prolonged, but this was not observed in the patients with heart failure. The mechanisms responsible for this phenomenon have not yet been elucidated, but are currently under investigation. It is unlikely that increased sympathetic vasoconstrictor activity can be held responsible, since local vasodilator metabolites are known to be capable of overriding neurogenic effects. It would appear that the heart failure state in some manner increases the rigidity of the resistance vessels, and their altered mechanical properties do not allow them to respond in a normal fashion to the vasodilator metabolites that accumulate during circulatory arrest.

Studies on the Mechanism of Action of Digitalis

The digitalis glycosides are unquestionably the most important drugs used in the treatment of heart failure. Their mechanisms of action have been studied in this Branch since its establishment and these investigations were continued during the past year.

Several investigators have proposed that the positive inotropic effect of digitalis on the heart is due, at least in part, to norepinephrine (NE) released from cardiac NE stores by the glycosides. Since this proposal is based on the finding of a decreased inotropic effect of digitalis after depletion of cardiac stores of NE by reserpine or dichloroisoproterenol, the possibility was considered that this finding was due to a direct action of these antiadrenergic drugs rather than the depletion of NE. To examine this question of the responses of papillary muscles depleted of NE by chronic cardiac denervation and those in which the NE depletion was produced by reserpine pretreatment were studied. The muscles depleted of NE by chronic denervation respond normally to oaubain and strophanthidin while those equally depleted of NE by reserpine exhibited a reduced inotropic response to these glycosides. It is concluded that cardiac NE stores are not essential for the positive inotropic effect of strophanthidin or ouabain on the heart and that reserpine may interfere directly with the inotropic action of these cardiac glycosides.

While digitalis glycosides improve the failing myocardium, their effects on the normal heart remain controversial. Newer methods developed in this laboratory permitted resolution of this controversey. The effects of ouabain (0.01 mg/kg) on ventricular force-velocity relations were studied in six patients following corrective cardiac operations. A beat-to-beat analysis of ventricular force-velocity relations was performed by relating the velocity of movement of roentgenopaque markers previously sutured to external surfaces of the ventricles and intraventricular pressure at constant ventricular dimensions. It was observed that ouabain always augmented myocardial contractility as reflected in the force-velocity relation. Velocity of shortening increased an average of $77\pm5\%$ above control while intraventricular pressure rose by an average of $23\pm6\%$. Despite this improvement in contractility, no consistent changes in cardiac output were observed. Analogous changes in force-velocity curves were obtained when a cardiac glycoside was added to isolated papillary muscles removed from normal cats. It is concluded that the fundamental action of digitalis glycosides is to augment the contractile state of the human heart, whether normal or failing, but that in the absence of heart failure this improvement is not translated into an increase in cardiac output.

There has been considerable dispute concerning the effects of digitalis on myocardial O_2 consumption (MVO_2) and efficiency. Analyses of previous data suggested that the interpretation of the results was complicated by the changes in circulatory dynamics induced by the drug. Accordingly, the effects of acetylstrophanthidin (Avg. dose=0.26 cat units/

kg) were studied in 6 non-failing, canine, right heart bypass preparations in which heart rate, stroke volume, and mean aortic pressure were held constant. MVO_2 increased in all experiments, by an average of 2.56 ml/min, while calculated cardiac efficiency declined by an average of 24.4% of control. Even though mean arterial pressure was held constant, the glycoside reduced the integrated systolic tension by an average of 40% and the peak systolic tension by an average of 18%, chiefly as a consequence of a small decline in left ventricular end-diastolic volume. However, the velocity of myocardial fiber shortening increased considerably, the peak left ventricular ejection rate rising an average of 36% and the peak ventricular dp/dt increasing by an average of 82%. Acetylstrophanthidin did not alter MVO_2 in two hearts which were studied in an identical manner, but in which left ventricular end-diastolic pressure was initially elevated. However in those experiments the large fall in end-diastolic volume resulted in a marked fall in systolic tension. In addition, in one experiment, following acetylstrophanthidin the left ventricular end-diastolic pressure and consequently myocardial wall tension were re-elevated to near control levels by increasing stroke volume. Under these circumstances, with no change in myocardial wall tension, oxygen consumption was increased with digitalis. It is concluded that digitalis tends to increase MVO_2, but that its strongly positive inotropic effect frequently results in a reduction of ventricular wall tension which tends to oppose and to mask this effect. It is proposed that the increase in MVO_2 is related to the increased velocity of contraction induced by this agent.

It is generally assumed that patients with mitral stenosis are, like patients with other cardiac disorders, benefited by digitalis. As a result, digitalis therapy was instituted in most patients with mitral stenosis, regardless of rhythm. However, the major impairment of cardiac performance in these patients results from mechanical obstruction to blood flow rather than from imparied myocardial function. Based on this consideration, it was hypothesized that digitalis would not benefit patients with mitral stenosis in normal sinus rhythm. On the other hand, relatively high mean left atrial pressures occur in patients with atrial fibrillation and rapid ventricular rates as a result of a greatly abbreviated diastolic filling time. In these subjects it would be expected that digitalis, by increasing the atrioventricular functional refractory period and thereby decreasing the ventricular response, would improve cardiac performance.

Seven patients with pure mitral stenosis in normal sinus rhythm were studied at rest and at mild to intense levels of treadmill exercise. A Cournand catheter was placed in the pulmonary artery for measurement of pressure and withdrawal of mixed venous samples for cardiac output determination by the direct Fick method. Arterial pressure, oxygen consumption (VO_2), EKG, and heart rate were continuously recorded. After control values were obtained at rest and during exercise, ouabain, 0.01 mg/kg was administered intravenously and the study was repeated. Several patients were given digoxin orally, and the studies repeated 4 to 7 days later. In 3 patients both the acute and chronic studies were performed. The hemodynamic response to exercise was assessed by comparing cardiac output, VO_2, pulmonary arterial pressure, and mixed venous oxygen saturation before and after the administration of digitalis. In patients with normal sinus rhythm all of the measured parameters of cardiac performance were essentially unchanged by either acute or chronic administration of digitalis. Thus, these findings do not support the position that digitalis favorably influences the circulatory dynamics of patients with mitral stenosis and sinus rhythm.

Studies on Circulatory Control of the Autonomic Nervous System

The specific role played by the autonomic nervous system in circulatory control has been of interest to investigators in this Branch for a number of years now. This interest has continued, and studies carried out on the autonomic nervous system are included among the other headings of this report. In addition to the aforementioned investigations, four others were carried out during the past year.

It is generally agreed that norepinephrine is the mediator released at the sympathetic neuroeffector junction. In addition, it is believed that the norepinephrine released from nerve terminals as a result of sympathetic nervous stimulation acts at the same sites as humorally transported norepinephrine. Furthermore, it is felt that blockade of neurally evoked vasoconstriction is as easily accomplished as blockade of humorally mediated vasoconstriction.

In order to study these aspects of neurotransmitter activity as they pertain to vasomotor control of the circulation, a canine preparation in which the skeletal muscle vascular beds of the hindlimb were perfused at a constant flow was utilized. Changes in perfusion pressure therefore reflected changes in vascular resistance of the limb. Reflex vasoconstriction in the limb was produced by bilateral carotid artery occlusion and by hemorrhagic hypotension. The changes produced by these reflex mechanisms were then compared with the changes in resistance brought about by the intra-arterial injection of norepinephrine.

In the dogs that served as controls, both carotid sinus hypotension and intra-arterial norepinephrine caused large increases in vascular resistance. The other animals were pretreated with phenoxybenzamine 15 mg/kg, a powerful alpha adrenergic blocking agent. In this group of animals, carotid sinus hypotension once again produced a reflex vasoconstriction in the perfused hindlimb, although not to as great an extent as in the normal limb. Intra-arterially administered norepinephrine, however, now produced an opposite effect, causing vasodilation. Thus, the pretreatment with phenoxybenzamine blocked the vasoconstriction normally produced by norepinephrine as a result of its alpha receptor stimulating properties and unmasked its beta receptor stimulating effects which produce vasodilation. This vasodilation could then be blocked by treatment with propranolol, a powerful beta-adrenergic blocking agent, thereby confirming that it was indeed a beta effect which had been unmasked.

These results indicate that humorally transported norepinephrine and neurally released norepinephrine act to a large extent at spatially different sites. In addition, contrary to statements in the literature, it seems clear that the alpha receptors at the neuroeffector junction are not reached by blocking agents as effectively as are alpha receptors at other sites. Finally, since reversal of *reflex* vasoconstriction was never seen, it seems reasonable to assume that the population of beta-receptors in the region of the neuroeffector junction is smaller in comparison to the population of alpha receptors than this ratio elsewhere.

Since the demonstration by Marey in 1859, it has been known that heart rate varies inversely with the arterial blood pressure. It is also firmly established that these changes in heart rate are mediated reflexly, the afferent arm of the reflex originating in the baroreceptors located in the carotid sinus and aortic arch areas. It was felt that it would be of interest to define the relative roles of the two main baroreceptor areas in the control of heart rate.

In nine dogs the carotid arterial circulation was isolated from the rest of the vascular bed and was perfused by a donor dog. Changes in arterial pressure could then be produced independently in the carotid sinus and in the aortic arch baroreceptor areas. It was found that both baroreceptor regions are important in the control of heart rate, although the aortic arch areas often predominate. It was also shown that a positive input, produced by raising the perfusing blood pressure is a more adequate stimulus than the negative input of lowering perfusion pressure. Finally, as shown by large changes in blood volume in the trunk which occur when carotid sinus pressure is raised, reflexes originating in this area exert a profound influence on the volume of the peripheral vascular bed.

The central nervous system controls heart rate by varying the impulse traffic in sympathetic and parasympathetic nerve fibers terminating in the sino-atrial node. Although there has been considerable interest in the mechanisms by which the heart rate is altered in response to exercise, postural changes, and stimulation of the baroreceptors, the relative role of the two divisions of the autonomic nervous system in mediating these changes in rate in conscious man have not been clarified. Further-

more, litle information is available concerning the manner in which the heart rate response to baroreceptor stimulation is modified during exercise.

The relative roles of the efferent pathways whch mediate the heart rate response to supine exercise and tilting were investigated by observing the separate and combined effects of beta-adrenergic blockade and parasympathetic blockade on cardiac acceleration in normal subjects. From these studies it appears that in the supine resting state, parasympathetic restraint is the dominant influence on heart rate, and the accelerating effects of sympathetic stimulation are minor. The speeding of the heart in response to mild exercise appears to result largely from withdrawal of parasympathetic inhibition, since cardiac acceleration was found to be essentially unimpaired by sympathetic blockade but to be inhibited by parasympathetic blockade and double blockade. At higher levels of exercise, however, cardiac acceleration must result in part from sympathetic stimulation, since sympathetic blockade reduces the augmentation of heart rate in comparison to the control study. The finding that the increments in rate during heavy exercise are smaller after double blockade than after parasympathetic blockade alone further identifies the contribution of the sympathetic system. In contrast to light supine exercise, substantial speeding could still occur during tilting when the parasympathetic system was blocked. Thus cardiac acceleration in response to mild supine exercise appeared to depend predominantly on parasympathetic withdrawal, whereas that produced by tilting involved a relatively greater degree of sympathetic stimulation. No alteration in baroreceptor sensitivity was found on transition from rest to exercise, and at rest, baroreceptor induced alterations in heart rate are moderated by stimulation or withdrawal of either efferent system. However, during exercise, heart rate changes are primarily dependent on changes in the activity of the sympathetic nervous system.

The presence of baroreceptors in the walls of the two atria has been postulated by many investigators. However, the importance of these proposed receptors in the reflex control of vascular resistance and venous tone in intact man has not been elucidated. An investigation was carried out which was designed to determine the effects of stimulation of the atria, by electrically pacing the right atrium with a catheter electrode, on the vascular dynamics of the forearm, and on the total peripheral vascular resistance. In 8 patients with normal or near normal cardiovascular dynamics at rest, stimulating the atria at a rate above the basal rate resulted in a striking augmentation of forearm blood flow and reduction of calculated forearm vascular resistance, indicating that arterial dilatation had taken place. In addition, a decrease of venous tone was produced, indicating venodilatation. Thus, it appears that as postulated, there are receptors in the walls of the atria, the stimulation of which are capable of producing vasodilatation of the normal human forearm.

Improvement of Cardiac Diagnostic Methods

The direct measurement of oxygen saturation of blood in the central circulation was made possible by the in vivo oximeter system utlizing fiber optics, as described in the 1965 annual report. Although that catheter system permitted the rapid continuous and accurate measurement of oxygen saturation, its clinical usefulness was markedly limited by the necessity of introducing a second catheter to measure pressure, to inject indicator, or to withdraw blood. Accordingly, attention was directly at encouraging and aiding in the development of a catheter which incorporates both fiber optics and a lumen. This catheter is now being utilized in the precise diagnosis of certain forms of congenital heart disease and in studying the oxygen saturation and blood pressure in the pulmonary artery during and following exercise in patients with varying degrees of impairment of cardiac performance.

In patients with congenital heart disease, the simultaneous measurement of oxygen saturation and pressure is making possible a more rapid exploration of these variables within the central circulation. It has been used to localize precisely and to quantify both left-to-right and right-to-left shunts by oxygen saturation

changes at these sites. The catheter lumen also permits the measurement of pressure, the central injection of indicator for peripheral arterial sampling and the withdrawal of blood for in vitro measurements. The execution of these maneuvers with a single catheter has proven useful and the simultaneous and direct measurement of oxygen saturation and pressure has been found particularly valuable in diagnostic procedures.

In normal erect subjects pulmonary blood flow is greater to the dependent zones of the lung than to the apices, and this pattern may be reversed in mitral stenosis. External scintillation scanning of intravenously administered I^{131} labeled macroaggregated of human serum albumin (MAA) was employed to evaluate distribution of pulmonary blood flow in the erect posture in 13 normal subjects and 35 patients with mitral valve disease. The upper/lower third ratio of blood flow (U/L) to the right lung averaged 0.43 in normals and was significantly greater (1.01) in patients with mitral valve disease ($p<0.001$). In the absence of heart failire U/L did not exceed 0.65 until MLAP exceeded 14 mm. Hg and was less than 0.80 whenever MLAP was less than 15 mm. Hg. The correlation between U/L and MLAP was significant ($r-Z$ 0.91, $p<0.001$) and from the regression equation (MLAPZ=14.0 U/L5.32), MLAP could be predicted to within 5 mm. Hg at the 95% confidence level.

It is concluded that the close correlation between the U/L ratio and the mean left atrial pressure indicated that it is possible to predict the latter within reasonable limits in patients with mitral valve disease by means of simple lung scanning. The method has been found especially useful in the screening of asymptomatic patients with the clinical findings of mitral valve disease, and, in the preoperative study of patients so ill that left heart catheterization was considered to be unusually hazardous. Scanning is also of value in determining whether the pulmonary *venous* pressure is elevated in patients with known severe pulmonary *arterial* hypertension, so that the presence of potentially correctible lesions such as mitral stenosis or cor triatriatum may be detected.

The technique is safer, produces less discomfort, and provides more quantitative data concerning the distribution of pulmonary blood flow than either pulmonary arteriography or differential bronchospirometry. The necessary equipment for the application of the scanning method is far simpler than that required for the radioactive gas techniques and it is readily available.

Scintillation scanning of the lungs after administration of I^{131} labeled macroaggregates of human serum albumin has also been employed to delineate the patterns of pulmonary blood flow in patients with congenital heart disease. It was found that in patients with tetralogy of Fallot the blood flow through a subclavian-pulmonary arterial anastomosis is directed principally to the lung on the same side as the anastomosis and that the relative disparity in flows to each lung provides an index of the efficacy of the anastomosis and of the development of pulmonary atresia. Also, the presence of anomalies that create pulmonary venous hypertension may be detected by demonstrating a reversal of the normal increase in perfusion from lung apex to base when the patient is in the erect posture. Avascular areas of the lung secondary to atresia or marked narrowing of the pulmonary arteries may also be detected by lung scanning. Right-to-left shunting through a patent ductus arteriosus may be diagnosed if the accumulated radioactivity in the capillary bed of the feet exceeds that in the hands after intravenous injection of MAA.

Clinical Studies

Idiopathic Hypertrophic Subaortic Stenosis

A detailed anatomic, hemodynamic, angiographic and clinical review of idopathic hypertrophic subaortic stenosis (IHSS) was undertaken in relation to possible mechanisms responsible for the intraventricular pressure gradient that occurs in this disease. The features were considered in particular relation to the recent proposal that cavity obliteration, rather than obstruction, is responsible for the pressure gradient in these patients. Anatomic observations at necropsy and at operation have revealed "bars" or "bands" of hypertrophic

muscle which appear to obstruct the left ventricular outflow tract; incision of these hypertrophied areas of the interventricular septum appears to abolish a sphincter-like action of the ventricular outflow tract. Review of hemodynamic observations, using a flowmeter at the time of cardiac surgery, has revealed that approximately 70% of the stroke volume is ejected during the time that a pressure gradient exists. Angiograms were reviewed in an effort to clarify the location of the site of obstruction, and it was concluded that a linear radiolucent area can frequently be identified, presumably at the point where the hypertrophied septum impinges upon the anterior leaflet of the mitral valve in late systole. Combined hemodynamic and angiographic measurements were made in 14 patients. It was shown that an abnormally elevated left ventricular systeolic pressure could be recorded in the inflow tract of the ventricle, while the tip of the recording catheter clearly lay within the pool of contrast medium. In addition, in many patients it was shown that when a catheter inserted into the left ventricle by the transseptal route was withdrawn from the area of the inflow tract into the left atrium, it never traversed a zone of low systolic pressure, i.e., one in which left ventricular systolic pressure equaled aortic pressure. Indeed, the tracing always reverted directly from an elevated left ventricular systolic pressure to left atrial pressure. These findings, together with clinical evidence, such as the presence of a systolic murmur in patients without mitral regurgitation and the marked reduction or elimination of the pressure gradient by operations designed to relieve obstruction, support the concept that obstruction plays a significant role in IHSS.

It is now well established that the discrete forms of obstruction to left ventricular outflow remain constant during hemodynamic interventions, and that striking variations in the severity of obstruction may be induced in IHSS by a variety of physiologic and pharmacologic stimuli. The effects of changes in heart rate on the severity of obstruction in IHSS have not been clarified. However, since ventricular volume is reduced and the contractile state of the myocardium is improved when cardiac rate increases, the possibility was considered that this intervention might intensify the obstruction in IHSS. The effects of increasing heart rate with atropine were determined in patients with IHSS and compared to the responses in patients with discrete forms of obstruction to left ventricular outflow. It was observed that an increase in heart rate, presumably through a decrease in left ventricular end-systolic volume, increased the trans-left ventricular pressure gradient and the severity of obstruction in IHSS. In patients with discrete obstruction the gradient was reduced and the orifice size was unaltered by increased heart rate. Therefore, the importance of heart rate in IHSS has been defined and, in addition, a useful diagnostic test for the differentiation of dynamic and fixed obstruction to left ventricular outflow has been provided.

Changes in body position have been shown to influence the degree of obstruction in IHSS. Tilting to the head-up position augments the left ventricular outflow tract gradient and tilting to the head-down position has been found to ameliorate the obstruction. Although the resting apex cardiogram of patients with IHSS is often quite characteristic of this condition, frequently physiological and pharmacological stimuli must be used in order for the characteristic second systolic wave to appear. A study was undertaken to determine if the apex cardiogram recorded during tilting could be used as a simple diagnostic procedure to establish the diagnosis of IHSS. Eight of 17 of the patients studied had a supine apex cardiogram characteristic of IHSS. Characteristic changes of IHSS could be elicited in 6 of the remaining 9 patients by use of the tilting maneuver. It is concluded that the use of the apex cardiogram during tilting is a simple and valuable adjunct in the diagnosis of IHSS.

Exercise is a potent stimulus to angina pectoris in certain patients with IHSS. Exercise has been shown to activate cardiac adrenergic receptors and to increase both the exercise and post-exercise outflow tract gradients. Beta-adrenergic blockade has previously been shown to cause a decrease in the left ventricular out-

flow tract gradient during exercise when compared with the pre-blockade exercise state. An investigation was undertaken to determine if beta-adrenergic blockade induced by oral propranolol would be of benefit in preventing exercise induced angina pectoris in patients with IHSS. Five of six patients with exercise-induced angina pectoris were improved on the days when propranolol was administered. These 5 patients walked an average of 10.5 minutes on the days when propranolol was administered in contrast to 6 minutes when placebo was administered. These differences are highly significant statistically, and propranolol appears to be a valuable drug in the treatment of IHSS.

Other Clinical Studies

Only limited information is available concerning the symptomatic and hemodynamic results of operative correction of atrial septal defect (ASD) in older patients. Moreover, in patients with ASD beyond the fourth decade, in whom the lesion is commonly associated with significant symptomatic disability, the criteria for selection for operative treatment have not been well established. Accordingly, the clinical and hemodynamic findings in 48 patients with ASD over 40 years of age have been reviewed. The majority of patients (92%) reported significant symptomatic disability, and at cardiac catheterization they exhibited mild to moderate pulmonary arterial hypertension in the presence of a moderate to large left-to-right shunt. Thirty-four patients underwent operative correction of the ASD, since 1960, however, only one has died as the result of operation. Following operation, all 12 Class III and IV patients, and 10 of 14 Class II patients were substantially improved. Postoperative cardiac catheterization revealed a substantial reduction in pulmonary arterial pressures in 15 of the 16 patients in whom it was present preoperatively. These results suggest that in the presence of a large left-to-right shunt, with or without moderate pulmonary arterial hypertension, age should not contraindicate operative correction of an ASD when symptomatic disability is present.

Two studies have been carried out on the function of the left atrium in patients with mitral valve disease. In the first, the determinants of the height of the left atrial contraction wave (*a* wave) were analyzed in 53 patients with pure mitral stenosis and sinus rhythm. Ratios of the height of the *a* wave to the *v* wave, the height of the *a* wave to the mean left atrial pressure, and the *a* wave pulse pressure to the mean left atrial pressure were related to a number of variables which are related to the severity of the mitral stenosis, i.e., to the mean mitral valve pressure gradient, the mean left atrial pressure, the mean pulmonary artery pressure, and the left atrial size, estimated roentgenographically. It was observed that the relative height of the *a* wave varies inversely with the hemodynamic severity of the mitral obstruction, indicating that as the disease process progresses, left atrial contraction may become weaker and make a smaller contribution to left ventricular filling.

The occurrence of an atrial gallop sound is an unusual finding in patients with mitral regurgitation. The development of atrial fibrillation in many patients with severe mitral regurgitation precludes the occurrence of an atrial sound and standard tests and reviews have not called attention to this finding even in patients with mitral regurgitation and sinus rhythm. The frequency of atrial gallop sounds was determined in a group of 51 patients with pure mitral regurgitation in whom the diagnosis was established at open operation. Nine patients demonstrated atrial gallop sounds and all nine had ruptured chordae tendineae. None of six patients with primary rheumatic mitral valvular regurgitation and sinus rhythm demonstrated this finding. A history of rheumatic fever was uncommon in patients with ruptured chordae tendineae but a history of subacute bacterial endocarditis was common in both groups of patients. The duration of a history of a heart murmur and of symptoms were shorter in patients with ruptured chordae tendineae. The left atrial chamber was generally smaller in patients with ruptured chordae tendineae, and was evidently capable of contracting forcefully and causing a ventricular filling sound.

From the clinical point of view it appears that in patients with mitral regurgitation whose cardiac disability is sufficiently severe to require mitral valve replacement, the finding of normal sinus rhythm should raise the suspicion of ruptured chordae tendineae. If, in addition, an atrial gallop sound is present the diagnosis of ruptured chordae tendineae is even more likely.

Detailed preoperative and postoperative catheterization studies were carried out in 12 patients with advanced mitral and aortic valve disease in whom surgical replacement of both valves was performed utilizing ball-valve prostheses. Since the prostheses effectively relieve the mechanical burden on the left ventricle, any residual impairment of circulatory function observed postoperatively can be attributed to an impairment of myocardial function. It was observed that replacement of both valves resulted in restoration of normal or nearly normal hemodynamics, recorded in the resting state. However, studies of left ventricular function during exercise, as assessed by the relationship between cardiac output and oxygen consumption, between cardiac output and left ventricular end-diastolic pressure, and between stroke volume and left ventricular end-diastolic pressure, indicated that significant abnormalities of myocardial function and dynamics exist that were not alleviated by combined valve replacement. Therefore, it appears likely that replacement fibrosis secondary to underlying myocardial disease and coronary artery disease, or myocardial dysfunction consequent to a chronic severe hemodynamic burden, contributes to the preoperative disability in these patients, and, in addition, may prevent complete symptomatic recovery following insertion of valvular prostheses. This postoperative impairment of myocardial performance appears to be more pronounced than that observed in patients with isolated replacement of either the mitral or aortic valve.

The study of phasic arterial blood flow patterns in intact, unanesthetized man has not previously been possible because of practical limitations in flow-measuring techniques. Recent improvements in the design of electromagnetic flowmeter circuits and the miniaturization of flow transducers, suggested the possibility that such instruments might prove suitable for these measurements. The characteristics of phasic and instantaneous mean brachial arterial (BA) blood flow, measured with the electromagnetic flowmeter, have been investigated in 35 patients undergoing cardiac catheterization. Phasic BA flow patterns were characteristically altered in the presence of valvular aortic stenosis, aortic regurgitation, and in hypertrophic subaortic stenosis, when compared with patterns recorded in patients without left heart disease. All patients exhibited spontaneous, often large variations in mean BA flow at rest. During forearm muscular contraction, striking reductions in BA flow occurred, and, during maximal muscular contraction, actual cessation of flow was observed. Reflex forearm vasoconstriction consistently occurred during the Valsalva maneuver, and with unilateral carotid occlusion, while forearm vasodilatation was demonstrated during carotid sinus stimulation. Thus this technique has permitted the first description of the normal pattern of phasic BA flow in unanesthetized human subjects, as well as the alterations in phasic flow contour accompanying various cardiac lesions. In addition, it has allowed investigation of rapid, transient alterations in peripheral arterial blood flow not previously accessible to measurement.

Although the overproduction of serotonin remains the hallmark of the carcinoid syndrome, recently attention has been focused on other biologically active substances elaborated by carcinoid tumors. The effects of bradykinin, epinephrine, and reflex stimulation of the sympathetic nervous system, were compared on the arteries and veins of the forearm in carcinoid patients and normal subjects utilizing a plethysmographic technique. In contrast to the normal subjects, in the carcinoid patients, epinephrine produced flushes, profound arteriolar dilatation, and resulted in a rise of brachial arterial bradykinin and the kinin-forming enzyme kallikrein; sympathetic stimulation produced flushes, attenuated vasoconstriction, and elevated plasma bradykinin and kallikrein. Therefore, this study provided

evidence to implicate the kallikrein-kinin system in the genesis of the carcinoid flush.

Idiopathic infantile hypercalcemia, a disease probably related to deranged vitamin D metabolism, may be a feature of the syndrome consisting of supravalvar aortic stenosis, mental retardation, and a peculiar "elfin" facies. It is not known if the observed multiple-system involvement is genetically determined or if any or all of the features are related to deranged maternal vitamin D metabolism or fetal vitamin D metabolism, or to a combination of the two. Accordingly, a study was undertaken to determine if vitamin D crosses the placenta, and to explore the relationship between hypervitaminosis D in the mother and the development of supravalvular aortic stenosis in the offspring.

Pregnant rabbits were divided into groups so that in addition to the stock diet some were given large amounts of vitamin D throughout gestation. Vitamin D bioassays revealed 7 and 8½ times higher values of vitamin D in the mothers and the newborns, respectively, than in the controls, providing direct evidence of transplacental passage. A total of 14 anatomic abnormalities of the aorta was noted in 34 offspring whose mothers received 1.5 million units of vitamin D throughout pregnancy. Definite similarities were noted between the aortic lesions in 8 rabbits and supravalvular aortic stenosis in the human. Thirty-five control offspring showed no abnormalities of the aorta. It is concluded that the vascular toxic effects of vitamin D cross the placenta. It seems reasonable to suggest that an *in utero* derangement in vitamin D metabolism on the part of mother and/or fetus may be responsible for supravalvular aortic stenosis, especially when the latter is associated with infantile hypercalcemia.

Section of Clinical Biophysics

The major activities of this section are concerned with exploration of basic mechanisms in the physiology of the lung, heart and vascular system. These objectives require strong programs in instrument development and development of computational procedures as well as a strong laboratory program. These activities will be described under three major headings: Vascular Mechanics, Myocardial Mechanics, and Pulmonary Mechanics.

Vascular Mechanics

Several moderately long term studies were concluded this year which gave us much better insight into the gross features of vascular dynamics. If we are to understand the mechanisms determining circulatory behavior, it is necessary to gain a clear picture of the mechanical behavior of both the vessel wall and the underlying hydrodynamics. A large number of special devices and instruments have been devised which have permitted isolation of various vascular segments in the larger arteries, such that discrete measurements of pressure, forces, flows, displacements, and dimensions could be recorded continuously on multi-channel F.M. tape. Rather formidable data processing programs have been developed which permit examination of these simultaneous variables for unique relationships among them. The major findings to date can be summarized in the following listing:

1. The distensibility of the aorta decreases progressively along the aorta from the root to the bifurcation.
2. The "elastic" component of this visco-elastic system dominates the mechanical behavior.
3. The vessel wall is incompressible.
4. The vessel wall demonstrates anisotropic properties, i.e. the elastic constants are different in the three different directions.
5. The longitudinal vascular tethering (the mechanical properties of the constraining tissues surrounding the vessel) have been measured and can be represented by a linear mathematical model consisting of 1 elastic element, 1 visco-elastic element, 1 viscous element, and an inertance.
6. The harmonic content of both the pressure and the flow curves in the major arteries seldom exceeds about 10 cycles per second whereas the harmonic content of the pressure gradient and the time derivative of pressure usually exceeds 25 cps.
7. The hydraulic input impedance to most vascular beds, including the pulmonary artery

and ascending aorta, are similar. The impedance spectrum is dominated by the constant term in each case. The time dependent terms decay rapidly with frequency.

8. The estimation of instantaneous blood flow, using the pressure gradient technique, has been validated in physical models as well as in the living pulsating vessel. The mean deviation of computed flow from monitored flow is of the order of 5% in the physical model, and of the order of 10% in the living pulsating vessel.

9. It has been established that the simple single degree of freedom model of pulsatile flow in a vessel segment proposed originally by this laboratory fits the experimental data as well as the more complicated and elaborate multidegree of freedom systems such as that suggested by Womersley.

10. The relationship between the time derivative of pressure and flow is a very indirect one which depends on the lumped properties of the entire vascular bed and, thus, contrary to the assertions appearing in the literature, it cannot be assumed to bear a unique relationship to the pulsatile flow at a point in the vascular bed.

11. Flow in the major arteries frequently can be shown to exceed the apparent critical Reynold's number, suggesting that arterial blood flow may be unstable or at the borderline between stable and turbulent flow during a significant portion of the cardiac cycle.

12. Pressure or flow disturbances are propagated along the arterial tree at a velocity which is dependent on both frequency and on position along the tree. Pressure moduli appear to increase with distance along the aorta in a manner consistent with the observation noted above that the arterial tree becomes progressively stiffer peripherally. The lower harmonics of both the pressure and flow curve demonstrate strong reflections.

The foregoing observations have clarified our picture of circulatory dynamics and have produced a number of useful mathematical models whose validity has been shown to hold at particular points along the vascular system. It remains to be seen whether an appropriate integration of these "segmented" models can predict over-all system behavior under widely varying conditions. This question can be approached only by "driving" the entire vascular bed with known flow functions and observing the degree to which such an integrated model will permit us to predict the corresponding pressure functions, vessel displacements, etc. A special hydraulic flow generator is being designed for this purpose.

With the foregoing major features of circulatory dynamics reasonably well defined, we are now in a position to turn to questions concerning the "fine-structure" of the pressure-flow relationships such as measurement of intravascular velocity fields. These studies will allow us to probe more deeply into mechanisms associated with vessel wall "injury," i.e. the processes related to the "wear and tear" of aging and disease.

To this end we began developing specialized measuring techniques about two years ago in conjunction with the Engineering and Mechanics Staff at Catholic University of America. If we are to gain insight into the fine structure of pulsatile flow, it is necessary to develop a velocity sensing probe which can be used to explore the entire flow field in an artery. Dr. Ling of Catholic University is a specialist in the area of "hot-wire anemometry," and over the past year has developed a needle blood-flow probe which will permit us to map these flow fields. Studies validating the fidelity of this instrument are currently being concluded. The device is linear and has a dynamic response extending beyond the range of our monitoring capabilities. Calculated extrapolations indicate that it is useful far into the kilocycle per second range. Therefore, it should be possible not only to map the "time smoothed" velocity profiles in major arteries but also to estimate the frequency spectrum and energy content of the associated turbulence when present. Preliminary studies in animals using this device indicates that the velocity profile is not symmetrical in the descending thoracic aorta.

The rather consistent topography of atheroma distribution in the human aorta suggests that hemodynamic events may be associated with the genesis of these lesions. The localization of atheroma does not appear to be

related to patterns of pressure distribution, but rather, to areas where one might expect high shearing stresses and/or turbulence. This observation has suggested a "working hypothesis" which can be used as a scaffold upon which to direct future research in this program.

It is hypothesized that a flux of lipoprotein molecules is driven toward the subendothelial tissue space from the blood phase by a concentration gradient. This gradient may be increased by raising the blood concentration of fat. The "diffusivity" of the lipoproteins will depend primarily upon their molecular weight and their temperature in laminar flow and will depend in addition upon the "eddy" viscosity in turbulent flow.

Factors assumed to act against this concentration gradient and diffusivity are the mechanical resistance of the endothelial cell wall and perhaps also an electrical field of opposite polarity. This potential field is assumed to emanate either from a fixed charge system in the endothelial wall or from selectively absorbed materials at the blood-wall interface.

To test this hypothesis two pilot studies have been completed. The first was designed to produce localized turbulence (to increase diffusivity) in dog aortas. The objective was to measure an associated increased deposition of fat and/or endothelial damage in the area of turbulence. Turbulence was produced by introducing a polished aluminum cylindrical plug into the descending thoracic aorta of dogs. The plug contained 2 patent longitudinal grooves placed axially along opposite sides of the outer surface. The plug size was chosen to occlude the unstretched aorta. Blood flow thus was diverted into the two longitudinal channels formed on three sides by the walls of the groove and on the fourth side by that portion of the vessel wall covering the groove. Dimensions and pressure drops were such that the Reynold's number of the flow in the groove was about 1000 (critical). Intravenous infusions of emulsified fat were then given and the animal sacrificed after two hours. Serial sections were made of the longitudinal strips of vessel which had formed one wall of the high-velocity flow-channel. These were examined for endothelial cell injury and fat deposition. It was somewhat surprising to note that in nearly half of the dogs marked endothelial damage occurred in the area of high-shear flow. In all animals a very high correlation was demonstrated between the calculated hydraulic shearing stress on the endothelial wall and the associated degree of endothelial damage. At times the shearing stress appeared to erode completely the endothelial cells down to the basement membrane. Evidence of fat infiltration roughly parallels the degree of cellular damage. Clear cut evidence of fat infiltration under the basement membrane was found in about ¼ of the animals, strongly suggesting that fat was actually driven from the blood phase into the subendothelial region. These studies are being repeated employing a better controlled "shearing stress" so that both moderate degrees of exposure as well as heavy degrees of exposure may be compared.

The second type of preliminary study was to explore the magnitude of any electrical barrier that might exist at the endothelial surface. A device was made which could be placed around the living pulsating blood vessel such that the position of electrodes introduced through the wall could be sensed electrically and recorded. Silver-silver chloride electrodes were used with high impedance amplifiers to sense the voltages across the blood vessel lumen as well as along the lumen.

The intravascular voltage distribution was found to be constant throughout the blood phase at about −2 millivolts with respect to the uninjured adventitial tissue of the vessel. At the intimal surface the voltage suddenly drops about 10 millivolts. Histologic examination of the electrode site in these instances showed no endothelial damage. The inference to be drawn is that this voltage represents some sort of "fixed charged" system on the endothelial surface and is not an "injury potential." As the electrode is advanced into the wall the voltage becomes initially somewhat more negative; however, then becomes progressively more positive usually climbing to a value somewhat positive with respect to the

voltage in the blood phase and occasionally positive even with respect to the adventitial surface of the blood vessel. The voltage on the ventral aspect of the aorta is about 4 mv more negative than that on the dorsal aspect of the aorta. Voltage differences also could be detected along the intimal surface of the aorta but no consistent trend could be established. Thus, it appears that there is, indeed, an electrical field probably associated with some "fixed charged" system at the endothelial surface. The significance of this as a barrier to passage of lipoproteins remains to be evaluated. Its significance in repelling the negatively charged blood cellular elements is obvious.

Myocardial Mechanics

The problem of myocardial function has been approached by three different avenues. The first has been study of cat papillary muscle preparation in an effort to devise empirical laws which will allow us to characterize the mechanical behavior of small units of muscle fibers. The second has been anatomical study of specially fixed total heart preparations from which dimensions, fiber distribution, and orientation have been determined microscopically. The third has been to study the sequence with which the various muscle masses are recruited into contraction under varying conditions of pressure and flow.

The papillary muscle studies were greatly hindered by Dr. Feigl's move to the University of Pennsylvania where he has only recently been able to resume them. Moreover, efforts to derive empirical mathematical models for the data that we already have on hand have been hindered by the repeated delays in activating the hybrid computer unit. A three component model to describe myocardial fiber behavior has been devised and checked out on the large Honeywell 800 computer, however, use of this approach is extremely slow and expensive, therefore, we have deferred further analysis of the data until the high-speed hybrid facility is available.

The anatomical studies are approaching completion and have demonstrated that the canine heart may be considered to have a gross configuration somewhere between a cylinder and an ellipsoid revolution. Microscopically we have obtained clear cut evidence of consistent patterns of fiber orientation in the left ventricular wall. The inner 40% of the wall contains fibers running primarily in an axial direction. The middle 50% of the wall has fibers running in a circumferential direction and the outer 10% again in the axial direction at about 60° from the axis symmetry of the heart.

Study of the sequence of contraction of the various muscle masses has indicated that a spectrum of behavior can be expected depending upon the particular animal and the pressure presented to the ventricle. During systolic ejection about one-third of the hearts appear to contract primarily in a circumferential direction whereas one-third demonstrates primarily an apex to base contraction. The remaining third demonstrated various combinations of circumferential and longitudinal contraction depending upon the phase of contraction. Attempts to correlate these modes of contraction with the distribution of fibers and their orientation have been inconclusive primarily because of the small samples of anatomical data available. These studies will be extended such that a better sampling will be available and hopefully a relationship can be established between the sequence of contraction and the distribution and orientation of fibers in the muscle mass.

Lung Mechanics

The major activity over the past year in the area of pulmonary mechanics has been directed toward solution of two difficult practical problems: automatic data processing techniques to handle the tremendous volumes of multi-channel analog information necessary in defining the mechanical behavior of the lung, and secondly, development of new instrumentation for the study of pulmonary mechanics in small animals, such as the rabbit. Although studies have continued in the area of mechanical autoregulation of flow and flow through collapsible conduits, the pressing nature of the foregoing problems plus the continued unavailability of

the hybrid facility have made significant advances in this latter area impossible.

Analysis of the results of a study in pulmonary mechanics can present an overwhelming problem of data reduction. In any meaningful or purposeful approach to these kinds of experiments, it is necessary to have the results analyzed from a particular experiment before proceeding to the next stage. Therefore, it is essential to streamline our methods of data analysis so that we may accomplish this objective. In a typical experiment 3, and usually more, variables are measured continuously with time as various prescribed respiratory maneuvers are carried out. Although it is possible to record these data on a strip chart and then do a laborious graphic analysis, this frequently requires one man-week per experiment. This is obviously impractical, and, therefore, we have spent considerable effort developing processing techniques which will do these sorts of analyses automatically.

General programs have been written and are now operational which will perform both editing functions as well as data sorting routines. The editing routines compare the channels of data against certain editing criteria which may be specified, such as the absence of unwanted noise, the adequacy of the prescribed maneuver, and so on. Once the data are edited to delete unwanted and redundant material, the data are then "sorted."

"Sorting" consists of exploring the relationship between two variables at a time for selected constant values for all remaining variables. If one has, in fact, captured all of the primary variables describing the system, then a meaningful relationship between the two selected variables will emerge. For example, in the case of pulmonary mechanics if one measures instantaneous flow, volume and pressure, one can ask for the relationship between pressure and flow for selected values of instantaneous volume. In this situation unique isovolume pressure flow curves emerge. At the experimenter's option, certain statistical fitting procedures then can be performed on the resulting data to allow the general form of the functional relationships to emerge immediately as smooth curves.

Using these techniques it is now possible to perform an experiment one day and have the essence of the results displayed graphically the following day. The scientific economy of this is obvious.

Standard techniques for measuring the variables for pulmonary mechanics in laboratory animals have not existed. This, therefore, has been our major effort in the area of pulmonary mechanics over the past year. A small body plethysmograph has been designed and fabricated in which instruments have been developed for the accurate measurement of instantaneous respiratory flow, volume and pressure. The plethysmograph itself consists of a rigid plastic chamber in which it is possible to ventilate artifically an anesthetized and/or curarized animal. A positive-displacement electrical recording-spirometer continuously senses the volume changes of the animal. Instantaneous flows are measured by a specially designed linear flow resistance device. The recording fidelity of these systems has been established in excess of 20 cps. Thus, adequate techniques are now available for the measurement of instantaneous flow and volume.

The measurement of intrathoracic pressure in small animals however is quite another story. Detailed studies of the relationship of intrathoraic and intraesophageal pressure have been carried out in an effort to establish the conditions under which the latter could be used as an estimate of intrathoracic pressure. The obvious physiologic merit as well as simplicity of the esophageal approach makes considerable effort in this direction worthwhile. It has been found that over the upper two-thirds of the vital capacity the esophageal route is adequate for most purposes. However, there are a number of notable exceptions to this which must be evaluated.

With the development of these better measuring techniques for small animals it is hoped that we can proceed to more detailed and better controlled studies of pulmonary mechanics in small animals as well as the development of suitable experimental animals for the study of the pathogenesis of chronic obstructive pulmonary disease such as emphysema.

SURGERY BRANCH

Clinic of Surgery

The investigative efforts of the Surgery Branch have, as in past years, principally related to studies of normal and abnormal circulatory physiology, particularly in relation to the methods and results of operative treatment in patients with congenital or acquired heart disease.

A major proportion of patients referred to the Surgery Branch now require operations involving the use of prosthetic cardiac valves, and a number of clinical and laboratory investigations have centered about the general problems associated with intracardiac prostheses. The principal late complication which accompanies the use of all presently available prosthetic valves is the formation of thrombus on them, and subsequent embolization. The anatomy of the calf heart and coagulation mechanisms of the calf closely resemble those of man, and various means of inhibiting thrombus formation on valves have been studied in this species. Standard Starr-Edwards valves universally collected thrombus in both the early and late postoperative period, while similar valves coated with graphite-benzalkonium-heparin seemed thrombus-resistant for one month, but later also clotted. In other calves with standard valves maintained on warfarin, thrombus was prevented throughout the observation period of one year. The temporary thrombus-inhibiting effect of GBH coating is explained by other studies in which the coating was prepared with radioactive (H^3) heparin. With *in vivo* implantation, 71% of the heparin was eluted from the surface within four hours, and more than 95% of it had disappeared within 36 days. The Melrose prosthesis in the tricuspid position has been used as a standard means of promoting thrombus. All such valves clotted within 48 hours in calves not given intravenous fluids postoperatively; animals given dextrose and saline survived for this period, but their valves revealed thrombus. In others given infusions of low molecular weight dextran during the first two days, 6 of 7 valves were thrombus-free at sacrifice. Studies are continuing to determine whether the effect of dextran in preventing thrombus formation is a specific one, or whether the effect is simply hemodilution. Initial observations indicate that the latter mechanism may be of principal importance. A controlled clinical study of the effectiveness of dextran after mitral valve replacement is underway, and most patients are treated with the agent for the 48–72 hours postoperatively.

Seven patients with prosthetic mitral valves have died early with massive thrombosis of the prosthesis and atrium, and two others have died in the late postoperative period as the result of thrombus formation. A clinicopathologic study of the patients who died early indicated that the thrombosis probably resulted from a discrepancy between the size of the prosthetic valve and the size of the left ventricular cavity. In each, the muscular ventricular septum protruded into the cage of the valve, prevented full descent of the ball, and obstruction to left atrial emptying resulted. This observation has indicated the necessity for an unusually small Starr-Edwards valve, or a valve of the discoid type, in patients with mitral stenosis and small or normal left ventricular cavities.

A new method for detecting intracardiac thrombus either pre- or post-operatively, has been studied in both animals and man. A rabbit antibody specific for fibrinogen is labelled with I^{131} and administered intravenously. The compound is concentrated where thrombus is in contract with blood. In dogs in which left atrial thrombi were produced experimentally, the radio-activity of the thrombus was sufficient to permit its detection by precordial scanning. In preliminary clinical trials, specific labelling of left atrial thrombi was achieved in two patients with mitral valve disease, and in another with a thrombus in the left ventricle.

The design and durability of the Starr-Edwards prosthetic valve has been proved in more than five years of clinical application but, as noted, thrombus formation on it is frequent. Past experience has proved that thrombus and emboli were never associated with the use of prostretic valves constructed of Teflon fabric, apparently because the valves quickly became

covered with host tissue. This principle has been applied to rigid Starr-Edwards valves, all metal parts of which were covered with a loosely woven fabric. Within six weeks of implantation all were covered with glistening tissue and none revealed thrombus. Continuing observations must be made to determine whether excessive tissue growth may result in dysfunction of the valve. Initial assessments have also been made of a new type of prosthetic valve which may have advantages over those currently available. The valve consists of a ring-like body within which a streamlined disc is held by an eccentric hinge. The opening angle of the valve can be controlled by the aerodynamic profile of the disc, and flow through it is largely laminar. Initial animal implantation is encouraging, and the valve will be subjected to detailed *in vivo* testing within the coming year.

A major problem which may complicate the course of any open operation on the mitral valve is associated aortic regurgitation, and methods of detecting the aortic regurgitation before operation were assessed retrospectively in 156 patients with mitral valve disease. No troublesome regurgitation occurred when an aortic diastolic murmur was absent, but when such a murmur was present neither its intensity, the pulse pressure, nor the diastolic arterial pressure indicated the severity of the leak. The magnitude of regurgitant flow could be determined accurately only by cineaortography and this study is, therefore, indicated in every patient with mitral valve disease in whom a blowing diastolic murmur is heard.

Abnormal left ventricular pressure pulses have previously been observed in patients with severe mitral regurgitation, indicating an abnormal pattern of ventricular ejection and aortic flow. Instantaneous aortic flow was measured at operation in patients with severe mitral regurgitation before and after valve replacement, and in dogs in which mitral regurgitation of controlled magnitude was produced. The pattern of aortic flow during mitral regurgitation was abnormal, and was characterized by an abbreviated systolic ejection period and time to peak flow, an increase in peak flow and mean ejection rate, and increases in peak acceleration of flow and the volume ejected during the first half of systole. Normal flow patterns were recorded in the patients after mitral valve replacement.

Another clinical study was made of the effectiveness of operative treatment of atrial septal defect. Data were available in 175 patients who were catheterized, operated upon, and followed thereafter. Six patients, four of whom were older than 40 years and were in congestive heart failure, died. Of 154 patients studied postoperatively, all but 11 were proved to have complete abolition of the left-to-right shunt. A number of patients had severe pulmonary hypertension postoperatively, and with few exceptions elevated pulmonary arterial pressure and resistance persisted, even though the defect was closed. Residual pulmonary hypertension rarely caused symptoms, however.

Approximately one-third of patients in have both clinical and hemodynamic evidences of severe tricuspid regurgitation, and in some clinics tricuspid valve replacement is frequently performed at the time of mitral replacement. Twenty-nine patients who had severe tricuspid regurgitation at the time of mitral valve replacement were managed conservatively; 24 had no operation on the tricuspid valve, and in five a tricuspid annuloplasty was performed. The operative mortality in this group (17%) did not differ from that in patients without tricuspid regurgitation, and late clinical and hemodynamic studies revealed spontaneous regression of the tricuspid lesion as heart size and right ventricular pressure decreased. The experience indicates that tricuspid regurgitation which occurs in association with mitral valve disease is usually of functional nature; the valve will become competent as the size of the heart decreases and tricuspid replacement is seldom indicated.

The effects of tachycardia, induced by atropine, were assessed in patients with discrete aortic stenosis and in others with IHSS. With rate increases, the systolic pressure gradient was unchanged or fell in patients with fixed obstruction, while in those with IHSS the gradient always increased, and the calculated orifice area fell. It appears that the dimensions

of the left ventricle decrease with tachycardia in patients with IHSS, and that the administration of atropine may prove a useful provocative test in patients with this form of muscular subaortic obstruction.

The usefulness of the left atrial pressure pulse in assessing the severity of mitral stenosis was determined in 53 patients who were in regular sinus rhythm. It was observed that the height of the atrial *a* wave varied inversely with the hemodynamic severity of mitral obstruction, suggesting that in advanced stages of mitral stenosis left atrial contraction is weaker and contributes relatively less to left ventricular filling. Certain patients, who are operated upon after long periods of congestive heart failure do poorly postoperatively, possibly because myocardial norepinephrine stores are depleted. An attempt was made to identify such patients by preoperative studies of leg vascular resistance, determined after ipsilateral femoral arterial injections of tyramine or norepinephrine. Resistance was twice as high in patients who had been in failure as in asymptomatic ones, and the norepinephrine dose-response curve was distinctly steeper in the heart failure group. Each patient with heart failure was proved to have exceptionally low cardiac norepinephrine content.

Several laboratory investigations have been related to cardiac arrhythmias and anti-arrhythmic drugs. It is recognized that patients in atrial fibrillation who are receiving digitalis may show evidence of digitalis toxicity following restoration of regular rhythm by countershock. This clinical observation suggested a study of the effects of atrial fibrillation on digitalis tolerance. In dogs, the toxic dose of acetylstrophanthidin was determined on successive occasions by titration. Later, and in the same animals, atrial fibrillation was produced by atrial pacing and acetylstrophanthidin titration repeated. The animals tolerated 10–15 percent more of the drug before developing toxic manifestations during atrial fibrillation. In other dogs some hemodynamic effects of atrial fibrillation were assessed. When fibrillation was induced and ventricular rate was not controlled, systemic arterial pressure and cardiac output fell, and atrial pressure increased. In other animals measurements were made during atrial fibrillation and compared to those obtained during paced regular sinus rhythm at the same ventricular rate. Arterial pressure remained unchanged, but systemic flow was significantly lower during atrial fibrillation than during regular rhythm at the same rate, an effect which can be attributed to impairment of atrial transport function.

When ventricular tachycardia was induced, either by digitalis intoxication or by an exogenous electrical pacemaker, the rate could be slowed in every animal by paired or coupled pacing. In these situations, ventricular tachycardia originated from a single ectopic focus. In other animals ventricular arrhythmias were produced by ligation of coronary artery branches, and multifocal ventricular contractions resulted. With this arrhythmia neither paired nor coupled pacing was effective, and either usually caused ventricular fibrillation.

Both lidocaine (Xylocaine) and procaine amide (Pronestyl) are widely used to control ventricular arrhythmias, but their effects on the contractile function of the heart have not been documented. In dogs in which heart rate, aortic pressure, and stroke volume were controlled, contractile force and left ventricular dp/dt were measured after these agents were administered. With lidocaine, either 1.5 or 3 mg./Kg., contractile force fell 18% after three minutes, but returned to control levels within 10 minutes. Either 6 or 12 mg./Kg. of procaine amide caused a progressive decrease in contractile force (maximum 15%) for 12 minutes, and force remained depressed after 30 minutes. Diphenylhydantoin (Dilantin) was evaluated in a similar manner. Contractile force decreased 32%, and in other animals depression of ventricular function curves was always observed. The drug also decreased total peripheral vascular resistance in steady arterial bow preparations.

A continuing laboratory and clinical interest of this unit has been the development, evaluation, and application of various biologic adhesives. On the basis of previous experimental studies, methyl-2-cyanoacrylate monomer has been found a satisfactory but not

ideal hemostatic agent. It is now undergoing systematic use in patients to reinforce closures of sutured incisions in the heart and great vessels, and to control bleeding from raw dissected areas. The courses of these patients, and the pathologic findings in those that die will be analyzed. A new adhesive system, consisting of gelatin, resorcinol and formaldehyde (GRF) has been developed. It permits a strong bond to be achieved in the presence of moisture, and has a high initial viscosity. Original preparations were found to be toxic to tissue because of excess formaldehyde, a defect largely corrected in more recent formulations. In the dog, GRF was used to close incisions in the ileum, and all animals survived. In other dogs it effectively controlled bleeding from the bisected spleen, the transsected kidney, and the incised bladder. It is contemplated that GRF will be found suitable for clinical application within the year.

Previous reports have described studies of the effects of morphine on the heart and peripheral circulation, and recent experiments evaluated the mechanism of action of this drug in the treatment of pulmonary edema. Pulmonary edema was produced in dogs by techniques which simulated the clinical disorders in which pulmonary edema commonly occurs. Morphine was administered and, as pulmonary edema subsided, strikingly and parallel decreases in pulmonary arterial flow and pressure, and left atrial and left ventricular enddiastolic pressures were recorded. It appears that the principal beneficial effects of morphine in pulmonary edema result from an increase in vascular capacitance, and an associated decrease in systemic venous return.

Mitral stenosis is one of the most frequent cardiovascular malformations encountered in patients, but no satisfactory experimental counterpart has been previously devised. In dogs, a suture was passed from the tip of the left atrial appendage, through the mitral valve, and out the posterolateral wall of the left ventricle. A cylinder of plastic sponge was attached to the atrial end of the suture, and the through the mitral valve ring. In dogs studied in both the early and late postoperative periods, left atrial and pulmonary arterial pressures were uniformly elevated, and diastolic gradients between the left atrium and left ventricle were present in all dogs. Long-term studies of the effects of mitral obstruction on the pulmonary vasculature can now be made.

Other techniques for producing and studying the pulmonary vascular changes which result from various congenital cardiovascular malformations have been devised. The end of a lobar pulmonary artery is anastromosed to the distal end of an adjacent pulmonary vein; the lobe is then perfused with oxygenated blood at low pressure. In other similar preparations lobes are supplied with oxygenated blood under high pressure, or venous blood under high pressure. In this manner the relative effects of oxygen content and perfusion pressure on the pulmonary vascular bed can be assessed.

The strain gauge arch is widely utilized to evaluate ventricular performance but the hemodynamic variables which may influence the indicated contractile force have not been systematically investigated. In dogs, recordings of the contractile forces of both ventricles were made as heart rate, stroke volume, and afterload were separately altered. Force increased with heart rate, but did not change over a wide range of stroke volumes. At constant rate and stroke volume, left ventricular force was a direct function of aortic pressure, whether the ventricle was empty or doing external work; this response was shown to result from changes in coronary flow. The experiment indicat that measurements of contractile force afford an accurate indication of ventricular performance only when rate and afterload are kept constant.

Several clinical studies have originated from the pathology section of the Branch. The kidneys of 132 patients who died of valvular heart disease were examined, and four were found to have extensive renal hemosiderosis. Each patient had a fixed calcified aortic valve which was both stenotic and regurgitant. Renal hemosiderosis was discovered in the kidneys of seven additional patients who had had prosthetic aortic valves inserted. These findings indicate that either a prosthetic valve or a diseased aortic valve can be responsible for intravascular hemolysis and, although renal

hemosiderosis was often severe, there was no evidence that impairment of renal function resulted. The hearts of 24 patients who had the carcinoid syndrome were examined, and 16 were found to have carcinoid heart disease. In six, typical lesions were present in the left side of the heart as well as the right. The presence of cardinoid heart disease was evidenced before death only by the presence of a murmur, unless detailed diagnostic studies were performed. Sections of the livers of 123 patients who died of valvular heart disease were examined and each was shown to have a brownish-yellow pigment in the centrolobular hepatic cells. In none, however, was the pigment found to contain iron. In 24 patients of the group, however, selective stains were positive for hemosiderin and this finding was attributable to massive blood transfusions in the period immediately preceeding death.

LABORATORY OF CLINICAL BIOCHEMISTRY

Amine Biogenesis and Metabolism

It is now generally accepted, as we originally proposed, that hydroxylation of tyrosine represents the rate-limiting step in the production of norepinephrine. For this reason, inhibitors of this enzyme are far more effective in depleting tissues or norepinephrine than are inhibitors of dopa decarboxylase or dpamine-β-hydroxylase. Several classes of inhibitors of tyrosine hydroxylase have been found among tyrosine analogues and catechol derivatives. The former compete with tryosine the latter with the tetrahydropteridine cofactor. α-Methyl-tyrosine has proved to be a most potent inhibitor *in vivo* as well as *in vitro*. Using αmethyl-tyrosine, it has been possible to show that when animals are subjected to stressful conditions such as severe exercise or exposure to cold, tissue levels of norepinephrine, even in brain, are maintained by virtue of a rapid resynthesis. In other words stimulation of sympathetic tissues causes them not only to release norepinephrine but also to increase the rate of synthesis. These findings were corroborated by experiments with labeled tyrosine. It was shown that exercise and exposure to cold result in the incorporation of 2 to 4 fold larger amounts of administered tyrosine-^{14}C into norepinephrine. The procedures have no effect on the amount or specific activity of the free tyrosine-^{14}C in the tissues. We have obtained some evidence of increased synthesis in isolated stimulated tissues. Other laboratories have obtained even more evidence for such an effect in isolated organ preparations. The mechanism for this increased synthesis is of interest. It appears that the increased synthesis is due to increased tyrosine hydroxylase activity. However, we have some evidence that neither exercise nor cold increase the absolute amount of the enzyme in tissues. It would appear, therefore, that the enzyme *in vivo* under normal conditions is not operating at maximal capacity and that some factor or factors increase its activity. One possibility is that norepinephrine itself, which we have shown to be an inhibitor of tyrosine hydroxylase, controls the initial reaction by end-product inhibition. Several other laboratories have reported findings consistent with such a mechanism.

We have obtained definite evidence that all three enzymes involved in norepinephrine synthesis are associated with a similar subcellular fraction within tissues. We feel, however, that the homogenization and sedimentation techniques which were used are not adequate for unequivocal determination of intracellular localization. Because of this limitation, we have turned to fluorescent antibody methods. We have purified beef dopamine-β-hydroxylase and prepared potent antibody to it in the rabbit. A contract was awarded to Microbiological Associates to produce enzyme antibodies, purify them and assist us in labeling them with fluorescent dye.

Inhibition of tyrosine hydroxylase has been carried to the clinical level in joint studies with the Experimental Therapeutics Branch. α-Methyl-tyrosine was administered to patients and was shown to decrease the formation of norepinephrine. At doses of 1.5 to 3 gram per day about 70% inhibition was attained in a large number of patients with essential hypertension and pheochromocytoma. In the latter group, blood pressure was lowered to the normal range as the excretion of norepinephrine and its metabolites fell to normal values. It

appears that α-methyl-tyrosine may become the treatment of choice for patients with malignant pheochromocytoma.

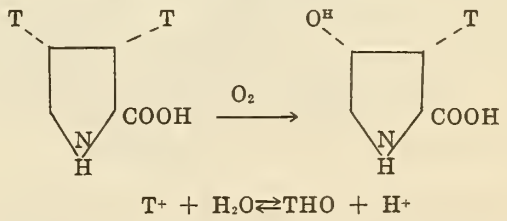
Another collaborative effort with the Experimental Therapeutics Branch concerns patients with phenylketonuria (PKU). Attempts will be made to answer two questions: (1) Is the small amount of conversion of phenylalanine to tyrosine which occurs in patients with PKU due to residual liver phenylalanine hydroxylase or is the latter enzyme completely lacking and the conversion carried out by the action of tyrosine hydroxylase in sympathetic nerves? If the latter is the case, then α-methyl-tyrosine, which specifically inhibits tyrosine hydroxylase, should abolish conversion of phenylalanine to tyrosine in patients with PKU but have no effect on the conversion in normals. (2) Do the large amounts of phenylalanine in tissues of patients with PKU inhibit tyrosine hydroxylase sufficiently to disturb peripheral and central sympathetic activity? If so, then we may expect to find similarities between phenylalanine and α-methyl-tyrosine in their pharmacologic effects in patients without liver phenylalanine hydroxylase. Attempts are also being made to produce animals deficient in liver phenylalanine hydroxylase activity by administering p-chlorophenylalanine.

Collagen and Hydroxypyroline

Out laboratory was the first to demonstrate formation of collagen hydroxyproline in cell-free systems and to present evidence for hydroxylation of peptidyl proline. Until last year several laboratories claimed that hydroxylation occurred at the level of t-RNA-proline. In the last year these contrary claims have been answered and withdrawn and it is now clear from work in three other laboratories (Prockop, Lukens, Meister) that we were correct in the mechanism we proposed. Continuing these studies, we were able to show that all those tested of a number of tissues which form collagen are capable of accumulating a protein deficient in hypro-^{14}C when incubated with proline-^{14}C. This protein is formed when hydroxylation is inhibited for any reason, e.g. lack of oxygen, chelation of Fe^{++}, lack of ascorbic acid, destruction of proline hydroxylase. The protein can be extracted along with collagen by the use of neutral salt solutions or acetic acid and can be degraded to peptides by specific bacterial collagenase. More recently we showed that the solubilized newly formed hypro deficient "collagen" obtained from guinea pig granuloma, fetal rat skin and chick embryo can all be hydroxylated by chick embryo proline hydroxylase. The latter enzyme has now been demonstrated in soluble preparations obtained from rat liver, fetal rat skin, and guinea pig granuloma. Previously the chick embryo system was the only cell-free system in which proline hydroxylation could be demonstrated.

Soluble proline hydroxylase, purified several fold, is stimulated by Fe^{++}, ascorbic acid and an unknown dialyzable substance found in boiled tissue extracts. It does not hydroxylate free proline or tripeptides containing proline. Proline, hydroxyproline, gly-pro-pro and gly-pro-hypro do not inhibit the hydroxylation of peptidyl-proline. This represents further evidence that smaller residues are not intermediates in the hydroxylation. Two potent inhibitors of peptidyl-proline hydroxylase are poly-proline-MW 10,000 and $(gly\text{-}pro\text{-}pro)_n$-MW 5,000. The former has been shown to inhibit competitively with solubilized protein substrate. Attempts are being made to produce inhibitors which are not only specific, but small enough to penetrate into cells and inhibit the enzyme *in vivo*.

Studies on peptidyl proline hydroxylase have been time consuming because the method of assay took about two days. Several years ago, we had considered using tritium labeled proline to follow proline hydroxylation by oxidative displacement of a tritium atom and we persuaded Dr. Bernhard Witkop to prepare 3,4-tritio-proline from 3,4-dehydroproline.



$T^+ + H_2O \rightleftarrows THO + H^+$

In the crude cell-free chick embryo systems, we could not use this procedure because of

many interfering side reactions. However, Meister succeeded in using it in following hydroxyproline formation in whole cell preparations from guinea pig granuloma. Recently, we have been able to prepare soluble hydroxyproline deficient substate, labeled with proline-^{3}H, for studies of proline hydroxylase. This material was purified and shown to contain traces of hydroxyproline-^{3}H (less than 1% of the proline). On incubation with proline hydroxylase we have demonstrated a stoichiometric displacement of tritium from substrate proline into water which is collected by a rapid distillation procedure. The method is extremely rapid, the blanks are essentially zero and the labeled substrate is stable indefinitely. With this procedure, we hope to purify the enzyme, characterize it and develop useful inhibitors. In collaboration with Dr. Arieh Berger, we are looking into syntehtic polypeptide substrates and inhibitors.

Actinomycin Biosynthesis

Although attempts to obtain synthesis of this peptide antibiotic in cell-free systems have failed, it has been possible to obtain more information on the nature of the synthetic process. 4-Methyl-3-hydroxyanthranilic acid (MHA) has been identified as a normal intermediate in the formation of actinomycin. Presumably this compound adds on the amino acid present in the pentapeptide chain. It has also been shown that the addition of D-valine to the medium results in the accumulation of MHA. This D-amino acid known to be an inhibitor of antibiotic production, inhibited either the formation or the attachment of the pentapeptide chain.

Other studies have shown that actinomycin inhibits the organism that produces it, suggesting that the production of the antibiotic may be a way by which the organism regulates its metabolism. Highly-labeled actinomycin has been produced and its distribution in tumor-bearing mice was investigated.

Cobamide Dependent Methionine Synthesis

The cobamide-dependent enzyme catalyzing methyl transfer from N^5-methyl-folate to homocysteine has been purified 200-fold. It is salmon-red in color and studies are being initiated on the binding of radioactive substrates to the enzyme. The unique function of S-adenosylmethionine (AMe) in this reaction has been investigated. It appears that AMe methylates the enzyme since other methyl donors (methyliodide, etc.) can replace Ame in the catalytic reaction. Studies are continuing in the formation of boloenzyme.

$$\text{apoenzyme} + \text{cobamide} \longrightarrow \text{holoenzyme}$$

A specific enzyme is necessary for this reaction if the cobamide employed is either hydroxy or deoxyadenosyl-B_{12}. Attempts to purify this enzyme as well as apoenzyme are in progress.

Formation of Formyl-methionine sNRA

The synthesis of N-formyl-methionine sRNA may be an important reaction in the initiation of protein synthesis. The enzyme that catalyzes the reaction

$$N^{10}\ \text{formyl} - \text{THF} + \text{methionine sRNA} \longrightarrow \text{N} - \text{formyl methionine sRNA} + \text{THF}$$

has been purified 2000-fold. The characteristics of the reaction are under study. The specificity for the substrate resides in the sRNA molecule. Ethionine and norleucine, when attached to the methionine sRNA, are also formylated.

Aromatic Hydroxylation

5-Tritio-tryptophan has been synthesized and used to develop an assay for tryptophan hydroxylase. There is a marked isotope effect in a hydroxylase system from mast cells although none was observed in whole cells of *Chromobacterium violaceum.*

Studies on the mechanism of ether cleavage by a microsomal system have shown that O^{18} is not incorporated into the phenolic product. This type of reaction does not involve a single displacement of the methoxy group by molecular oxygen.

Serine Metabolism

A simple and sensitive method for the assay of serine hydroxymethylase has been developed.

It is based on the isolation of formaldehyde released as the dimedon derivative.

2,3-Dihydroxybenzoylserine has been isolated as a metabolite of stationary cells of *E. coli*. It is synthesized by the following reaction:

2,3 — dihydroxybenzoic acid + serine + ATP⟶2,3 — dihydroxybenzoylserine.

The enzymatic reaction and the role of this compound in the metabolism of *E. coli* are under investigation.

Studies on Amino Acid Transport

Three aminonaphthylalanines were prepared and shown to have fluorescence characteristics which were discernible in the presence of animal and bacterial cells. Two of these amino acids were found to be concentrated by Sarcoma-37 ascites cells in a manner comparable to that of naturally occurring aromatic amino acids. The fluorescence of the amino acid after its active transport into cells did not differ in excitation spectrum, emission spectrum, quantum yield and temperature coefficient. Examination with the fluorescence microscope indicated uniform distribution through the cytoplasm. Furthermore, rupture of the cells released the amino acids completely. By contrast atabrine, eosin and anilinonaphthalenesulfonic acid, which are known to bind to macromolecules, exhibited changes in many parameters and were not released from the cell on rupture. Since intracellular concentration of the aminonaphthylalanines does not alter fluorescence characteristics which are normally changed when a small molecule binds to a large one it is reasonable to assume that the amino acid exists in a free form. These findings represent direct evidence that actively transported amino acids exist free in the cell.

Peptides and Peptide Linkages

The major kinin in wasp venom has been purified and subjected to sequence analysis. Preliminary data indicate that glutamic acid is at the N-terminal end. The sequence of two amino acids remain in doubt (in parenthesis) but the following structure is fairly certain:

glu (asp, thr) lys lys leu arg lys gly
arg pro pro gly phe ser (bradykinin) pro phe arg (sequence)

The demonstration of β-aspartyl linkages in fibrin has been established.

Brain Nucleic Acid

Studies with rats in which the liver is ligated have shown that simple precursors such as aspartic acid, orotic acid and glucose do not yield appreciable labeling in brain RNA. These results agree with studies on brain slices which indicate that brain requires preformed nucleosides for its synthesis of RNA and presumably obtains them via the blood from the liver.

Bacterial Phenylalanine Hydroxylase and Its Cofactor

Induced phenylalanine hydroxylase of *Pseudomonas sp.* has been purified to a high degree. It has been shown to have an absolute requirement for Fe^{++} but can be activated by many other metals. The pteridine cofactor required for activity is also synthesized by the organism and it has been shown that it is derived from guanosine phosphate. Some of the intermediate enzymes required for synthesis of the pteridine have been isolated and studied.

Other aspects of control of aromatic amino acid biosynthesis in *Pseudomonas* have also been investigated.

Nitrogen Containing Lipids

The significance of palmitylethanolamide is still not clear. On the one hand it is normally made and found in tissues. On the other hand its synthesis in *in vitro* preparations seems to represent an artifact formed by the reaction of ethanolamine with some form of "activated" fatty acid. The nature of this activated fatty acid in tissues remains to be determined.

Enzyme Mechanisms

Glutamic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, tyrosine hydroxylase and dopamine-β-hydroxylase have been subjected to careful kinetic studies. Antibodies

have been prepared to some of these and the mechanism of interaction studied.

EXPERIMENTAL THERAPEUTICS BRANCH

A broad spectrum of research has been continued over the past year with a major orientation toward findings which may be of immediate or at least potential clinical significance. Data obtained will be considered under three headings: (1) Biochemistry and Pharmacology of Aromatic Amines, (2) Studies of Selected Proteins, and (3) Miscellaneous.

Biochemistry and Pharmacology of Aromatic Amines

It has been our impression that, with the exception of amines in urine collected following the ingestion of amine-containing foods, the urinary aromatic amines are of endogenous origin. Evidence has been presented in the literature, however, that the action of intestinal bacteria may contribute to urinary tyramine and tryptamine. Our studies on the effects of glucose diets and intestinal sterilization have reconfirmed the conclusion that tyramine, tryptamine and metanephrine are of tissue origin and reflect endogenous metabolism. Rather than a decrease in the excretion of tyramine and tryptamine during gut sterilization in patients receiving a constant diet, a significant and consistent increase in excretion of these amines was observed. This latter finding has not been explained as yet.

Previous work showed that α-hydrazino-histidine is a potent and selective inhibitor of histidine decarboxylase in animal systems both *in vitro* and *in vivo*. Clinical trials of the compound were initiated in three patients. It was quickly established that the drug is poorly tolerated because of the development of emesis and further clinical use is not anticipated. Concomitantly, a former member of the department has administered another histidine decarboxylase inhibitor, 4-bromo-3-hydroxy-benzyloxyamine (NSD–1055), to patients with urticaria pigmentosa and has observed rapid and striking clinical improvement. Similar studies are planned in this department with NSD–1055 which is also a potent inhibitor of dopa decarboxylase and dopamine β-oxidase. It was shown previously that up to 1.0 gm per day of this compound is well tolerated by human subjects.

A sensitive radioassay has been developed for measuring the activity of tryptophan hydroxylase; the reaction catalyzed by this enzyme appears to be the rate limiting step in the biosynthesis of serotonin. The assay was perfected using as a prototype the tryptophan hydroxylase from neoplastic murine mast cells. Preliminary attempts to study tryptophan hydroxylase in mammalian brain tissue have indicated low levels of this enzyme in rabbit brain stem in some experiments. Unlike mast cell tryptophan hydroxylase, active preparations of the brain stem were not stimulated by tetrahydropteridine or ferrous iron. The mast cell preparation is in use both here and elsewhere in screening programs for the discovery of the inhibitors of tryptophan hydroxylase. Such compounds should be useful in the treatment of patients with the carcinoid syndrome. First stage clinical testing is planned with one such agent, 3,4-dihydroxyphenyl-β-propylacetamide (dopacetamide), a potent inhibitor of tryptophan hydroxylase both *in vitro* and *in vivo*.

Tryptophan deficiency has been suspected of causing African cardiomyopathy and serotonin of causing endomyocardial fibrosis. A number of studies of serotonin metabolism in tryptophan deficient rats have been done with inconclusive results. Peculiarly, such animals show a greater uptake of injected serotonin by the heart than do controls. Further studies are planned relative to reports in the literature that patients with African cardiomyopathy have elevated plasma serotonin concentrations and that tryptophan deficient rats have increased amounts of 5-hydroxyindole-acetic acid in the urine.

Hydroxylation of tyrosine to dopa has been found to be the rate limiting step in the biosynthesis of the catecholamines. In collaboration with members of LCB a number of studies have been performed in laboratory animals. Rats, pretreated with the tyrosine hydroxylase inhibitor α-methyl-tyrosine (αMPT) and then stressed by exercise on a treadmill or exposure

to cold (3° C), show a great reduction in brain, heart, spleen and adrenal catecholamines. There is little change with stress or αMPT alone. The findings are interpreted as indicating increased rates of synthesis of norepinephrine and epinephrine due to increased sympathetic nerve activity. The effect of sympathetic nerve activity on norepinephrine synthesis was evaluated more directly in a newly-designed, isolated nerve, perfused-heart preparation of the guinea pig. Nerve stimulation increased norepinephrine synthesis as judged by the difference in concentrations of the amine in the heart in the presence and absence of αMPT. Thus far, however, it has not been possible to demonstrate an increased incorporation of radioactivity into norepinephrine from C^{14}-tyrosine in the perfusing fluid. It may be necessary to add a monoamine oxidase inhibitor to prevent metabolism of the amine and to study the effect of varying concentrations of tyrosine in the perfusing fluid. A number of tyrosine hydroxylase inhibitors have been compared for their catecholamine depleting potency *in vivo* in the hope that at least on compound in addition to αMPT might be available for clinical studies. One possibility is L-3-iodo-α-methyl-tyrosine which is a more effective inhibitor than αMPT. Another is α-methyl-phenylalanine which is about as potent as αMPT and has the advantage of a much greater solubility in water. Clinical studies with αMPT have been concerned with its absorption, metabolism, chemical effects and pharmacologic effects in patients with pheochromocytoma, and essential hypertension. About 30 patients have been studied, 17 of whom had pheochromocytoma. In oral doses of 400–400 mg/day of αMPT, inhibition of catecholamine synthesis was shown as indicated by the urinary excretion of catecholamines and their metabolites. Reductions of synthesis up to 60–90% were achieved by the larger doses in both types of patients. In cases of pheochromocytoma reductions of catecholamine synthesis were accompanied by marked clinical improvement; subjects with essential hypertension showed only a minimal blood pressure reduction while receiving the drug. It was shown that after oral administration of αMPT 45–90% is absorbed. After single oral doses of the drug maximum blood levels occur within two hours and 50% of the dose is excreted within 4 to 6 hours though detectable amounts of drug (radioactive label) are excreted for up to 3 days. Almost all the orally absorbed drug is excreted unaltered though small amounts appear as the decarboxylated product, αmethyltyramine, and as catechol products of which α-methyl-dopa and α-methyl-dopamine have been identified. The drug appears to have definite value in the management of cases of malignant pheochromocytoma and in preparation of benign cases for surgery. In a few cases of the latter type the tumor has been rendered functionless by the drug. While a slight lowering of blood pressure is demonstrable in the patients with essential hypertension receiving large doses of the drug, it is doubtful that the hypotensive potency of the compound is sufficient to be therapeutically useful. Most patients develop readily recognizable nervous system effects during treatment with αMPT. Typically the patients show sedation during the first two days of therapy; this may be pronounced but is usually greatly diminished within three days. At dosages of 3 gm or more per day, occasional patients develop tremor and agitation. Upon discontinuation of drug most patients show some degree of insomnia and heightened alertness and energy for several days before resuming their usual behavior pattern. Overt depression has not been observed. It is possible that αMPT would be therapeutically useful in other diseases such as thyrotoxicosis, Raynaud's disease, anxiety states, angina pectoris and endotoxin shock; further investigations are planned along these lines.

The tyramine pressor test for pheochromocytoma has now been evaluated in 20 patients with this tumor. The incidence of false negative tests is in the range of 25–30% which is comparable to the incidence of false negatives with the histamine test. In contrast to the histamine test, however, there appears to be no hazard in the use of tryamine as a provocative agent. A continuing interest in catecholamine metabolism has resulted in extensive clinical experience in the medical and surgical

management of patients with pheochromocytoma. This has permitted a number of other studies to be performed. Contrary to data reported in the literature, no diminution in red cell mass or plasma volume has been found except in cases of severely ill patients with metastatic disease. In spite of the apparently normal blood volume it has been possible to obviate the typical severe hypotension following removal of tumor by vigorous transfusion with volume expanders (blood, plasma or albumin in saline solution). Experimental use of the β-adrenergic blocking drug propranolol has shown it to be effective in the management of cardiac arrhythmias occurring during surgery. Several of the pheochromocytoma patients studied during the past year are members of a single family and have exhibited the recently recognized triad of hyperparathyroidism, thyroid medullary carcinoma with amyloid deposits and bilateral adrenal pheochromocytomas. The huge increase in the work load of analyses of catecholamine metabolites has led us to set up a computer program for the calculation of results.

Studies on the turnover rates of radioactive dopamine, and norepinephrine and its metabolites following the intravenous administration of H^3 dopa have been expanded considerably. The half time for turnover of dopamine, norepinephrine and vanilylmandelic acid averaged 6 hours, 8 hours and 14 hours respectively in normal subjects and hypertensive patients. No significant difference between normals and hypertensives was noted. During treatment with αMPT the specific activity of norepinephrine was increased, a finding consistent with a small dopa pool. Treatment with a monoamine oxidase inhibitor also increased the degree of labelling of norepinephrine and VMA but slowed their rates of decay. Reserpine therapy greatly increased the rate of decay. The latter finding is one of the most convincing demonstrations of an effect of reserpine in clinical doses on catecholamine metabolism in the human.

Studies of Selected Proteins

Measurements of the urinary excretion of hydroxyproline (HOPr) peptides as an index of endogenous collagen metabolism have been continued in a number of clinical circumstances. Hydroxyproline excretion was found to be normal or slightly elevated in patients with scleroderma. When dimethylsulfoxide (DMSO) was applied topically to almost the entire body (in doses as high as 70 ml of 90% DMSO) of 5 patients with sclerodema, urinary hydroxyproline was unchanged. Healing of ischemic ulcers was noted in 2 patients concomitant with DMSO therapy. Urinary hydroxyproline excretion was also found to be unchanged in patients with scleroderma receiving up to 12 gm per day of potassium paraaminobenzoic acid or 2 gm daily of D-penicillamine. In one patient with severe scleroderma who received β-aminopropionitrile (BAPN) for 4 weeks at a dose of 3 gm daily, urinary HOPr increased about 50%.

During the past year a number of techniques have been adapted to determination of a "collagen profile" in minced specimens of dermis obtained by punch biopsy. This profile consists in part of determination of water content, total collagen, percent of collagen extractable into 0.5 molar acetic acid, and the degree of cross-linking in soluble collagen as estimated by disc electrophoresis on acrylamide gel. One hundred twenty biopsies from patients with various clinical disorders in soluble collagen was observed along with a decrease in cross-linking, associated with a high water content. Young normal scars had a similar collagen profile while scars older than 6 months resembled normal skin. D-penicillamine, used in the treatment of Wilson's disease, cystinuria and (by one investigator) rheumatoid arthritis, was found to produce an accumulation of soluble, poorly cross-linked precursors of insoluble collagen in the skin. This is the effect seen in animals given lathyrogenic agents such as BAPN. In several patients with Wilson's disease treated with large doses of penicillamine for more than 4 years, levels of soluble collagen were found to be 5 times those in normals (patients = 20%; normals $<3.5\%$). Possible therapeutic use of penicillamine in scleroderma is suggested by the finding that in this disease soluble collagen in dermis is consistently less than normal. Heritable diseases of connective

tissue were also evaluated. Patients with Marfan's syndrome, Ehlers-Danlos syndrome and osteogenesis imperfecta were found to have normal collagen solubility and cross-linking. Patients with Ehlers-Danlos syndrome were found to have less total collagen expressed as μg/HOPr/mg dry weight than normals. Several patients with homocystinuria—a condition with excess circulating and tissue levels of sulfhydryl-containing compounds similar to penicillamine—have had significantly increased solubility and decreased cross-linking in dermal collagen. Normal collagen profiles have been found in acromegaly and iatrogenic Cushing's disease. Adult patients with isolated growth hormone deficiency have less soluble collagen than normal. This is in contrast to the high levels of solubility with normal cross-linking in collagen of adolescents and children, reflecting phases of active growth and protein turnover. Earlier hypotheses that Marfan's syndrome and osteogenesis imperfecta represent abnormalities in the maturation of collagen thus were not supported by these studies. On the other hand penicillamine produces a true "collagen disorder" in man.

The lathyrogenic agent BAPN has been administered carefully to five patients with advanced scleroderma. Maximum dose administered was 3 gm per day and maximum duration 4 weeks. A method was developed for measuring BAPN in biological materials using the automatic amino acid analyzer. The major metabolite of BAPN, cyanoacetic acid, has been measured by either gas or paper chromatography. After single or multiple oral dosage, 7–15% of the drug can be recovered unchanged in the urine, and 50–75% as cyanoacetic acid. The half-life of BAPN in the blood is about 2.5 hours and no accumulation is observed on a 6 hour schedule of dosage. Mild allergic reactions developed in two of the patients, disappearing rapidly when the drug was discontinued. With the addition of the dermal collagen profile it is felt we can now proceed more rapidly and safely with trials of BAPN since the effect of the drug on collagen can be more accurately monitored and is thought to be the main basis of toxicity in experimental lathyrism. The incidence of allergic reactions to the drug is unknown but thus far these have not been of sufficient severity to deter us from further investigations. Probably the BAPN studies will be slowed somewhat by the recent findings that penicillamine, a current drug, also has powerful effects on collagen cross-linking. Since the cross-linking in collagen appears to involve the oxidative deamination of terminal amino groups of lysine residues, importance of a soluble monoamine oxidase is suggested. We have proceeded on the basis that plasma monoamine oxidase may be the enzyme involved and accordingly have developed a method of radioassay for this enzyme. Preliminary findings indicate that BAPN is a competitive inhibitor of plasma monoamine oxidase and that the monoamine oxidase levels in plasma of patients receiving pencillamine is markedly reduced.

The conversion of radioactive proline to urinary peptide-bound hydroxyproline has been studied in young adult rats and in a single patient with scleroderma. The rats were studied several weeks after the administration of a single pulse label and at a time when the specific activity of urinary hydroxyproline was almost constant, reflecting metabolism of previously labeled and now insoluble collagen. When parathyroid hormone was administered to these rats, urinary hydroxyproline excretion increased to twice normal levels while hydroxyproline specific activity remained unchanged. This indicates that parathyroid hormone exerts its effects on the metabolism of insoluble collagen since the increase in urinary hydroxyproline originated from the labeled insoluble collagen pool. Similar studies with thyroid hormone indicate a mixed action, that is, both on synthesis and degradation. The attempt to label the collagen of a patient with scleroderma was successful. The specific activity of his plasma hydroxyproline was found to have a half-life of about 6 days and the graph of his urinary hydroxyproline specific activity indicated contributions from at least two different pools of collagen. The first had a half-life of 0.5 days and probably represents the soluble collagen pool while the second had a half-life of over 60 days, representing the insoluble collagen pool. It will not be possible to do several studies on the effects of drugs on

human collagen in this patient whose collagen pools have been labeled with C^{14} in hydroxyproline.

Studies have continued on the small nonheme iron containing proteins which serve as electron carriers in low redox potential reactions in certain bacteria. These include the clostridial ferredoxins and a new protein termed "rubredoxin" which was first isolated and crystallized from C. pasteurianum in this laboratory. According to the literature ferredoxin is a one electron carrier but conclusive evidence has now been obtained that clostridial ferredoxin is a two electron carrier. A search for ferredoxin-like proteins in mammalian systems has so far been unsuccessful. Studies on rubredoxin indicate that it has a molecular weight of about 6,000, contains a single iron atom and has a redox potential (minus 0.05 v) considerably higher than ferredoxin (minus 0.42 v). Studies on the reactivity of the cysteines of the molecule as well as electron spin resonance studies indicate that the iron in ferredoxin is ionically bound in contrast to the apparent covalent iron-sulfur bonding in ferredoxin. Similarity of the protein to ferrichrome A, transferrin and a number of ferric iron chelates is suggested. Although the metabolic significance of rubredoxin is unknown it would appear to be useful in the study of iron binding mechanisms.

Miscellaneous

The only study in this category which has potential of generating a new research program is that concerned with oxidative phosphorylation in mitochondria. The work was an outgrowth of attempts to extend the ferredoxin studies into mammalian systems. Of several different findings the most interesting were those indicating that administration of large depot doses of catecholamines in rats produces an uncoupling of oxidative phosphorylation in heart mitochondria studied *in vitro*. This catecholamine affect cannot be shown *in vitro* using mitochondria from untreated animals. The time course of catecholamine accumulation by myocardium was found to be similar to the P/O ratio depression produced following catecholamine administration. Results following pretreatment with adrenergic blocking agents provided further evidence of a correlation between uncoupling of oxidative phosphorylation and elevation of myocardial catecholamines. Although the mechanism of the catecholamine effect *in vivo* is not yet defined, the phenomenon itself may contribute to our understanding of oxygen "wasting" that can be produced by catecholamines in animals and in man.

LABORATORY OF TECHNICAL DEVELOPMENT

Fluorescence Methods

Methods in current use for measuring the absolute quantum yield of fluorescence (ratio of quanta emitted to number absorbed) are unsatisfactory. The methods are either imprecise or require special equipment, thus making it difficult to check on claims of accuracy. It seemed to us that the Aminco-Bowman spectrophotofluorometer could be used for this purpose, since our instrument was calibrated last year. To demonstrate that quantum yields could indeed be measured with this instrument, studies were undetraken to measure corrected spectra of compounds which had been reported on in the literature as well as compounds which had never been investigated with respect to fluorescence efficiency. By publishing our results, it was hoped that it could be established that commercially available instrumentation was capable of performing a determination hitherto thought possible only with specially-built components.

The spectrophotofluorometer was evaluated with regard to quantum yield determinations by making these determinations for some 30 solutions, for some of which there were literature values. In general, the agreement with established values were good. Temperature dependence of quantum yield, and corrected emission spectra were determined. Sources of error, advantages, and disadvantages of the system were listed.

Quantum yields of tyrosine, tryptophan, and phenylalanine were found to be about 33% lower than reported by Teale and Weber. Our

values of 0.15, 0.14, and 0.027 were recommended for use as standards in protein fluorescence work. Quantum yields of fluorophenylalanines (3 isomeric forms) were found to be about 0.19.

Quantum yields and fluorescence polarizations were determined for a large number of dye-conjugates of bovine serum albumin. These are the first measurements of this type for such commonly used (in immunology, for example) dyes such as fluorescein and 1-dimethylaminonaphthalene-5-sulfonate. It was shown that dye-dye energy transfer occurs and causes a decrease in fluorescence yield with degree of labeling. Perrin plots showed that the depolarization was due to energy transfer in heavily labeled conjugates. Föster distances were calculated for four types of dyes, and the results indicated that dipole dipole interaction was probable on a molecule of the size of BSA.

The fluorescence quantum yield of 1-dimethylaminonaphthalene-5-sulfonate was found to be lower than reported by Weber and Teale. In addition, the fluorescence was quenched by bicarbonate. It was pointed out that some workers had used the erroneously high figure of Weber and Teale in their calculations.

Quantum yields of pyridoxamine-5-phosphate, pyridoxamine, and pyridoxal were found to be 0.14, 0.11, and 0.048 at room temperature in neutral aqueous solutions. Fluorescence polarization spectrum of pyridoxamine-5-phosphate and the temperature dependence of quantum yields of the three vitamin B_6 compounds were determined. The results clearly conflicted with a literature report that claimed the quantum yield of pyridoxamine-5-phosphate was 0.55 under these conditions.

While measuring the quantum yield of tyrosine, it was noted that phosphate was a strong quencher of fluorescence. The quenching of tyrosine fluorescence was investigated in some detail and Stern-Volmer constants were determined. The tyrosine fluorescence of proteins was also found to be quenched by phosphate. Evidence from fluorescence polarization, temperature-dependence curves, the relative sizes of the quenching and association constants, and data from a larger quencher (propionate) showed that the quenching was mainly collisional. The use of phosphate as a probe of the accessibility of tyrosines in proteins was discussed.

DPNH and TPNH fluorescence can be assayed in the Aminco-Bowman instrument at high concentrations (up to 3×10^{-4}M) by the use of a microcell and excitation at 395 mμ. Under usual conditions of assay employed by many workers, the limit is no higher than 10^{-5}M. It was shown that non-linearity of the fluorescence vs. concentration curves was due to absorption of the incident light rather than absorption of the emitted light.

A single horizontally-oriented polarizer in the excitation beam was found to reduce drastically the amount of light scatter reaching the detector. It was shown that this was effective for protein emission spectral work, where the intense scattering of the UV excitation is a problem.

To demonstrate the effectiveness of horizontally polarized excitation in spectral work with proteins, the emission spectra of human serum albumin at pH's near 4 were determined. It was discovered that there are marked changes over a narrow pH range, which had been established by other workers using a variety of physical techniques to coincide with an unfolding of HSA. Analysis of the spectra showed that acidification was accompanied by a quenching of tryptophan fluorescence and an enhancement of tyrosine fluorescence. This can be understood in terms of a decrease in tyrosine-to-tryptophan energy transfer.

In addition, a small number of experiments on the fluorescence of dye-labeled glutamic dehydrogenase and polynucleotides were performed in continuation of projects started the previous year. These results still must be evaluated but they indicate that the extended sensitivity we have achieved in polarization measurements may increase the usefulness of the spectrophotofluorometer.

All of the material above has either been published or submitted for publication.

Emission spectra of bovine serum albumin and the interaction of this protein with a dye-

anilinonaphthalene-sulfonate, are under investigation. The use of such dyes as molecular probes of conformational change has been postulated by others and confirmed by us.

Protein chemists are interested in amino acids labeled with 1-dimethylaminonaphthalene-5-sulfonate ("dansyl amino-acids") since his dye locates amino acids on chromatograms with about 1000-fold more sensitivity than ninhydrin. The fluorescence of these derivatives has never been characterized, but we have prepared about 23 dansyl amino acids and purified them by thin layer chromatography. Quantum yields and corrected spectra have been determined. These results will be part of a paper in preparation.

Ultramicro Methods

The helium glow photometer for the simultaneous analysis of Na and K at the picomole (10^{-12} moles) level has been further improved by the addition of operating convenience and proven by the application to several problems in the analysis of renal tubule micropuncture samples in cooperation with the LKEM.

The extension of the method to calcium and magnesium was explored and preliminary results indicated that sensitivity was more than adequate being in the 10^{-13} mole range. Conditions for calcium measurements have been worked out to eliminate interference to the point that accuracy in the $\pm 2\%$ range is confirmed for calcium at the picomole level with good possibility of extension to 10^{-13} moles. The magnesium method has not been explored sufficiently to predict performance.

A fluorometric method for determining nanograms of inulin obtained by micropuncture of renal tubules has been developed. The reaction of fructose, the subunit of inulin, with dimedone in concentrated o-phosphoric acid produces a moderately fluorescent product which has its emission peak near 400 nm and excitation peak near 360 mm. A concentration of 1 μg/ml of fructose or inulin gives a signal that is twice the blank signal.

The method has been shown to be more sensitive than the colorimetric method commonly used. However, an additional order of magnitude increase in sensitivity seems desirable. We will attempt to improve the optical arrangement to reduce the scattered light and to reduce the volume of reagent required.

A method of producing luminescence by treatment with ozone has been discovered to be a remarkedly convenient and highly sensitive method of analysis of a wide variety of compounds of biological interest. Although it has been known for many years that ozone can induce luminescence in a few compounds, it has never been explored as an analytical method for biochemical application.

Our exploration has revealed that some compounds on suitable substrates emit enough light on exposure to ozone gas to permit clean assay at the level of 10^{-14} grams and a large proportion of fluorescent substances can be excited to luminescence with ozone to permit assay at the 10^{-12} gram level.

Several dozen compounds have been discovered to have useful luminescence by this method with some indication that several compounds with relatively poor sensitivity for ultraviolet fluorescence excitation have high sensitivity by ozone excitation. For example, the dye Safranine is weakly fluorescent, but emits strongly when excited by ozone. Ozone chemiluminescence methods are being compared with fluorescence methods for specific problems to discover what advantages the method has over fluorescence or other analytical techniques.

The spectrum of emission appears to be generally related to that of fluorescence and provides some specificity but the low level and evanescent character of the emission makes spectral measurements tedious.

Excitation can be produced in solution or dry so that chromatographic plate spots can be reacted for quantitative assay. The compound is usually consumed in the process with the emission of a number of photons proportional to the quantity present. The apparatus is very simple, consisting of a photometric system and a suitable chamber for exposing the sample to ozone enriched oxygen provided by passing tank oxygen through an electric discharge produced by a high voltage transformer.

Automation of Bacteria Counts and Antibiotic Sensitivity Tests

Consideration of the burden of counting bacteria in body fluids and the determination of antibiotic sensitivity by the clinical laboratory indicated a need for a simplification of the methodology amenable to automation and reduction in time required before a definitive report can be rendered. A capillary tube method is based on the idea that nutrient agar in a capillary tube will permit the growth and multiplication of individual organisms to a multiplet of organisms in less time than it takes to form a recognizable colony and that a simple scanning and memory system can be used to recognize and count only those organisms that have demonstrated their viability by beginning to multiply.

The capillary tubes replace not only the petri dishes but serve as measuring pipettes and provide a linear array of organisms which can be counted with a much simpler system than a planar array.

The capillary tube is exposed to a sharply defined plane of illumination provided by the light of a neon laser. The neon laser light is a continuous high intensity coherent source of simple design available at reasonable cost that can be focused so fine that the resolution of the system can be relatively independent of the radial position of the organism in the capillary.

A simple magnetic tape recording of the scattered light from the initial population including debris is recorded, the capillary tube incubated and a new recording indexed to match the previous recording is made while the first signal is subtracted so that only those organisms that have multiplied are counted. Variation including repeated counts and incubations as well as histograms of growth rate per organism can be easily obtained to increase the information obtainable. Antibiotics incorporated at specific dilutions in the medium can easily be assayed for more specific information than can be obtained by clear zone methods. For example, it is apparent that the latent period before division can be measured for a large number of individual organisms in a short time in contrast to the tedium of such observations by watching single organisms for a first sign of division under the microscope.

The apparatus has been constructed and tested for small particles and methods developed for eliminating noise due to defects in the capillary tube and light scattering from the agar. Resolution and sensitivity seem adequate but application to actual counts and determination of accuracy have not been completed.

Artificial Organs

The membrane oxygenator developed previously is still not in production because of the manufacturer's delays in getting satisfactory production methods. Some experimental miniature oxygenators were constructed for isolated organ or tumor perfusion are now undergoing experimental evaluation in the NCI in a cooperative project.

The biventricular cardiac assistor development has progressed through some 18 modifications to delineate the important design parameters. In situ tests on fibrillating as well as normally beating dog hearts were performed. Normal blood pressure and normal pulse wave forms were achieved. Defibrillation promptly restored the normal beat after 4 hours of fibrillation in the assistor. The longest duration of massage was 11 hours. A system to apply the assistance in synchrony with a normal beat has been developed to determine the ability of the assistor to augment the function of a failing heart. It is presumed that maintenance of the integrity of the circulation will permit or accelerate healing of a damaged heart that would otherwise be unable to sustain the circulation. The assistor has been applied to dog hearts and the chest completely closed to confirm its potential for longer periods of support.

Some improvements in the mechanical design of the "artificial kidney" hemodialysis unit have improved the efficiency to permit high clearance at low dialysate flow. The addition of adsorbants such as activated carbon further increases the effective concentration gradient for adsorbable substances. Most small

molecule metabolites with the exception of urea are probably adsorbable. Hippuric acid dialysate capacity is increased 14 times. This represents a highly efficient compact disposable unit not requiring any priming blood, and using a minimum of dialysate.

Fast Reaction Methods

The developments in instruments and methods for the study of fast chemical reactions in solution include the introduction of a new mixer, a fast pH detector system, and a new thermocouple amplifier. The new mixer permits 99% mixing to be achieved within 40 microseconds or less even in the mixing of 92% glycerol and water.

A small stopped flow apparatus has been constructed for us by the Biomedical Engineering Branch which permits the use of optical, fluorescence, pH, and thermal detectors. Time resolution for pH work is about 50 milliseconds and sensitivity is 0.001 pH unit with the presently available glass electrodes. Thermal and optical time resolution is in the range of 2 to 5 milliseconds.

Our instrument built by Science Products Corporation has improved time resolution with the new mixer system of 40 microseconds for a continuous flow thermal detector. Optical detection in stopped flow is 100 microseconds.

Work has progressed on several fast thermal detectors to where a one millisecond response time has been achieved with glyptal coated copper-constantan thermocouples. Thin film sputtering techniques are being developed by Victory Engineering Corporation to coat the junctions with quartz in order to still further reduce the response time.

The twin cell differential microcalorimeter has been under test for six months and is satisfactory for heats of reaction which produce 30 millicalories or more in 4 ml of solution. The Tris-HCl reaction is being developed as a solution calorimetry standard and a commercial version of the instrument has been manufactured by Science Products Corp.

The data restoration program developed for the differential calorimeter has been put into operation and critically tested. It has been shown to be able to restore electrical heater data from the calorimeter to better than 2 accuracy regardless of the heat input function shape. Thus the adiabatic losses from the calorimeter can be corrected for, even when reactions run for twenty minutes.

Using the simulation of physical laws method of solving partial differential equations developed here, a theoretical treatment of the thermocouple response time measurements obtained using different thermoelectric materials and dimensions as well as different coating materials has been achieved which agrees within the experimental error of 2–5%.

Chromatographic and Ultrasonic Methods

Ultrasonic methods of measuring the content of sample in gas chromatography effluents developed and applied last year have been suitably modified and tested for performance in liquid chromatographic effluent streams. The system tested measures the change in phase of a 20 MH_z sound wave in a 1 ml cell to 0.01° due to a velocity change in response to a few micrograms of sample in 1 ml with the sensitivity limited by temperature fluctuations of the sample. An order of magnitude or two increase in sensitivity is available by better thermal stabilization.

A variation of the method of sound velocity measurements has been applied to permit very accurate determination of sonic velocity in solutions. Here an interferometric technique based on standing wave measurements using a variable sound frequency which can be very accurately measured. The sound frequency is changed until the standing wave is re-established by accurately identifying the position of the standing wave peaks. Sonic velocity is computed electronically and is related to density and compressibility.

As density is easily measured the method offers a very accurate measure of compressibility. Some preliminary measurements have been made to examine the possibility that changes in compressibility of a solution of large molecules would reflect the degree of dipole association and the influence of dielectric constant of the solvent on the association. The unfolding of albumin produced by low pH

values was disappointing but other systems may be more suitable.

Miscellaneous

A system of measuring low gas flows (1 to 100 ml/min) where the soap bubble meter is not applicable due to solubility or permeation of the soap film was developed, applied and published. A microperfusion pump that delivers $25x10^{-6}$ ml/min. by programmed controlled thermal expansion of the oil in a micropipette has been developed for micro puncture experiments. This pump eliminates the need to support a heavy mechanical piston drive mechanism on the micromanipulator to provide greater freedom of motion and versatility.

An air bearing turbine-operated grindstone was devised and tested to provide a very simple method of sharpening micropuncture pipettes.

Recovery of microgram quantities of labeled fatty acids on T.L.C. films without solvent extraction and the current problems of reconcentrating the sample was demonstrated by vacuum distillation in a simple apparatus.

The utility of activated carbon for the removal of residual fatty acids bound to pure samples of albumin was demonstrated.

LABORATORY OF CARDIOVASCULAR PHYSIOLOGY

This report represents the last to be submitted by the Laboratory of Cardiovascular Physiology of the National Heart Institute. Since its inception in 1954 under the direction of Dr. S. J. Sarnoff until its inactivation on July 1 of this year, the laboratory has made many major contributions to cardiovascular physiology. Besides the high quality experimental work which was considered by many to characterize the laboratory, the laboratory provided a fertile field for the training of many young men, many of whom subsequently went on to senior positions in research teaching and medicine. The contributions of the laboratory which, to a large extent, represent the contributions of Dr. S. J. Sarnoff who retired as the result of illness in August of last year are best summarized in the citation which accompanied the Gairdner Foundation Award presented to Dr. Sarnoff in 1962, . . . "in recognition of his contribution to the knowledge of cardiac physiology and especially for his demonstration of the interrelated roles of the nervous system, hormones, and heart size in the control of cardiac performance, thus establishing physiological principles which have assisted medical scientists to better understand the action of the heart in normal and diseased states."

Studies on Myocardial Mechanics

The contribution of physical and chemical factors to the adaptation of the heart under varying conditions has continued to be of substantial interest. Several years ago this laboratory clearly defined the phenomenon of homeometric autoregulation, i.e., the increase in myocardial contractility associated with an elevation of ventricular outflow resistance. It was shown that homeometric autoregulation was associated with a loss of myocardial potassium. Since it was known that decreasing intramyocardial K^+ was associated with an increase in myocardial contractility in isolated cardiac muscle, we suggested that the appearance of homeometric autoregulation was causally related to the potassium loss. At the same time the possibility existed that homeometric autoregulation was caused by physical changes in the heart such as shape or compliance changes. The former possibility was of particular interest since we had observed that when aortic pressure was increased isovolumic systolic expansion often increased. This observation suggested the possibility that a two-step Frank-Starling effect may occur when aortic pressure is increased; the first step occurring at end-diastole and the second step occurring with the expansion of the circumferential fibers. We had also observed, however, that if heart size was increased the extent of isovolumic systolic expansion decreases. We therefore undertook experiments in which, when aortic pressure was increased, heart size also increased, in order to cancel the effects of the two interventions on isovolumic systolic

expansion. Such experiments showed that an increase in isovolumic systolic expansion was not necessary for the appearance of homeometric autoregulation. In these same experiments we also observed that a change in ventricular compliance occurs when aortic pressure is increased. The compliance change however could not explain the changes in myocardial performance. We have concluded therefore that while physical factors may contribute to the adaptation of the heart when aortic pressure is elevated, they do not appear to be necessary for homeometric autoregulation. With respect to the possible contribution of compliance changes to ventricular adaptation, investigations were carried out to determine if ventricular compliance changes during single or paired ventricular pacing. In the ejecting heart in which aortic pressure and heart rate were maintained relatively constant, no discernible influence of either type of stimulation on the relation between left ventricular end-diastolic pressure and circumference was observed. In contrast, paired ventricular stimulation was observed to increase the compliance of the isovolumic ventricle in which developed tension increases during stimulation. Also, the increase in compliance paralleled the increase in developed tension. These experiments taken in conjunction with the experiments in which homeometric autoregulation was induced suggest that paired stimulation has no direct effect on ventricular compliance but will alter compliance if developed tension is changed significantly.

An understanding of the mechanisms whereby the output of the heart is controlled is, to a large extent, an understanding of the factors controlling the volume of blood in the heart at the end of diastole and at the end of systole. While it is appreciated that inotropic interventions modify the fraction of blood ejected by the ventricle at each beat the role of physical factors in controlling stroke volume is not always appreciated. Studies were carried out therefore to determine the influence of hemodynamic factors on ejected ventricular fraction. It was observed that increasing cardiac filling increased the fraction of blood ejected by the ventricle while an increase in aortic pressure decreased the ejected fraction. These experiments show therefore that hemodynamic variables can modify the ejected fraction and point out the inadequacy of this measurement as an index of myocardial contractility.

Despite the large amount of published data describing the phenomenon in isolated tissue, it was reported recently that the classical Bowditch Staircase or "Treppe" could not be demonstrated in the intact dog atrium. This finding was unexpected, at least to us, for it would not seem appropriate for a change in frequency of stimulation to produce the well established increase in ventricular contractility without an accompanying increase in atrial contractility. The problem was studied therefore in this laboratory with special emphasis on observing the influence of heart rate on atrial force and rate of development of force over a broad range (approximately 60–200/min). The results of the studies have demonstrated conclusively that the contractility of the intact dog atrium is changed with respect to both force and velocity when heart rate is modified. Also, potentiation of contraction is produced by extrasystoles and by continuous post-extrasystolic potentiation. Thus, the increase in the force of atrial contraction produced by increasing heart rate can significantly augment ventricular filling during tachycardia.

When heart rate was increased to high levels in the above study pulsus alternans was occasionally observed. Since the experimental design was such that force gauges were usually placed on both the atrium and ventricle it was possible to observe the influence of alternans on both chambers. Of interest has been the observations of what might be termed asynergistic alternans. This asynergism has been observed on the same side of the heart between atrium and ventricle as well as between the right and left side of the heart. Of further interest is the observation that during alternans the strong beat occurs from a lower tension than the weak beat suggesting that pulsus alternans may be the result of alternating diastolic compliance.

Myocardial Oxygen Consumption

Studies concerning myocardial metabolism have been directed primarily towards determining those factors which can modify myocardial oxygen consumption. Previous studies using isolated cardiac muscle have been extended to the whole heart in which it was found that developed tension is a major determinant of myocardial oxygen consumption. Studies in both the whole heart and isolated cardiac muscle have been the first to demonstrate conclusively that at a constant inotropic background the amount and/or rate of shortening of cardiac muscle can influence oxygen consumption. In the working heart this influence is small while in isolated muscle it is somewhat greater. When calcium is added to the bath containing isolated cardiac muscle while maintaining heart rate, preload and afterload constant, substantial increases in oxygen consumption are observed. Although the increase in oxygen consumption is associated with increases in the extent and rate of muscle shortening, it is also possible that a direct metabolic effect of the calcium is contributing to the oxygen consumption changes. When calcium is administered to the whole heart working from a high end-diastolic pressure, substantial increases in contractility occur without a consistent change in oxygen consumption. The failure to show a change in oxygen consumption under these conditions may be due to the cancelling effect of the decrease in developed tension which would decrease myocardial oxygen consumption and a direct metabolic effect which would increase oxygen consumption. The experiments mentioned above in the whole heart would indicate that the extent or rate of shortening would play only a small role. In any event, it is of interest that calcium, as we have shown also for acetyl strophanthidin and norepinephrine, can produce substantial increases in myocardial performance without a net oxygen cost. With respect to acetyl strophanthidin it is of interest that preliminary experiments suggest that this drug has an oxygen cost which does not necessarily correlate with changes in shortening and/or rate of shortening.

Influence of Drugs and Electrolytes on the Heart

Previous studies from this laboratory have shown that the myocardial effects induced by various agents are associated with changes in myocardial potassium balance. These studies have been extended to determine the influence of calcium and sodium bicarbonate on myocardial performance and potassium balance. Like those of norepinephrine, the inotropic effects of calcium chloride have been found to be associated with a net gain of myocardial potassium and no consistent influence on myocardial oxygen consumption. The extent of the potassium change is directly related to the amount of calcium administered. The influence of calcium on K^+ balance is in contrast to the loss of myocardial K^+ associated with the administration of cardiac glycosides. These experiments have suggested the possibility that under inotropic influences the myocardial movements of K^+ and calcium occur in the same direction. This would then lead to the possibility that agents such as cardiac glycosides which cause a loss of myocardial K^+ may also produce an associated loss of myocardial calcium.

Experiments have also been completed which characterize the myocardial response to sodium bicarbonate infusion. This acid-base disturbance produces an initial decrease followed by an increase in myocardial performance. These changes are not dependent upon changes in myocardial potassium balance, coronary blood flow or changes in the effective catecholamine background. Rather they appear to be the result of changes in intracellular hydrogen ion concentration. It was demonstrated that during sodium bicarbonate infusion the bicarbonate ion concentration of cardiac muscle is increased.

In view of the extensive use of Dicumarol and Coumadin as oral anticoagulants it was of interest to determine if these agents have a direct effect on myocardial performance when administered to the dog in therapeutic doses. Preliminary, although consistent, results indicate that over a wide dosage range Dicumarol depresses, while Coumadin appears to have little influence on the myocardium.

Studies on Electrical Stimulation of the Heart

Previous studies have shown a minimum energy threshold for cardiac stimulation in chronic and acute preparations at pulse durations between 0.5 and 1.0 milliseconds, using a constant current stimulus source. Histological sections of cardiac tissue from these chronic preparation implantation sites have shown >0.2 mm fibrous ring thicknesses around the electrode. Thus, stimulation occurs in excitable tissue beyond this fibrous ring, i.e., beyond 0.2 mm from the electrode surface.

Analysis of the electrochemical impedances at the electrode-electrolyte interface shows three components. Two of these impedances increase with time during the pulse, (activation and concentration impedances) while the third (ohmic) is constant. All vary with current density (not current) but only ohmic impedance is directly proportional to current density. By studying the immediate stimulus electrode environment in electrolyte solutions and isolated heart tissue using glass microelectrodes, it has been shown that activation and concentration impedances are distributed within 10 microns from the electrode surface. This is not the depolarizing region, and stimulus energy dissipated in this region does not contribute to stimulation. Only the ohmic impedance is distributed in the region where tissue is depolarized in chronic preparations. Since this impedance is directly proportional to the current density at the point of deplorization, it is proposed that the electrical influence which leads to depolarization is also proportional to the current density in these preparations.

The distribution of ohmic impedance in myocardium is not uniform. It has been demonstrated in the isolated cat heart papillary muscle that this impedance is greater across fibers than along fibers. This provides a means of investigating "local disturbance" as a function of potential gradients, since for a constant current pulse the potential gradient is steeper across fibers than along fibers.

Studies on Water and Salt Regulation

Previous studies demonstrated a substantial contribution of intracardiac and intravascular receptors to the control of body water. The extent to which non-cardiac vagal fibers contribute to the control of body water is not known. Experiments were carried out therefore to determine the influence of section of the vagal nerves on water diuresis in the dog in which cardiac vagal nerves had been ablated. In these animals vagal nerve section is always associated with a delayed antidiuresis which can be attributed to a decrease in free water excretion. No consistent change in sodium excretion is seen. In some animals the antidiuresis is sustained while in others it is only transient. This variability appears to be related to the extent of water loading and thus the extent of the water diuresis. The findings suggest that extracardiac vagal pathways can modulate free water excretion and thus possibly the circulating levels of ADH. At the same time, the failure of dogs given a heavy water load to show a sustained antidiuresis following vagotomy indicates that other controlling mechanisms are operating. The latter may reside, at least in part, in the carotid sinus area.

Studies on the Peripheral Circulation

The contribution of potassium to the control of skeletal vasculature resistance has continued to be of substantial interest. Previous studies demonstrated that the vasodilation of simulated muscle exercise or reactive hyperemia is associated with a loss of potassium from the muscle. When the resting muscle was perfused with K^+ so as to effect the same increase in venous K^+ as that which occurred during exercise or hyperemia, less vasodilation was observed. Studies were undertaken therefore to determine if changes in venous pO_2 can influence the vasodilator response to a given level of venous K^+. The results of these experiments demonstrate that the influence of potassium on vascular resistance is determined to a large extent by the oxygen supplied to the muscle. Thus, the lower the pO_2 of blood the greater the vasodilator effect of a given level of blood potassium. The experiments indicate that both oxygen and potassium contribute substantially to the control of skeletal muscle blood flow and that they can reinforce

each other in influencing vascular resistance in both active and resting skeletal muscle.

The interrelationship between the vascular resistance changes induced in skeletal muscle by sympathetic stimulation and ionic balance has been studied. Substantial alterations in the total calcium concentration of the blood perfusing the muscle does not influence the degree of vasoconstriction produced by the infusion of norepinephrine and epinephrine even though the calcium concentration was altered within broad extremes. The injection of $CaCl_2$ failed to increase or prolong the vasoconstriction induced by the single injection of these two catecholamines. It was found that these agents, norepinephrine and epinephrine, did substantially increase the venous pO_2 level (at constant arterial pO_2 and blood flow) when they produced vasoconstriction but exerted no effect on pO_2 when they produced vasodilation such as could be produced by epinephrine following an alpha receptor block. Of note, it was observed consistently that beta adrenergic stimulation resulted in an uptake of potassium by the muscle whether or not vasoconstriction or vasodilatation occurred. Chemically blocking the beta receptors prevented these potassium changes. Such findings indicate that these changes might be used in the study of adrenergic receptors and could aid possibly in their chemical and morphological classification.

It has been suggested by others that kinins act as physiological mediators of functional vasodilatation in glands. To test this proposal experiments were carried out in which the influence of carboxypeptidase B on the vasodilatation of the cat salivary gland produced by stimulation of the chorda tympani nerve was determined. The direct infusion of kallidin into the artery of the salivary gland produced vasodilatation which was blocked by the simuletaneous administration of carboxypeptidase B. In contrast, Carboxypeptidase B did not block the vasodilatation associated with nerve stimulation. The vasodilatation was essentially blocked however, by a combination of atropine and a beta blocking agent. These experiments indicate therefore that the kinins play little or no role in the functional vasodilatation of the salivary gland and that the well known "atropine resistant" vasodilatation seen with nerve stimulation is the result of beta adrenergic stimulation.

Studies on the Kallikrein-Kininogen-Kinin System

Investigations have been continued on the isolation and purification of the various components of this system. During the past year special emphasis was given to the isolation of kininogen, the substrate in plasma. Two different kininogens have been isolated from human plasma. Both are glycoproteins containing hexose, sialic acid and glucosamine and have a molecular weight of approximately 50,000. Although immunologically identical they are clearly different by several physical, clinical and biological criteria. It appears that kininogen I has kallidin at the C-terminal sequence and kininogen II has kallidin near the C-terminal end of the molecule.

LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM

The research activities of the Laboratory of Kidney and Electrolyte Metabolism encompass six general fields of interest: (1) The function of the intact kidney as determined by micropuncture analyses of luminal fluid in dogs and smaller animals. (2) Water and electrolyte transport in isolated perfused portions of the rabbit nephron in vitro. (3) The characterization of electrolyte transport in red cells and the associated biochemical processes. (4) Analogous studies in epithelial membranes such as toad bladder. (5) Characterization of a protein-globulin complex in mammalian plasma. (6) The role of renin-angiotensin-aldosterone system.

Micropuncture Studies in the Kidney

Considerable progress has been made in the past year concerning the nature of the countercurrent system for urinary concentration and dilution. Implicit in this theory has been the view that an osmotic gradient develops between the ascending and descending limbs of the loops of Henle in the deeper portions of the kidney by virtue of the transport of sodium chlor-

ide out of the ascending limb without water (thereby reducing the osmotic pressure) and the loss of water without solute by osmotic flow from the descending limb (thereby increasing the osmotic pressure). Although most workers had subscribed to this view, no direct supportive evidence had been available, and in fact a number of previous attempts limited to portions of the loop very close to the turn had failed to demonstrate any significant difference in the osmotic pressure of the two adjacent limbs.

In the past year advantage has been taken of a partial nephrectomy technique developed in this laboratory which permits direct visualization of the deeper portions of the papilla in the rat (see last year's report), and thereby allows direct micropuncture of previously inaccessible portions of Henle's loop. Using this preparation, evidence has been obtained for the presence of an osmotic gradient between adjacent portions of the descending and ascending limbs consistent with the original countercurrent hypothesis. Thus, the osmotic pressure of fluid in the thin ascending limbs of loops of Henle is significantly lower than that of adjacent thin descending limbs. Similarly, the sodium concentration is lower in fluid from the ascending limb as compared with that of descending limb fluid. The difference in sodium chloride concentration between the two limbs accounts for most of the difference in osmolality, and is consistent with the idea that active transport of sodium chloride out of the ascending thin limb is the driving force for the creation of an osmotic gradient in the deeper portions of the medulla and papilla. These findings support the view that the thin loops of Henle participate actively as countercurrent multipliers in the production of a hypertonic renal medulla.

In an earlier report it had been noted on the basis of micropuncture sampling and analysis of fluid from proximal tubules in the dog, that antidiuretic hormone appeared to stimulate sodium reabsorption in this portion of the nephron. Although these results have been reported, we have remained skeptical about this interpretation since an effect of antidiuretic hormone in the proximal segment on sodium transport was inconsistent with other information regarding its site of action. Studies were therefore carried out in dogs in which samples were collected from the same site (the recollection technique) in proximal segments first during water diuresis and again after administration of antidiuretic hormone. A new remarkably sensitive and accurate technique for inulin determination based on fluorescence that was developed in the Laboratory of Technical Development was used. The tubular fluid/plasma-inulin ratio, an index of water and sodium chloride reabsorption in the proximal nephron, did not change significantly when diuresis was interrupted by the injection of vasopressin. It was concluded that the hormone has no effect on proximal sodium transport, and that the earlier conclusions were probably erroneous.

The recollection technique is to be pursued in a variety of studies in the future in an effort to determine whether the maximal sodium gradient developed in the proximal tubule during administration of osmotic diuretics can be altered by administration of diuretic agents thought to interfere with sodium transport in this segment of the nephron. No effect on sodium reabsorption as determined by changes in the tubular fluid/plasma ratio of inulin had been noted in earlier studies. However, further analysis and study are required before final conclusions can be reached regarding the proximal sites of action of these drugs.

In collaboration with the Department of Medicine of Duke University, an investigation of electrolyte transport in the distal nephron of the dog is also in progress. The recognition of the presence of distal nephrons in the superficial cortex of the dog has heretofore been a difficult problem. In the past year, however, it has been possible to detect the presence of an appreciable number of distal nephrons in the accessible portions of the superficial cortex by injecting a dye (lissamine green) which makes it relatively easy to locate these segments of the neprhon. In addition to the information being acquired concerning the normal composition of fluid in the distal tubule, it has been found that the administration of furosemide increases the tubular fluid/plasma ratio of sodium in the accessible portion of the distal

nephron and lowers the tubular fluid/plasma ratio of inulin. No effect on potassium concentration has been noted. These results support the view that furosemide limits sodium transport in the loops of Henle and permits the delivery of a greater volume of less dilute urine to the distal convolution.

A number of other analogous studies, in progress in both dogs and rats, are directed at characterizing transport and homeostatic mechanisms in individual accessible segments of the kidney.

Studies of Isolated and Separated Tubules in Rabbits In Vitro

In earlier reports the results of kinetic analyses of electrolyte transport in slices of renal cortex and suspensions of renal tubules were discussed. On the basis of these studies it was concluded that a minimum of two sodium and two potassium compartments are present within tubule cells. Thought the interpretation was reasonable on the basis of the available evidence, it was not possible to exclude with certainty that the pools of these electrolytes were located in different cells or tubule segments. Recently a technique for direct isolation and study of individual and viable segments of rabbit tubules has been perfected. Kinetic studies of isotopic exchange have been repeated under a variety of conditions, and it has been concluded that the existence of several potassium compartments represents inhomogeneity of the tubule population in slices of cortex and in suspensions of cortical tissue, or in other words that each nephron segment contains only a single potassium compartment. Similar analyses with respect to Na, however, revealed the presence of at least two compartments in individual segments.

Portions of collecting tubules have also been dissected freehand from rabbit kidneys and perfused; unidirectional and net transport of water and solute have been measured under different experimental conditions. We had previously proposed that the permeability changes induced by vasopressin in toad bladder and tubule are mediated by the intracellular formation of cyclic 3′5′ AMP. This compound produces all the effects of antidiuretic hormone in toad bladder and its concentration within the tissue is increased following incubation with hormone. No direct evidence of an effect of antidiuretic hormone on the permeability of the kidney tubule to water and solute had been developed in the past, although all investigators had agreed that the effect must be similar to that in skin and bladder. Furthermore, no effect of cyclic AMP on H_2O permeability in the kidney in vivo had been obtained. Using the microperfusion technique with isolated collecting tubules it has now been demonstrated for the first time by direct measurement that antidiuretic hormone does in fact increase the permeability of the tubule membrane to water in fashion consistent with the idea that the hormone increases either the number or size of aqueous channels or pores within the limiting membrane. It has also been found that cyclic 3′5′ AMP mimics antidiuretic hormone insofar as permeability change is concerned in the collecting tubule, again suggesting that this compound may mediate the action of antidiuretic hormone. No effect on urea permeability has been noted with either ADH or cyclic AMP, a finding which contrasts with observations in toad bladder. This and other studies of electrolyte transport, the electrical characteristics of the membrane in proximal tubule and collecting tubule are in progress.

The naturally occurring fatty acid, prostaglandin (PGE), first discovered by Von Euler and subsequently characterized as to chemical composition by Bergstrom at the Karolinska Institute in Stockholm, appears to be a major component of "medullin," an extract made from renal medulla which lowers blood pressure in animals with reno-prival hypertension. Some time ago it was reported from this laboratory that PGE interfered with the permeability changes in toad bladder inuced by antidiuretic hormone and a physiological regulatory role in the kidney was suggested. No effect on the changes in permeability induced with exogenous cyclic AMP was noted, a finding consistent with the view that PGE interferes with the mechanism by which antidiuretic hormone induces an increase in the concentration of cyclic AMP in the responsive tissues. PGE

has now been shown to inhibit the effects of antidiuretic hormone on the permeability of the collecting tubule, adding credence to the view that this naturally occurring fatty acid may be physiologically significant in renal tissue.

Red Cell Studies

Studies of the characteristics of electrolyte transport across red cell membranes of a variety of species have been a continuing effort in this laboratory. Hemolyzed preparations of red cells (ghosts) as well as intact red cells have been studied. On the basis of earlier work, it had been assumed that transport of ions as measured by unidirectional fluxes can be separated, operationally, into at least three distinct components; active transport, that is, transport against an electrochemical gradient; passive diffusion; and so-called exchange diffusion. Exchange diffusion is assumed to involve a one-for-one exchange of an ion with a counterion of the same species, that is, sodium for sodium or potassium for potassium. Recent studies have cast considerable doubt on the operational definition of exchange diffusion. Exchange diffusion is by definition symmetrical, and any manipulation which modifies one of the unidirectional fluxes must exert a similar effect on the reverse flux. This has not turned out to be the case in all instances of what had been assumed to be exchange diffusion. Further evidence has developed that essentially eliminates the possibility that true exchange diffusion, sodium for sodium, or potassium for potassium, does in fact occur in the human red cell. It now appears that sodium-sensitive sodium efflux, previously considered to represent exchange diffusion, and potassium-sensitive potassium influx both are effected by a hitherto underscribed component of active transport.

In the past year progress has been made in further characterizing this second active pump system. It has been established that sodium-sensitive sodium efflux is, in fact, active, but differs from the usual form of the potassium and sodium active transport mechanism (Pump I) since it is not inhibited by digitalis glycosides, does not utilize ATP as its proximate energy source, does not require external potassium, and is not stimulated by an increase in internal sodium concentration. Active sodium efflux of the second type (Pump II), though not inhibited by ouabain alone, is inhibited by a combination of ethacrynic acid and ouabain and is sensitive to external sodium. A component of active potassium influx has similar characteristics. This transport mechanism has been demonstrated both in ghost preparations and in intact human red cells. It appears that under normal conditions approximately 90% of the sodium movement out of the red cell is by active transport and approximately 90% of the potassium entering the cell moves by active transport. The classical active transport process moves approximately 60 to 70% of the sodium and potassium while the newly described mechanism transports the other 20–30%. It is not clear whether the newly discovered mechanism should be interpreted as requiring a second carrier or whether an entirely new conceptualization of the transport process is required.

The role of the active transport in the regulation of red cell metabolism has also been studied in both human red cells and ghost preparations. A successful attempt has been made to delineate the mechanism of interaction between cation pump activity and the control of glycolysis in human red cells. The flux of K^{42} and changes in enzyme activity in the glycolytic cycle were examined with and without ouabain and under a variety of other experimental conditions. It was observed that when the internal sodium of human red cells is elevated both potassium influx and lactate production rise, evidence of an increase in rate of glycolysis, and both become more sensitive to the inhibitory action of ouabain. This occurs with either glucose or adenosine as substrate. Fresh whole hemolysates enriched with sodium and magnesium will convert intermediates in the glycolytic chain above the triose-phosphate-dehydrogenase step to lactate at a rate which is slowed by ouabain. Metabolism of intermediates below this step, that is beyond the phosphoglycerate kinase step are not so affected by ouabain. The removal of membranes from the preparation eliminates the ouabain effect, evidence that the responsible enzyme is present in

the membrane. Hemoglobin-free ghosts alone were shown to contain both triose phosphate dehydrogenase and phosphoglycerate kinase activity. The reaction rate of this two enzyme sequence was measured by following the conversion of DPN to DPNH and was found to be a function of the ADP concentration in the medium. On the basis of this and other studies it was concluded that the reaction catalyzed by membrane phosphoglycerate kinase is the step at which the sodium-potassium transport system influences the metabolic rate in red cells, and this action is possibly exerted via a compartmentalized ADP which is an immediate substrate for ghost phosphoglycerate kinase. The availability of the ADP is presumably dependent upon the activity of the sodium-potassium activated APTase system which is inhibited by ouabain, thereby accounting for the inhibition of electrolyte transport and metabolism by this agent.

Studies in Toad Bladder

Vasopressin is known to have two characteristic effects on the urinary bladder of the toad: One, it increases the permeability to water, and two, it increases the rate of sodium transport. There is conflicting information concerning the energy requirement for each of these steps, and, in fact, as to whether metabolic energy was essential for the effect on permeability to water. As a first approach to the problem the effect of specific metabolic inhibitors on the responsiveness of the membrane to vasopressin was studied. The effect of these agents on the osmotic flow of water when stimulated by the hormone as well as on active sodium transport as estimated by short-circuit current were studied under a variety of experimental conditions. It had been known that many of the metabolic inhibitors interfered with the active transport of sodium induced by vasopressin, but as implied above conflicting evidence was present concerning the effect of these agents on the permeability response insofar as water is concerned. It was observed in the present studies that a number of metabolic inhibitors interfered with the response of sodium transport to hormone and also depressed the basal rate of sodium transport of toad bladder. However, in contrast to findings of other investigators, a number of these agents under varying experimental conditions also interfered with the action of the hormone on osmotic flow of water. Although the effects of the individual metabolic inhibitors were specific, as indicated by studies in which changes in lactate production, CO_2 production from labelled glucose and pyruvate, and tissue citrate concentrations were measured, it was not possible to pinpoint the precise metabolic steps involved in the osmotic permeability response. However, it was possible to conclude that metabolic energy is a necessary requirement for the alterations induced by ADH in the permeability of toad bladder to water.

In addition to the above studies a major effort has been directed at examining the control of glycogenolysis in the toad bladder as changes are induced by anaerobiosis, vasopressin, ouabain (an inhibitor of active sodium transport in this tissue) and the removal of sodium from the bathing medium. The crossover technique of Chance was used. In this instance, the technique involves the measurement of the concentration of all of the glycolytic intermediates under control and experimental conditions. Rate controlling steps are detected as the site of reciprocal alterations in the concentration of substrate and product in experimental as compared to control situations. When glycogenolysis is inhibited by ouabain or the removal of sodium from the incubation solution, an increase in the concentration of glucose-6-phosphate and fructose-6-phosphate and a fall in the concentration of fructose diphosphate and triose phosphate, pyruvate and lactate are observed, indicating inhibition of the conversion of fructose-6-phosphate to fructose diphosphate, a step catalyzed by the enzyme phosphofructokinase. The opposite pattern was obtained when glycogenolysis was stimulated by anaerobiosis. In this instance the concentrations of glucose-6-phosphate and fructose-6-phosphate were decreased, and the concentrations of fructose diphosphate, triose phosphate, pyruvate and lactate were elevated, indicating stimulation of phosphofructokinase. When vasopressin was

used to stimulate glycogenolysis, a small but significant rise in the concentrations of glucose-6-phosphate and fructose-6-phosphate and a further increase in the concentration of fructose diphosphate and triose phosphate were observed. This is evidence in support of the view that activation of both phosphorylase and phosphofructokinase is induced by vasopressin under the circumstances of these studies. Analogous studies using the crossover technique to pinpoint alterations in the rate controlling enzymes in the citric acid cycle are under way at the present time. It is also proposed to examine the metabolic effects induced by aldosterone in this tissue in a similar fashion.

Physiologic studies relating the permeability effects of antidiuretic hormone and its analogues to the concentration of divalent cations in the bathing medium are also in progress. It has been known for some time that an increase in the concentration of calcium in the bathing medium interferes with the water permeability response to antidiuretic hormone but does not eliminate the stimulation of sodium transport. No effect was observed on the action of cyclic AMP on either water or sodium movement. Presumably, the inhibitory step involving calcium precedes the formation of cyclic AMP within the tissue. The dissociation of the sodium and water permeability response to the hormone suggests the presence of two separate steps mediated by cyclic AMP, one controlling water movement, the other that of sodium. Preliminary results in a further investigation of this problem indicate that although the response to cyclic 3′5′ AMP is not affected by high concentrations of either calcium or magnesium, the increase in the rate of flow of water along an osmotic gradient in bladder in response to a variety of hormone analogues and to theophylline is inhibited by both of these divalent cations. The response to each hormone analogue apparently manifests a characteristic sensitivity pattern to one or the other cation. Further studies are in progress to delineate the effects of high concentrations of calcium and magnesium on the diffusional permeability of the bladder to water and urea with and without hormone.

The Chemical Characterization and Physiologic Significance of a Cardioglobulin System Present in Mammalian Plasma That Increases The Contractility of Isolated Frog Heart

This system is thought to contain three globulin components, all of which are necessary for the contractility response. In the past year, further purification of the factors has been accomplished revealing that certain of the components from plasma of different species (rat and human) differ in characteristics. This has permitted improvement of an assay for the presence of the cardioglobulin system in plasma, and it has been reestablished that a small increase in the fractions referred to as cardioglobulin A and C occurs in patients with long-standing hypertension, and that a decrease in fraction C is present in about 30% of patients with idiopathic myocardial failure. The significance of these changes is at present unknown. The most striking new finding, however, has been the uniform observation of a decrease in at least one of the three components of the cardioglobulin system in plasma of 22 of 24 patients with lupus erythematosus. Normal values, on the other hand, were observed in 17 other patients with other types of "connective tissue" disease. This aspect of the study will be pursued in an effort to determine the significance of the alterations in cardioglobulin composition in the plasma of patients with lupus erythematosus in the hope that it may yield information regarding the pathogenesis of the disease process.

The Renin-Angiotensin-Aldosterone System and Other Humoral Factors in Homeostasis and Disease

A major effort has been directed at examin- the renin-angiotensin-aldosterone system in normal, hypertensive animals and in those with experimentally induced heart failure and other causes of fluid retention. The major concern of this portion of the laboratory has been with establishing the mechanism of control of the secretion of aldosterone in normal and in diseased states. In addition, the nature and the function of certain extra-adrenal factors promoting sodium retention and excretion have

been investigated. Detailed background information concerning these problems has been presented in earlier reports.

In experimental hypertension in the dog, the relation of plasma renin to sodium balance and arterial pressure was examined in a number of studies. The results revealed a striking correlation between plasma renin concentration and sodium balance, but no relationship of these factors to variations in arterial pressure. The secretion of desoxycorticosterone in experimental heart failure was studied in an attempt to determine if this hormone contributes substantially to the total sodium retaining activities in this physiologic derangement. It was observed that desoxycorticosterone secretion was normal in experimental heart failure. Studies were also initiated in an attempt to define whether or not a hepatic hormone may lead to renin release. Thus far the results have been negative. Of some significance was the observation that the renin-aldosterone system is present in lower vertebrates, such as the opossum and the American bullfrog. In both of these animals renin and ACTH increased aldosterone secretion and studies of the juxtaglomerular apparatus were interpreted as indicating hyperplasia and hypergranulation during sodium depletion.

Evidence has been obtained in the past in this and other laboratories, on the basis of cross-circulation studies, that a humoral factor is involved in the natriuresis of saline loading. If one infuses saline into a donor animal and cross-circulates its blood into a recipient, a significant augmentation in sodium excretion occurs in the recipient animal. Attempts to determine the locus of secretion of this unknown humoral factor by cross-circulation of blood from hepatectomized, decapitated or nephrectomized dogs have thus far yielded negaive results. Further studies, however, are in progress. Attempts have also been made to obtain information concerning the afferent input into the system involved in the natriuresis of saline loading. The natriuretic response to the infusion of blood was compared to the response to infusion of saline. It was noted that blood as well as saline produced substantial increases in sodium excretion. Additional studies, however, are necessary to define adequately those factors which lead to certin differences in the responses to blood and saline which were observed in these studies and to determine whether or not the afferent input for the natriuretic mechanism is related mainly to volume expansion or to other factors as well.

LABORATORY OF BIOCHEMISTRY

Section on Enzymes

The Section on Enzymes continues to direct its attention to the following major areas of investigation: (1) the intracellular regulation of enzyme activities, with particular emphasis on the regulation of divergent metabolic pathways; (2) the uptake of amino acids by isolated cytoplasmic membrane preparations; (3) the metabolism of amino acids; (4) the metabolism of one-carbon compounds; (5) cystathionine synthesis and trans-sulfuration; (6) the mechanism of action of vitamin B_{12}, and; (7) the dissimilation of heterocyclic compounds. These studies embrace broad areas of enzyme function in metabolism and have led inadvertently to the discovery of several biochemical processes in which vitamin B_{12} derivatives and non-heme iron electron carrier proteins (ferredoxins) are of fundamental significance. These special processes have therefore been the target of intensive study since they represent model systems whose investigation may help to elucidate the mechanism of action of vitamin B_{12} compounds and non-heme iron proteins in intermediary metabolism.

The Regulation of Divergent Biosynthetic Pathways of Metabolism

Cumulative Feed-back Inhibition of Glutamine Synthetase.—Previous studies in this laboratory have shown that the glutamine synthetase of *Escherichia coli* is subject to feedback inhibition by eight different end-products of glutamine metabolism; namely, tryptophan, histidine, AMP, CTP, carbamyl-P, glucosamine-6-P, glycine and alanine. On the basis of kinetic studies it is concluded that each of these compounds is independent in its action. When tested individually at saturating

concentrations each product will cause only partial inhibition of the glutamine synthetase activity; however, these effects are cumulative, and when all eight compounds are present simultaneously, over 95% of the enzyme activity is inhibited.

In efforts to understand the mechanism of this unique cumulative feed-back inhibition pattern, the glutamine synthetase of *E. coli* has been isolated as a homeogeneous, crystalline protein and some of its properties have been determined.

On the basis of various physical and chemical measurements including sedimentation analysis, light scattering measurements, disc gel electrophoresis, amino acid analysis and fingerprint analysis of tryptic digests, it has been established that the enzyme has a molecular weight of about 680,000 and that it is composed of 12–14 probably identical subunits. Complete disaggregation of the enzyme into its catalytically inactive subunits (MW=49,000–53,000) is achieved by treatment either with 4 M guanidine or with 1.0 M urea plus 0.01 M EDTA. After complete disaggregation by the latter method (4°, pH 7.0), the addition of 0.02 M $MnCl_2$ causes reaggregation of the subunits to form an oligomer that is indistinguishable from the native enzyme with regard to sedimentation constant and disc gel electrophoretic mobility. This reaggregation is accompanied by restoration of up to 80% of the normal catalytic activity.

The enzyme contains 4 cysteine residues per 53,000 molecular weight subunit. At high protein concentrations, 300 μg/ml, these sulfhydryl groups are all inaccessible to titration with alkylating agents or with Ellman's reagent. However, quantitative titration of the sulfhydryl groups with Ellman's reagent is possible following denaturation with dodecyl sulfate or 4 M guanidine. On the other hand, in dilute solutions, 250 μg/ml, the enzyme does react slowly with alkylating agents such as p-chlor-mercuriphenyl sulfate (PCMPS) in the absence of protein denaturants. This alkylation is attended by dissociation into catalytically inactive subunits of undetermined size.

From considerations of the kinetics of glutamine synthetase inhibition and the diverse structures of the 8 different end-products of glutamine metabolism it was previously concluded that the enzyme (for that matter probably each of the identical subunits) possesses separate binding sites for each of the 8 feed-back inhibitors. Efforts to achieve differential inactivation of these separate binding sites by means of selective denaturation through controlled proteolytic digestion were unsuccessful. On the other hand, the existence of separate binding sites for each inhibitor is supported by investigations of the kinetics of inhibition and also from studies on the effects of the various feed-back inhibitors on the inactivation of the enzyme by PCMPS. For example, the inhibition of glutamine synthetase by tryptophan, glycine and CTP is competitive with respect to the substrate glutamate, yet these three inhibitors apparently do not react at a common binding site since tryptophan protects the enzyme from inactivation by PCMPS, whereas CTP enhances the inactivation and glycine has no effect. It is evident that histidine and glucosamine-6-P involve binding sites that are different from those that react with the other inhibitors since these two compounds are competitive only with respect to the substrate ammonia; yet histidine protects the enzyme from PCMPS inactivation, whereas glucosamine-6-P has little effect. Finally, the three end-products that are noncompetitive inhibitors of glutamine synthetase activity are differential in their effects on PCMPS inactivation. Thus, AMP protects against PCMPS inactivation, carbamyl-P enhances the inactivation and alanine has little effect. These results are most compatible with the conclusion that the enzyme possesses separate binding sites for each of the 8 feed-back inhibitors.

In order to determine the number of binding sites for tryptophan, some preliminary studies have been carried out in collaboration with Dr. Gregorio Weber at the University of Illinois. For these studies advantage was taken of the fact that the tryptophan analog, 6-nitrotryptophan (NT), will replace tryptophan as a feed-back inhibitor and protects the enzyme against PCMPS inactivation. Since NT is non-

fluorescent and possesses a strong light absorption band above 300 mμ, its binding to the enzyme is accompanied by a quenching of protein fluorescence at 335 mμ. By measuring the degree of quenching as a function of NT concentration, it has been estimated that there are about 33 moles of NT bound per mole of enzyme at saturation concentrations, and that the dissociation constant is 5×10^{-5} *M*. From these data it is concluded that between 2 and 3 equivalents of NT are bound to each subunit. The further observation that the quenching of protein fluorescence by NT is not influenced by saturating concentrations of alanine, glycine or glutamate, support the conclusion that the various feedback inhibitors are independent in their actions and that the binding of one inhibitor is not influenced by the binding of another. If the results of NT binding using the fluorescence technique can be confirmed by the more conventional method (i.e., by equilibrium dialysis) this fluorescence technique will be exploited further to study the interactions of the enzyme and the various end-products and substrates.

Finally, a new highly sensitive method has been disclosed which permits more careful kinetic measurements of glutamine synthetase activity. In this method the ADP formed as a by-product of the glutamine reaction (1) is coupled with reactions catalyzed by pyruvate kinase (reaction 2) and lactate dehydrogenase (reaction 3). The oxidation of DPNH in the over-all reaction 4 is followed spectrophotometrically and under appropriate conditions is a measure of the glutamine synthetase activity.

$$\text{ATP} + \text{NH}_3 + \text{glutamate} \longrightarrow \text{glutamine} + \text{Pi} + \text{ADP} \quad (1)$$

$$\text{ADP} + \text{p-enolpyruvate} \longrightarrow \text{ATP} + \text{Pyruvate} \quad (2)$$

$$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \longrightarrow \overset{+}{\text{DPN}} + \text{lactate} \quad (3)$$

$$\text{Sum: Glutamate} + \text{NH}_3 + \text{DPNH} + \text{p-enolpyruvate} + \text{H}^+ \longrightarrow \text{glutamine} + \text{lactate} + \overset{+}{\text{DPN}} \quad (4)$$

Using this method it was discovered that glutamine synthesis is not a linear function of time but exhibits a short lag of 1–2 minutes before maximum velocity is reached. The lag can be eliminated if this enzyme is given a prior incubation with very high concentrations of glutamate at room temperature. The concentration of glutamate (0.4 M) required for this effect is considerably greater than that needed to saturate the enzyme as a substrate in glutamine synthesis. The results suggest that glutamate at high concentration induces a conformational change in the enzyme to a form that is catalytically more active. Removal of the glutamate by sephadex filtration results in the immediate return to a preparation that exhibits a lag phase.

In order to determine the generality of the cumulative feed-back control mechanism in the regulation of glutamine synthetase, the inhibition patterns of the enzyme from various sources have been examined. Ten different microorganisms were studied including 5 aerobic bacteria representing 4 different generic types, and one species each of an obligately anaerobic organism, a photosynthetic bacterium, a mold, a yeast and a green alga. Although significant differences were found in the regulation patterns with respect to the nature and extent in inhibition, in all instances the glutamine synthetase activity was subject to inhibition by several or all of the end-products of glutamine metabolism. In most cases, a cumulative feedback mechanism seems to operate for majority of the inhibitors; however, in some instances certain pairs of inhibitors are antagonistic or synergestic in their action. Of special interest is the strongly synergistic effects of AMP and glutamine or of AMP and histidine that are observed with the glutamine synthetases of *Bacillus cereus* and *Bascillus Licheniformis*. The enzymes from these organisms promise to be of unique interest from the standpoint of allosteric interactions and purification of the enzyme from *B. licheniformis* is in progress.

The Uptake of Amino Acids by Isolated Cytoplasmic Membrane Preparations

In an effort to determine the mechanism of carrier-mediated transport, the uptake of amino acids and sugars by isolated cytoplasmic membranes from *Escherichia coli* is under investigation. The membranes are prepared from penicillin-induced spheroplasts by osmotic shock in hypotonic salt solution containing DNase, followed by the addition of EDTA, and are separated from trace amounts of intact cells and spheroplasts by sucrose density gradient sedimentation. Homogeneity of the preparations was established by electron and phase contrast microscopy, viability assays, chemical and enzymatic assays, and disc gel electrophoresis. The isolated membranes contain less than 5.0% of the DNA or RNA and less than 1% of the soluble cytoplasmic protein of the cell.

Previous studies showed that such membrane preparations from wild-type *E. coli W* catalyze the energy dependent uptake of glycine, whereas similar preparations from a mutant strain (*WS*), having a defective glycine uptake capacity, failed to do so. Although these results indicated the glycine uptake by the membranes is the manifestation of a physiologically significant transport process, interpretation of the results was complicated by the discovery that the glycine taken up by the membranes was largely converted to phosphatidyl ethanolamine, probably via serine and phosphatidyl serine. As a consequence, the intramembranal concentration of *free* glycine did not significantly exceed the external concentration. It was therefore not possible to determine if the energy requirement for glycine uptake is concerned solely with a glycine specific carrier mediated concentration system or if it is related also to the conversion of glycine to phospholipids.

In view of these considerations attention was turned to the uptake of proline whose potential metabolic fate is more restricted. It has been found that membrane preparations from *E. coli W6*, a proline auxotroph, catalyze the energy dependent concentrative uptake of proline; whereas comparable preparations from a mutant strain *E. coli W157*, that is deficient in proline uptake and exchange capacity, do not catalyze concentrative uptake of proline. In contrast to the results with glycine, the membranes from the parental strain (*W6*) establish an intramembranal concentration of proline strain (*W6*) establish an intramembranal concentration of proline that is 50 times greater than the external concentration. Moreover, more than 80% of the proline taken up can be accounted for as free proline.

Proline uptake by *W6* membranes exhibits saturation kinetics at low external proline concentrations, is oxygen dependent and is stimulated by glucose; it is inhibited by DNP, iodoacetamide, amytal and by hydroxyproline but not by other amino acids or by chloromycetin, or KCN. Fixed proline exchanges with external proline and hydroxyproline but not with other amino acids. Proline uptake by *W157* membranes exhibits linear kinetics over a 100,000-fold concentration range and is not stimulated by glucose; it is not inhibited by DNP, iodoacetamide, or hydroxyproline in concentrations sufficient to produce maximum inhibition of proline uptake by *W6* membranes. Proline taken up by *W157* membranes does not exchange with external amino acids. When membranes are allowed to accumulate proline, sonicated or solublized with detergent and then passed over a Sephadex G–50 column, less than 5% of the radioactivity is associated with the large molecular weight components coming off the column at the front. This finding indicates that the intramembranal proline is not bound to a membrane component by a covalent bond.

Finally, preliminary studies on thiomethylgalactoside uptake by membrane preparations from constitutive and deletion mutants for the lactose operon have yielded results thus far similar to those reported above for the proline system.

These results indicate that membrane preparations provide an excellent medium for studies on the mechanism of carrier mediated transport. In future studies efforts will be made to determine the nature of the energy dependent process and to establish the mechanism of permease action.

The Metabolism of Amino Acids

REDUCTIVE DEAMINATION.—Studies on the mechanism of the anaerobic phosphorylation associated with electron transfer during the reductive deamination of glycine by soluble enzyme preparations of *Clostridium sticklandii* have been continued. As noted earlier, the glycine reductase system is extremely complex, it has been resolved into at least eight separate protein components some or all of which are required for glycine reduction depending upon the nature of the electron donor system employed. Two of the protein fractions, Fraction GR and Protein A are always needed irrespective of the electron donor. When TPNH or DPNH are electron donors the respective TPNH or DPNH flavoprotein dehydrogenase are also required and in addition ferredoxin (the nonheme iron electron carrier protein), and a protein fraction B, (obtained by precipitation between 0.55 and 0.9 saturated ammonium sulfate), an arsenite sensitive protein fraction and an acetyl CoA dependent enzyme component are all needed. All of these protein components, with the possible exception of ferredoxin and DPNH dehydrogenase, which have not been tested, are required also when reduced methyl viologen is the electron donor. On the other hand, when a dimercaptan such as dimercaptothreitol is the electron donor only Fractions GR and Protein A and possibly the arsenite sensitive catalyst and the acetyl CoA dependent enzymes are needed. Thus, the artificial dimercaptan electron donors appear to bypass a highly complex electron transport system needed in the normal metabolism.

The partial purification of Protein A, an acidic low molecular weight protein component of the glycine reductase system, was described previously. Further studies show that this is a sulfhydryl protein that is inactivated by alkylation of its reduced form with iodoacetamide. The aggregated, less acidic, partially inactive form of protein A can be disaggregated and reactivated, to some extent, by prolonged treatment with dimercaptothreitol in 3 M guanidine. A possible phosphoryl group carrier function for protein A was suggested by the fact that orthophosphate partially protects protein A from inactivation and aggregation. However, in crude extracts no phosphorylation of protein A with P^{32}-labeled ATP or acetyl-P could be detected. On the other hand, it may be highly significant that under these conditions P^{32} is readily incorporated into the flavin nucleotide prosthetic groups of the TPNH and DPNH flavoprotein dehydrogenases whose isolation was previously reported.

LYSINE FERMENTATION.—The fermentation of lysine by cell-free extracts of *C. sticklandii* and Clostridium M–E leads to the formation of ammonia, acetate, butyrate and ATP according to the over-all equation:

$$\text{Lysine} + 2H_2O + Pi + ADP \quad ATP + 2NH_3 + \underset{\text{acetate} + \text{butyrate}}{}$$

Previous studies have shown this to be a complicated process requiring the participation of several enzymes, and a number of cofactors including CoA, DPN, Fe^{++}, α-ketoglutarate, pyruvate, acetyl CoA and vitamin B_{12}coenzyme.

From the standpoint of intermediary metabolism, the identification of β-lysine and 3,5-diaminohexanoic acid as early intermediates in lysine degradation discloses a very unusual biochemical mechanism. The conversion of α-lysine to β-lysine involves migration of the α-amino group to the β-position. This reaction was first described by Barker et al. and has now been shown to be the first detectable step in the fermentation of lysine by *Clostridium sticklandii* and Clostridium-ME. The reaction is sensitive to sulfhydryl reagents and probably involves a cofactor since it is inhibited by treatment of enzyme preparation with either charcoal or Dowex-1-formate.

The second detectable step is the conversion of β-lysine to 3,5-diaminohexanoic acid. This unusual reaction involves migration of the amino group from the 6 to the 5 carbon atom. From the biochemical point of view this transformation is of special interest since it involves participation of vitamin B_{12} coenzyme; in addition, ATP, Mg^{++} and probably pyruvate are needed.

When α-lysine or β-lysine are fermented by cell-free extracts at pH 7.0 (one pH unit below the optimum for the over-all fermenta-

tion), 3,5-diaminohexanoic acid accumulates in substantial amounts (30%) and has been isolated in good yield by chromatography on Dowex 50 and silicic acid columns. The enzymatic product was identified as 3,5-diaminohexanoic acid or the basis of the infra red and NMR spectra and by high resolution mass spectrometry of the enzymatic product and of its δ-lactam and N,N-dibenzoyl derivatives. Authentic samples of 3,5-diaminohexanoic acid and its δ-lactam derivative were prepared by reaction of ammonia with sorbic acid under high pressure. The synthetic products were shown to be identical with the enzymatic products by comparison of their chromatographic behaviors and their NMR and mass spectra and of their abilities to be fermented to acetate, butyrate and ammonia by cell-free extracts of *C. sticklandii.*

Future studies will be aimed at the purification of the enzymes involved in the synthesis of β-lysine and 3,5-diaminohexanoic acid. In particular the role of vitamin B_{12} in the conversion of β-lysine to 3,5-diaminohexanoic acid will be studied in detail.

One Carbon Metabolism

METHANE FERMENTATION.—A role of a methyl-B_{12} derivative in the enzymatic synthesis of methane is suggested by the previous studies in this laboratory showing that crude extracts of *Methanosarcina barkeri* catalyze the synthesis of methyl-B_{12} from methanol and vitamin B_{12s}, and in the presence of appropriate electron donors, they catalyze also the conversion of methyl-B_{12} to methane. It has now been established that formaldehyde, the carboxyl group of pyruvate and unidentified endogenous substrates present in crude extracts are also able to react enzymatically with B_{12s} to form methyl-B_{12}.

In order to elucidate the mechanism of methyl-B_{12} synthesis, the enzymatic reaction between methanol and B_{12s} has been studied in detail. Methyl-B_{12} synthesis is stimulated by a reducing atmosphere (H_2 vs argon) and is inhibited by aminopterin, arsenite, intrinsic factor and o-phenanthroline; all of which inhibit also the conversion of methanol to methane.

The enzyme system that catalyzes the synthesis of methyl-B_{12} has been resolved by DEAE-cellulose chromatography into three fractions, protein fractions R and A, and a fraction containing a heat stable cofactor, all of which are necessary in addition to ATP and Mg^{++} in order to obtain methyl-B_{12} synthesis. The active component of fraction R is probably a highly acidic red protein that has a spectrum typical of a B_{12}-protein. This component is enriched throughout a 30–50 fold purification of the active protein by means of chromatography on Bio-gel P-100 or P-200 and TEAE-cellulose or DEAE-Sephadex. Fraction A contains at least two components which are required for optimal methyl-B_{12} synthesis. One component has been identified as a ferredoxin-type protein by its spectrum in the oxidized and reduced states, and also by the fact that it will replace ferredoxin in the DPN-linked hydrogenase system of *Clostridium kluyveri.* Whether or not the ferredoxin-like activity of fraction A is identical with clostridial ferredoxins remains to be established. The function of the other protein component in fraction A that is required for methyl-B_{12} synthesis is not known.

The heat stable cofactor obtained in the effluent fraction from DEAE-cellulose columns has not yet been identified, but it has been partially purified and some of its properties have been determined. It is stable in 2 N HCl for 2 hours at 80° or in 0.1 N NaOH for 30 minutes at 100° C. It is anionic as shown by elution from Dowex-l-Cl with 0.1 N HCl. It is quantitatively adsorbed on charcoal and eluted with ammoniacal ethanol. The most highly purified preparations have no absorption in the visible spectral region, but do absorb at 260 mμ; it remains to be seen if the absorption is due to the active component.

It has not been possible to resolve the enzyme system catalyzing the over-all conversion of methanol to methane into its component parts, presumably because of the oxygen lability of the system. Nevertheless, when low concentrations of crude extract are used, sig-

nificant stimulation of the over-all conversion of methanol to methane is obtained by adding ATP, CoA and each of the resolved components (i.e., protein Fractions A and R and the heat stable cofactor) shown to be required for the synthesis of methyl-B_{12}. This is further evidence that the enzyme system catalyzing the synthesis of methyl-B_{12} is involved in the synthesis of methane. In addition to the purified heat stable cofactor described above, the conversion of methanol to methane appears to require still another heat stable cofactor present in boiled cell extracts.

Further studies will be made to characterize the heat stable cofactor and to establish the interrelationship of the various protein fractions involved in methyl-B_{12} synthesis and in the synthesis of methane from methanol.

SYNTHESIS OF ACETATE FROM CO_2.—Previous studies in this laboratory have shown that cell free extracts of *Clostridium thermoaceticum* catalyze the total synthesis of acetate from CO_2. A role of a methyl-cobalamin derivative in this synthesis is indicated by the fact that the incorporation of CO_2 into acetate is partially inhibited by intrinsic factor and by the fact that cell-free extracts catalyze the *de novo* synthesis of Co-methyl-cobalamin from CO_2, B_{12s} and pyruvate, and are able to utilize the methyl group of Co-methyl-cobalamin for the synthesis of the acetate methyl group.

As reported last year the enzyme system that catalyzes the latter reaction has been resolved into two protein fractions which together with clostridial ferredoxin from other sources are required for the utilization of Co-methyl-cobalamin in acetate synthesis. In continuing studies a third protein fraction has been obtained from crude extracts that will replace the ferredoxin requirement. This fraction possesses ferredoxin-like activity since it will substitute for ferredoxin in the hydrogenase system of *C. kluyveri*; however, it has an atypical chromatographic behavior on DEAE-cellulose columns that differentiate it from other ferredoxins. The possible relation of this ferredoxin-like compound to the recently reported favodoxin is under investigation.

In addition to the three protein fractions, the utilization of Co-methyl-cobalamin for acetate synthesis requires the presence of pyruvate and CoA; other α-ketoacids except α-ketobutyrate are not able to replace pyruvate. The specific role of pyruvate and CoA could be (1) to serve as a source of acetyl-CoA or acetyl-P; (2) to serve as a source of low potential electrons, as for example reduced ferredoxin; or (3) to serve as a source of an active carboxyl group via a transcarboxylation mechanism. Thus far attempts to demonstrate one or more of these functions have failed. The pyruvate requirement cannot be replaced by acetyl-P or acetyl-CoA or systems generating these compounds; nor can it be replaced by other sources of low potential electrons such as reduced methyl viologen nor by reduced ferredoxin generated by the hydrogenase system of *C. kluyveri*; moreover, a biotin dependent transcarboxylation function is contraindicated by the insensitivity to avidin of the over-all acetate synthesis or of the exchange of CO_2 with the pyruvate carboxyl group, that is also catalyzed by cell-free extracts.

In future studies further efforts will be made to identify the individual steps in the metabolism of Co-methyl-cobalamin and especially to establish the roles of pyruvate or of ferredoxin in the synthesis of acetate.

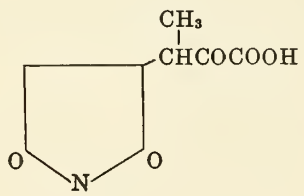
Cystathionine Biosynthesis and Transsulfuration

The major findings of the past year are in 4 areas. The first concerns the reactions mediating cystathionine synthesis from cysteine and homoserine (biosynthetic trans-sulfuration) in fungi. Two decades have elapsed since this process was first postulated to occur, and since 3 genes were defined which appeared to govern it in *Neurospora*. Mutation in one of these genes has now been found to result in: (*a*) failure to accumulate the acetyl ester of homoserine; (*b*) ability to respond to this ester nutritionally; and (*c*) lack of an enzyme catlyzing an exchange between the ester and free homoserine.

Second, one of the enzymes of bacterial biosynthetic transsulfuration, cystathionine γ-

synthetase, has been obtained in pure form from *Salmonella*, and its physical properties and subunit structure have been characterized. Third, the cystathionine γ-cleavage enzyme of *Neurospora* (reverse trans-sulfuration) has been found to be derepressed 60-fold by sulfur deprivation. Such striking derepression in this organism has previously been associated with enzymes that appear spontaneously only during sexual differentiation. However, in contrast to the latter enzymes, cystathionine γ-synthetase was not elevated after total starvation. A non-sporulating yeast showed no response to sulfur deprivation.

A unique kind of pyridoxal phosphate catalysis has been detected through the effect of added N-ethylmaleimide on γ-elimination reactions. Instead of α-ketobutyrate, a new product is formed, identified last year as structure I.



I

The structure has been confirmed by mass spectroscopy. The major findings of the past year were concerned with the mechanism of formation of compound I. The discovery that β-substituted 4-carbon amino acids also react to yield I has restricted the possible points of maleimide attack to intermediates in enamine-ketimine tautomerization. The possibility that free aminocrotonate was the reactive intermediate was emphasized by the discovery of a slow non-enzymic formation of compound I from α-ketobutyrate and maleimide, which specifically required ammonia as catalyst. Recent work has been concerned with the latter reaction. A comparison of the effects of ammonia and of a series of amines on the rate of solvent exchange with the β-hydrogen of α-ketobutyrate has made it unlikely that the non-enzymic reaction involves the formation of α-aminocrotonate. A possible clue to the catalytic action of ammonia has come from the finding that the rate of hydrolysis of maleimides is decreased in its presence.

The Mechanism of Action of Vitamin B_{12} Coenzymes

ETHANOLAMINE DIAMINASE.—It was previously established in this laboratory that the conversion of ethanolamine to acetaldehyde and ammonia by a clostridial enzyme is a vitamin B_{12} coenzyme dependent reaction. Purification of the ethanolamine deaminase was therefore undertaken in order to obtain a simple model system that could be used for investigation of the mechanism of cobamide coenzyme action.

A relatively simple three step procedure has been developed for the isolation of the enzyme from cell-free extracts of ethanolamine adapted cells. The isolated deaminase is homogeneous as judged by its sedimentation in the ultracentrifuge and its mobility on disc gel electrophoresis. The pure enzyme has an absolute requirement for cobamide coenzyme and for potassium ions. The pH optimum is from 6.8 to 8.2. Preincubation of the deaminase and coenzyme in the absence of substrate results in diminished activity within a few seconds and over 90% inactivation within five minutes. At low concentrations of the cobamide coenzyme there is a significant lag period in the deamination reaction which decreases with increases in coenzyme concentration.

The molecular weight of the deaminase is approximately 500,000 gm. as determined by the Yphantis sedimentation equilibrium method. The sedimentation coefficient is 14.5 Svedbergs. Preliminary studies indicate that 4 M guanidine causes the enzyme to dissociate into subunits of about 55,000 gm. molecular weight. The enzyme is stable indefinitely in liquid nitrogen and for several days at 4° C in concentrated solution. Amino acid analysis shows 3 half cystine residues per minimum molecular weight of 28,660 gm. Parachloromercuriphenylsulfonate is the only sulfhydryl reagent of several tested that inhibited the

enzyme. The enzyme is highly specific. Of a large number of structurally related compounds tested, ethanolamine is the only compound that will serve as a substrate.

The purified deaminase is pink to orange in color due to the presence of 1.35–2.1 equivalents of tightly bound α-adenyl cobamide derivative. The cobamide cannot be removed from the enzyme by charcoal or by dialysis, but it is removed by treatment with acid ammonium sulfate. In the presence of added cobamide coenzyme the resolved enzyme has a higher specific catalytic activity than the unresolved enzyme suggesting that the cobamide bound to the enzyme is a partially degraded, catalytically inactive compound that occupies some of the coenzyme binding sites. Preincubation of the enzyme with 10^{-8} M cyano-cobalamin results in significant noncompetitive inhibition.

The number and nature of the cobamide binding sites, the nature of the enzyme-coenzyme interaction, and the mechanism of the substrate-coenzyme interaction are subjects of future study.

The Metabolism of Heterocyclic Compounds

THE HYDROXYLATION OF NICOTINIC ACID.—The conversion of nicotinic acid to 6-hydroxynicotinic acid is the first step in the anaerobic decomposition of nicotinic acid by a clostridial species. Purification of the enzyme that catalyzes this step is being carried out with the ultimate objective of obtaining a model system to investigate the mechanism of anaerobic hydroxylation of aromatic compounds. This is a problem of unique biochemical interest since the hydroxylation of aromatic compounds in other systems so far investigated is an obligately aerobic process involving the incorporation of molecular oxygen into the hydroxyl group.

Previous reports have shown that TPN is a specific electron acceptor in the anaerobic hydroxylation of nicotinic acid as catalyzed by partially purified enzyme preparations, and that the enzyme catalyzed reaction is stimulated by Fe^{++} and by glutathione. It has now been found that the Fe^{++} and glutathione requirements can be replaced by dithiothreitol plus vitamin B_{12} derivative. These supplements are apparently needed only to remove trace amounts of oxygen since they can all be eliminated if the reaction mixture is made *strictly* anaerobic by exhaustive gassing with helium. In contrast to aerobic hydroxylation reactions, anaerobic hydroxylation of nicotinic acid is a freely reversible process. When supplemented with an appropriate TPNH generating system the clostridial enzyme catalyzes the reduction of 6-hydroxynicotinic acid to nicotinic acid.

THE REDUCTION OF 6-HYDROXYNICOTINIC ACID TO 6-OXO-1, 4, 5, 6-TETRAHYDRONICOTINIC ACID.—The second step in the fermentation of nicotinic acid is the reduction of the double bond between carbons 4 and 5. The enzyme catalyzing this reaction has been partially purified and some of its properties determined. Reduced ferredoxin appears to be the immediate election donor. The previously reported requirement or pyruvate and CoA in the metabolism of 6-hydroxynicotinic acid is probably associated with the fact that in crude extracts of the clostridium, pyruvate serves as an electron donor for the reduction of ferredoxin. This conclusion is supported by the fact that neither CoA nor pyruvate are needed for the reduction of 6-hydroxynicotinic acid when other sources of reduced ferredoxin are supplied, as for example, dithionite or molecular hydrogen plus the *C. kluyveri* hydrogenase system. Finally, it has been shown that reduced ferredoxin can be replaced by low potential dyes such as reduced methyl viologen, but not by the reduced forms of dyes with greater oxidation-reduction potentials.

THE CHEMICAL SYNTHESIS OF 6-HYDROXYNICOTINIC ACID.—Elaboration of the mechanism of nicotinic acid dissimilation would be greatly facilitated by the use of isotopically labeled 6-hydroxynicotinic acid. Therefore, various methods for the chemical synthesis of this compound have been investigated in an effort to determine a practical procedure for the synthesis of C^{14}-labeled derivatives. After several attempts, one reasonable method was developed. This method involves reaction of the

ester of nicotinic acid with benzyl bromide to form the quarternary salt which is then oxidized by alkaline ferricyanide to the corresponding α-pyridone carboxylic acid. By treatment with $POCl_5$ the α-pyridone was converted to 6-chloronicotinic acid which was finally hydrolyzed by strong acid to yield 6-hydroxynicotinic acid.

Section on Cellular Physiology

The research program of the Cellular Physiology Section continues to be directed toward the structural basis of the biochemical activities of proteins, their biosynthesis and functional relationships in the integrated activities of cell structure. This program is broadly divided into three main projects:

Studies on protein structure: In this area research is primarily focused on the fibrous proteins involved in muscle contraction and blood clotting with major emphasis on substructure and the relationship of primary structure to biochemical activity.

Protein biosynthesis: Investigations in this area are concerned with the role of cellular membranes in protein biosynthesis and the energetics of the process.

Biochemistry and cytology of cell transport: Principal attention in this area is focused on the cell membrane and its role in phagocytosis and pinocytosis.

Summaries of the individual projects follow.

Muscle Proteins: Myosin

Previous reports have summarized investigations which led to development of a three stranded rope model of the myosin molecule with a globular portion at one end. The three strands of this model are physically and chemically identical polypeptide chains all having the same sense in this rod shaped molecule with their amino terminal ends in the globular portion of the molecule. This portion also contains the enzymic sites. The latter are controlled by two cysteine residues in each polypeptide chain. Methods were developed in the past for individually labeling these two cysteine residues with a radioactive sulfhydryl reagent. Peptide fragments resulting from tryptic and chymotryptic digestion of the labeled protein have been isolated and the sequences of amino acids in the peptides determined. These two cysteine residues appear to be associated in the following twelve amino acid sequence:

$$Cys(S_2).Gly.Asn.Val.Leu.Glu.Gly.Ile.Arg.Ile.$$
$$Cys(S_1).Arg.$$

While it is not clear in this sequence how $Cys(S_2)$ affects the enzyme activity it seems possible that Cys (S_1) influences the binding of the polyanion substrate by the two positively charged arginine residues closely associated with S_1.

Studies on the Extraction of Actomyosin from Rabbit Muscle

Actomyosin, a complex of the two proteins—actinand myosin—appears to provide a model of the functional relationship of these two proteins in the contractile process.

While actomyosin can be prepared from its components, it is also extractable from muscle, under some conditions, particularly in prolonged extraction of the tissue. The present study was directed at the factors influencing the extraction of actomyosin from muscle and the nature of the low molecular weight proteins associated with the preparation. In confirmation of earlier proposals, it was observed that extraction of actomyosin occurs subsequent to breakdown of the intrinsic ATP of muscle. Extraction of actomyosin is strongly inhibited by phosphate ions. In the presence of 0.2 M phosphate no actomyosin appears in the extracts even after 24 hours extraction. This is not due to any suppression of ATP breakdown, which occurs at the same rate as in the absence of phosphate. It appears that high concentrations of phosphate ions may produce the same effect as ATP in maintaining the structure of the myofilaments and thereby inhibiting actomyosin extraction.

In the normal extraction of actomyosin low molecular weight components were detected in association with myosin before the extracted protein developed the characteristics of actomyosin. These low molecular weight com-

ponents appeared to be primarily either G-actin or F-actin of a very low degree of polymerization. Their presence in a myosin preparation, though not detectable by the usual criteria of purity, suggests the need for developing new means of evaluating homogenety in this protein.

Radiation Damage in Proteins

The previous report described an original technique for trapping and labeling free radicals formed in gamma-irradiation of protein using tritiated hydrogen sulfide. The amino acids residues susceptible to free radical formation can then be identified through the radioactive label. This technique has been applied to a number of proteins—ribonuclease, lysozyme, chymotrypsinogen, insulin, myoglobin, and gelatin. Methionine and proline were the residues most heavily labeled in all proteins—alanine, isoleucine, leucine, phenylalanine and valine were the least labeled. From examination of proteolytic digests of some of the proteins, it appears that the most heavily labeled residues are widely distributed in the amino acid sequence. Experiments with unfolded—denatured—proteins as compared to the native proteins indicate that conformation plays a role in free radical distribution. Repair of radiation damage by reaction of the tritiated hydrogen sulfide with free radical also occurs as judged by recovery of enzyme activity and other properties of the native protein.

Protein Biosynthesis

Previous reports have demonstrated the high metabolic activity of the lipid soluble amino acid and peptide fractions of hen oviduct. In the current work it was found that when water dispersions of radioactive lipo amino acids were incubated in an homogenate the amino acid moieties were efficiently incorporated into homogenate proteins. This incorporation was more than 10 times as efficient as incorporation of the free amino acid and its incorporation was not reduced by the presence of a large free amino acid pool indicating a direct transfer from the lipo amino acid moiety into homogenate protein. The active lipoamino acid material can be purified by thin layer chromatography (T.L.C.); however, a single radioactive amino acid will produce at least 9 different components on T.L.C., though only one or two of these appear to be active for incorporation into protein.

Nonphosphorylated Intermediates of Energy Transfer and Protein Biosynthesis

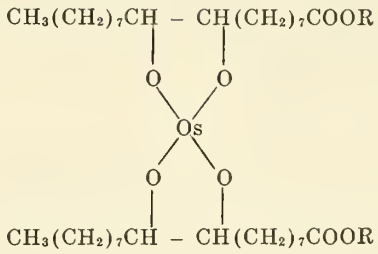
Recent work in a number of laboratories has indicated that the electron transport system gives rise to high energy intermediates that are available to a number of mitrochondial endergonic processes, including amino acid uptake, prior to their involvement of inorganic phosphate leading to ATP formation. In the present work, the generality of this non-phosphorylated intermediate concept has been examined in anaerobically grown yeast in which it was found that classical uncouplers of oxidative phosphorylation, dinitrophenol and sodium azide inhibit protein synthesis without inhibiting glycolysis or disturbing intra-cellular ATP levels. The uncouplers also inhibited RNA formation under anaerobic conditions, although RNA precursor pools and the enzyme believed to be responsible for RK synthesis, DNA dependent RNA polymerase, were unaffected by those agents. Although not conclusive, the studies suggest an effective link of the synthetic activities for RNA and protein in the cell with transient non-phosphorylated intermediates of energy transfer in mitochondria or other intracellular membrane systems.

Biochemistry and Cytology of Cell Transport

Continuing earlier studies on the uptake of fatty acids by amoebae, it has been found that unesterified fatty acids are taken up by energy-independent processes to give two forms of bound fatty acids. One is removable by washing the cells with serum albumin and the other is not. The latter class is converted to glycerides and phospholipids in a subsequent energy dependent step.

Extending earlier studies on the chemistry of some of the straining and fixation techniques used in electron microscopy, the structure of the reaction product of oxmium tetrox-

ide with oleic acid or methyl oleate has been determined as:



Demonstration that the osmic acid reacts with the double bonds in unsaturated fatty acids or their derivatives rather than with the polar portions of the molecules as widely assume, has many implications for the interpretation of electron micrographs.

Phagocytosis by amoebae has been studied using polystyrene beads. Conditions were established in which it was possible to study some of the kinetics of the process, the apparent kinetic parameters being independent of the size of the beads, large beads being taken up individually and the smaller beads accumulating on the surface and being taken up in clusters. Phagocytosis appears to be a highly discriminative process, for C^{14}-glucose and I^{131} albumin present in the medium are not taken up with the beads. The over-all process is very efficient; 10^6 cells (0.004 ml) can remove essentially all the beads from one ml of medium in 15 min and at saturation the average amoeba has taken up about 40 beads of 2.6 μ diameter. The amount of membrane surrounding the internal beads is about equal to the mass of membrane surrounding the cell. During phagocytosis no change in cell volume occurs. These appear to be the first quantitative studies on the process of phagocytosis.

Section on Comparative Biochemistry

The activities of the Section on Comparative Biochemistry are centered upon the investigation of mechanisms of lipid biosynthesis as well as a study of the control mechanisms in lipid biosynthesis. Previous work in this laboratory has established that the acyl carrier protein (ACP) plays an important function in fatty acid biosynthesis. Acyl groups involved in fatty acid synthesis are bound to ACP through thioester linkage with the sulfhydryl of the prosthetic group, 4′-phosphopantetheine. The mechanism of ACP involvement in this biosynthetic pathway has been further probed. Several of the reactions of this pathway have been studied in purified systems in order to understand the enzymatic mechanism involved. The total structure of ACP, which appears to be extremely important in determining the reactivity of acyl-ACP derivatives with the enzymes of fatty acid synthesis, has been further studied. The general occurrence of ACP in fatty acid biosynthesizing systems has been established by experiments which indicate its presence in mammalian enzyme systems. The general biochemical significance of ACP has been further studied by a search for new reactions in which ACP may be involved. Experiments suggest that it may be involved in both complex lipid synthesis and sterol synthesis. Thus ACP is the acyl carrier in a number of biosynthetic reactions in which CoA was previously thought to be involved. Therefore a study of the absolute concentrations of CoA and ACP was carried out in *E. coli*. In addition studies have been initiated which should explain the regulation of ACP concentration in the cell.

ACP Involvement in Lipid Metabolism

FATTY ACID BIOSYNTHESIS.—ACP functions as the acyl carrier in the biosynthesis of long-chain fatty acids. The condensation reaction of this pathway is complex and requires three enzymes in *E. coli*:

$$\text{Malonyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{Malonyl-S-ACP} + \text{CoA-SH} \quad (1)$$

$$\text{Acetyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{Acetyl-S-ACP} + \text{CoA-SH} \quad (2)$$

$$\text{Acetyl-S-ACP} + \text{malonyl-S-ACP} \rightleftharpoons \text{Acetoacetyl-S-ACP} + CO_2 + \text{ACP-SH} \quad (3)$$

$$\text{Sum: Malonyl-S-CoA} + \text{Acetyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{Acetoacetyl-S-ACP} + CO_2 + 2\text{CoA-SH} \quad (4)$$

Reactions 1, 2 and 3 are catalyzed by malonyl transacylase, acetyl transacylase and β-ketoacyl-ACP synthetase respectively. Malonyl transacylase was isolated as a homogeneous protein as shown by the presence of a single component upon disc-gel electrophoresis. Because this enzyme catalyzes the transfer of a malonyl group from the sulfhydryl of CoA to the sulfhydryl group of ACP and because the enzyme is a sulfhydryl protein studies were carried out to determine whether the transacylase contains a prosthetic group such as phosphopantetheine. The amino acid composition of the protein indicates that it does not contain this prosthetic group since there is no β-ananine or mercaptoethylamine in this protein. Thus, although the enzyme catalyzes the acyl transfer reaction, the sulfhydryl group of the enzyme involved in the transacylation is not a component of phosphopantetheine.

β-Ketoacyl-ACP synthetase was purified approximately 400-fold, 2-fold over the previously reported value. This enzyme catalyzes the condensation of acetyl-ACP with malonyl-ACP to form acetoacetyl-ACP, CO_2 and ACP–SH. This enzyme, like the acetyl and the malonyl transacylases, is a sulfhydryl protein. Since it had been shown previously that acetyl-ACP protects this enzyme against inactivation by N-ethylmaleimide, the hypothesis had been formulated that an acetyl-enzyme intermediate might occur during this over-all reaction. The 400-fold purified enzyme, although still containing 4 protein components upon disc-gel electrophoresis, was reacted with radioactive acetyl-ACP under various conditions. However all attempts to isolate an acetyl-enzyme or acetyl-ACP-enzyme intermediate failed. Although acetyl-ACP must bind to this enzyme, the binding may not be sufficiently stable to allow isolation of the substrate-enzyme complex.

Acetyl transacylase as well as the other enzymes of fatty acid synthesis in *E. coli* are being studied to determine whether other protein-bound prosthetic groups are involved in this pathway.

Complex Lipid Biosynthesis.—Membrane preparations of *E. coli* catalyze the synthesis of monopalmitin from α-glycerophosphate and palmityl-ACP. The monopalmitin was identified by column and thin layer chromatography. Since glycerol cannot substitute for α-glycerophosphate in this system, it is postulated that the primary reaction product is lysophosphatidic acid which undergoes secondary dephosphorylation. This same membrane preparation catalyzes the synthesis of lysophosphatidic acid from α-glycerophosphate and palmityl-CoA. It is not yet known whether separate enzyme systems are involved in the esterification of α-glycerophosphate by palmityl-ACP and by palmityl-CoA.

Sterol Biosynthesis.—A possible role for ACP in sterol synthesis through its involvement in β-hydroxy-β-methylglutarate (HMG) synthesis is suggested in experiments recently carried out with yeast condensing enzyme. Incubation of yeast condensing enzyme with acetyl-CoA and acetoacetyl-CoA gave rise as expected to HMG-CoA, whereas a similar incubation substituting acetoacety-ACP for acetoacetyl-CoA yielded HMG-ACP. Although the reaction rate was faster with acetoacetyl-CoA, the fact that acetoacetyl-ACP was metabolized is significant. *E. coli* ACP was utilized in these experiments and it is known that yeast enzymes discriminate against bacterial ACP derivatives. The possibility that ACP is involved in this reaction awaits experiments in which the enzyme and the ACP utilized are from the same source.

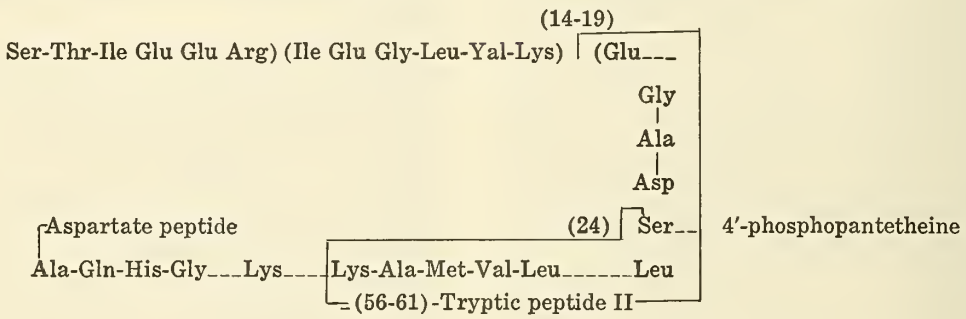
Mammalian ACP

ACP has been identified in several bacteria in this laboratory and in several plants by P. Stumpf (University of California). Its identification in more complex fatty acid synthesizing systems such as those of yeast, pigeons, and mammals is difficult since the ACP is contained within a protein complex in those systems. Injection of C^{14}-pantothenate into pantothenate deficient rats leads to the formation of C^{14}-ACP within the rat fatty acid synthetase complex. This labeled ACP can thus be studied and perhaps isolated from the complex. Using this technique of *in vivo* labeling of ACP, it was previously established that the rat fatty acid synthetase contains protein-bound C^{14} phos-

phopantetheine. Attempts to dissociate intact labeled ACP from this purified synthetase complex by treatment with deoxycholate, high pH, low ionic strength, urea, and guanidine have failed. Thus it is possible that ACP is part of the matrix of the enzyme complex in mammals. Further experiments to delineate mammalian ACP will be pursued.

Structure of E. coli ACP

Previous studies have demonstrated that the *E. coli* enzymes of fatty acid synthesis utilize as substrates acyl thioesters of ACP rather than acyl thioesters of CoA. Since both ACP and CoA contain 4′-phosphopantetheine as the terminal component to which the acyl group is bound, it is clear that some structural feature other than the prosthetic group accounts for the fact that ACP derivatives are the preferred substrates for these enzymes. Studies of the primary structure of ACP have indicated the following partial structure:



As shown above the prosthetic group is attached to serine which is about 24 residues from the amino terminus of the molecule. Peptides have been obtained by treating ACP with dilute HCl, CNBr and trypsin. Treatment of ACP with dilute HCl cleaves the protein at the aspartic acid residue which is adjacent to the serine to which phosphopantetheine is bound. The resulting peptide which contains the prosthetic group and the C terminus of the molecule was isolated and found to be inactive as tested in the malonyl-CoA-CO_2 exchange reaction. Tryptic digestion of ACP gave rise to a large peptide containing the prosthetic group and comprising about one-half the ACP molecule. This peptide was inactive as tested in the malonyl-CoA-CO_2 exchange reaction and in the β-ketoacyl-ACP synthetase reaction. However an acetoacetyl derivative of this peptide was active in the β-ketoacyl-ACP reductase reaction. Kinetic studies with this enzyme have indicated that acetoacetyl-ACP is the best substrate, but both acetoacetyl-peptide and acetoacetyl-pantetheine are active in the reaction. It is of particular interest that the pantetheine derivative is more reactive than the peptide derivative. These studies indicate the importance of the ACP protein structure in conferring the reactivity with the various enzymes of fatty acid synthesis. The specific sites of ACP responsible for the activity of ACP derivatives with these enzymes will be further studied.

Concentrations of ACP and CoA in E. coli

Evidence is accumulating which indicates that ACP rather than CoA is the acyl carrier in a number of biosynthetic reactions. Therefore it is of interest to study the concentrations of these two compounds in some biological system. A convenient system for this is a pantothenate-requiring *E. coli* auxotrophy. When this organism is grown on C^{14}-pantothenate both CoA and ACP are labeled and therefore can be easily measured. This auxotroph was grown on different concentrations of C^{14}-pantothenate. Cells grown on limiting concentrations of pantothenate contained about 0.65 mμmole ACP/mg protein and 0.04 mμmole of CoA/mg protein. When the bacteria were grown in an excess of pantothenate, extracts contained 3.0 mμmoles CoA/mg protein and 0.65 mμmole ACP/mg protein. Thus the concentration of ACP did not vary under these conditions whereas the concentration of CoA

was directly related to the concentration of pantothenate in the medium. When cells grown in an excess of pantothenate were transferred to pantothenate deficient medium, the CoA concentration decreased dramatically as the cells continued to grow normally. The ACP concentration did not change under these conditions. Thus it is clear that the ACP concentration of the cell is maintained at the expense of cellular CoA. These experiments also suggest that CoA can be the source of phosphopantetheine for ACP synthesis.

ACP Hydrolase

Study of the biosynthesis of ACP and investigation of additional reactions in which ACP is involved may be aided by the availability of ACP hydrolase (ACPase). This enzyme, purified from *E. coli*, catalyzes the manganese-dependent cleavage of 4′-phosphopantetheine from ACP and thereby inactivates ACP. One product of this reaction is 4′-phosphopantetheine which has been identified by column chromatography. The protein product, apoACP, has been isolated and shown to have approximately the same molecular weight and amino acid composition as ACP minus the prosthetic group. A hydrolytic cleavage is assumed in this reaction, by analogy to other phosphodiesterase reactions, but a β-elimination has not been ruled out. ACPase does not catalyze either the inactivation of any other protein that has been tested nor the cleavage of CoA. Its extreme specificity is demonstrated by its failure to catalyze the cleavage of 4′-phosphopantetheine from acetyl-ACP or from large peptides of ACP. It is also inactive against ACP of *Clostridium butyricum*, and it does not cleave phosphopantetheine from the mammalian fatty acid synthetase complex. This striking specificity suggests that ACPase may be involved in the rigid control of cellular holoACP concentration.

CLINICAL ENDOCRINOLOGY BRANCH

Studies in the Clinical Endocrinology Branch have included investigations of:

A. Studies of steroidogenesis by the adrenal cortex, with especial reference to biogenetic control mechanisms, the adrenogenital syndrome and the relationship of the renin-angiotensin system to aldosterone metabolism.

B. Studies of calcium and phosphorus metabolism, including *in vitro* studies of bone mineral and of solutions of calcium and phosphorus, *in vivo* studies in rachitic dogs, and clinical studies in patients with metabolic bone diseases.

C. Studies of renal physiology, with especial reference to dynamic measurements and analysis of the renal circulation, studies of the amino acid clearance in cystinuria with and without treatment, and measurements of adrenal factors affecting free water clearances.

D. Studies of neuro-endocrine relationships, including measurements of factors controlling catecholamine excretion, studies of hormonal and nonhormonal disorders of taste, smell and hearing sensitivity, and studies of steroid accumulation in the central nervous system.

Studies of Steroidogenesis by the Adrenal Cortex

Steroidogenesis was measured directly *in vivo* and *in vitro* in dogs. The rate of secretion of steroids into the adrenal vein as influenced by sodium deprivation, angiotensin infusion and potassium loading were measured over a two-day study period. The animals were then sacrificed and steroidogenesis was measured *in vitro* with and without the addition of precursors. The effect of stimulation at a rate-limiting step was observed by measuring the production of steroids below and above such a step. In this way, it was shown that sodium deprivation stimulated steroidogenesis at at least two steps, one before the production of corticosterone, and another apparently between the corticosterone and aldosterone. The stimulus to the first of these may be angiotensin, but the second appears to be clearly separate from angiotensin. A similar technique is being applied to the measurement of effects of potassium and of other agents acting on steroidogenesis.

Studies on the biogenetic defects for steroids in the adrenogenital syndrome were extended. Studies support the hypothesis that patients with the adrenogenital syndrome and congeni-

tal adrenal hyperplasia may have one or two biogenetic defects in hydroxylation of progesterone or of 17-hydroxyprogesterone as substrate. When a defect is present only in hydroxylation of 17-hydroxyprogesterone, there is hypersecretion of corticosterone and of aldosterone, suggesting an actual facilitation of hydroxylation of progesterone. It was further shown that this step was not maximally active in the subject on average sodium intake, but could be strongly stimulated by sodium deprivation and that it was dependent on ACTH.

Patients with hypertension were studied for involvement of the renin-angiotensin system by measurement of circulating renin and of renin secretion, as influenced by sodium intake and by posture, and by measurement of a wide variety of clinical indices, the most important of which appear to be the aldosterone secretion and metabolic clearance rate and the response of the serum potassium to sodium loading. The aldosterone secretion rate reveals the presence of aldosteronism and the response to sodium loading distinguishes those patients in whom over-production of aldosterone is autonomous in that secretion is not decreased by the sodium loading, and produces hypokalemia. Adrenal hyperplasia, autonomous in these respects, was found to be as common a cause of primary aldosteronism as tumor. In patients with tumor, renin concentration in the plasma is normal in recumbency with average sodium intake.

The relative role of renin and of the pituitary in the control of aldosterone secretion was investigated in patients with hypopituitarism. Aldosterone secretion and metabolic clearance rate and plasma renin were measured in patients with hypopituitarism before and after sodium deprivation. Early results suggest that effect of sodium deprivation on aldosterone secretion is normal in hypopituitarism (as opposed to reported results that aldosterone excretion is subnormal), but that the role of renin in stimulating this response is relatively much greater than that in normal subjects. Patients with panhypopituitarism are also under study for the effect of growth hormone upon production of renin and upon the secretion of aldosterone. Preliminary results suggest that aldosterone secretion rate is stimulated by purified human growth hormone.

The role of renin in steroidogenesis was further studied by measurement of steroidogenesis and of plasma angiotensin in normal subjects during infusion of angiotensin in subpressor quantities on the one hand, and during deprivation of sodium on the other. Results suggest that angiotensin stimulates production of aldosterone, corticosterone and of cortisol and that sodium deprivation is an effective stimulus to the production of angiotensin. The routes of secretion of circulating renin were further explored in normal dogs and compared with those found after constriction of the inferior vena cava, a procedure which increases renin secretion. It was found that whereas renal lymph delivery of renin increased markedly with caval constriction, it nevertheless never represented more than a very small fraction of the total renin secretion via the renal vein.

Studies of Calcium and Phosphorus Metabolism

Ionic exchange with bone mineral was reexplored in dynamic studies in which the time course of entry of calcium and phosphorous ion into bone mineral was explored, and the effect thereon of various ions in solution was measured. In this way, it was shown that uptake of calcium by the crystals is isoionic and can be characterized as a sum of four exponential curves; the same constants characterized loss of calcium from labelled crystals. The effect of ions upon the exchange was found to be closely related to the effect of these ions upon the organization of water molecules, and thus presumably upon exchange at the layer of hydration. The observations for calcium could be repeated with essentially identical results with phosphorus. These studies are continuing with observation of the effect of hormones and other agents upon exchange. Effects of calcium ions on stearylphosphate monolayers are being further explored. The observation that calcium ions contract the film area per mole provides a direct measure of calcium phosphate interaction in the solution phase. This tool may thus be used to explore the agents which affect this interaction.

The effects of Vitamin D on parathyroid hormone were further explored. In rachitic dogs, it was found that a state of endogenous hyperparathyroidism exists and removal of the parathyroid had its usual effects, including that of decreasing phosphate clearance. Administration of parathyroid extract promptly returned phosphate clearance to previous values, despite the continued absence of Vitamin D. Parathyroidectomized rachitic dogs were further treated with Vitamin D itself, and this was shown to have a parathyroid hormone-like action in increasing phosphate clearance. The role of the parathyroid and of Vitamin D on calcium clearance is being studied in a similar preparation. In such animals, parathyroidectomy increases calcium clearance, a finding consistent with the view that rickets represents a state of hyperparathyroidism. Whereas parathyroid extract was shown to be clearly effective in the Vitamin D deficient dogs, its action was quantitatively greatly augmented by addition of small doses of Vitamin D. The effects of thyrocalcitonin on renal clearance of calcium and phosphorus were studied in dogs with separate renal artery cannulas. The infusion of thyrocalcitonin in one side had no renal artery cannulas. The infusion of thyrocalcitonin in one side had no effect on phosphate clearance on that side as compared to the opposite side.

Studies in patients involve the development of a more satisfactory model for analysis of calcium accretion rate in patients subjected to various procedures and in patients with various metabolic bone diseases. Compartmental analysis for stable and isotopic calcium was laborated with the help of the department of Biomathematical Research. These methods show that our investigations obtain a great increase in resolution by the prolonged observation of radioactivity in the intact arm with a Packard-Armac counter. Analysis of results obtained thus far suggests that calcium accretion is markedly augmented in acromegaly and this result is apparent by any method of analysis. This finding probably has no relationship to the known increase in bone formation rate in acromegaly, because the locus of the bone formation is clearly different from the locus of the calcium accretion and because infusion of calcium into normal subjects produces equally striking increases in accretion rate. Studies of patients with osteoporosis receiving calcium 47 intravenously reveal an exponential decay characterized by at least three rate constants as measured over a 21-day span. The analysis of the curve suggests that patients with idiopathic hypercalciuria have a larger calcium "pool" than patients with osteoporosis, but that the loss of isotope from bones is more rapid with hypercalciuria than osteoporosis. In two patients with pseudohypoparathyroidism, together with hypothyroidism, the effects of parathyroid extract were measured with and without replacement of thyroid. In this way, a quantitative dependence of the action of parathyroid extract upon the status of thyroid function was shown. Preliminary results suggest that a similar dependence of the action of Vitamin D upon the presence or absence of thyroxin will be found in these patients.

Studies on the role of collagen metabolism in bone metabolism and of the possible use of urinary hydroxyproline as an index of bone metabolism continue. Human growth hormone was shown to increase hydroxyproline excretion in patients with hypopituitarism, as previously reported from several centers. Effects of cortisone on the rate of growth induced by human growth hormone and on the rate of hydroxyproline excretion are under study. Studies of the effect of calcium loading in osteoporosis have been greatly expanded to include measurements of the rate of bone formation and destruction as measured by microradiography and the rate of bone formation as measured by labelling with tetracycline as well as the effects on calcium dynamics and the effects on calcium and phosphorus balance. Control studies are completed in three patients and in two, the preliminary results from the calcium loading experiments are available, and suggest a clear effect on bone dynamics. The role of intestinal hyperabsorption of calcium as a determinant of hypercalciuria is under study in a large number of patients, with direct measurement of accumulation of isotopes in the blood and the bone tissue, when oral isotope is given. The result suggests that a syndrome of hyper-

absorption hypercalciuria may be clearly separable from other causes of renal stone formation.

Studies of Renal Physiology

Studies on the hormonal control of sodium and water excretion were continued in experimental animals, in normal human volunteers and in patients with pathological retention of sodium. Quantitative retention of dietary sodium was produced in adrenalectomized dogs receiving sodium-retaining steroids by the application of inferior vena caval cuff. The effect of adrenergic ganglionic blockade was then measured with infusion of pentolinium. The pentolinium produced decreases of blood pressure and less frequently marked decreases in filtered sodium load; it nevertheless consistently produced moderate to marked increases in sodium excretion. These results confirmed previous results indicating a role of the adrenergic nervous system in the control of renal handling of sodium. This was further explored in normal subjects and in patients with abnormal retention of sodium by the use of agents producing adrenergic blockade. Parahydroxyamphetamine was found to decrease blood pressure and to lower the post Valsalva overshoot of arterial pressure, and the cold pressor response. These results indicate that parahydroxyamphetamine can function as a "false transmitter", replacing norepinephrine at storage sites, and producing adrenergic blockade. It was further found that patients subjected to adrenergic blockade with hydroxyamphetamine lost sodium more readily and showed a more rapid escape from the effect of sodium-retaining steroids than they did before the drug was given. The effect of beta adrenergic blockade with propranolol and of alpha adrenergic blockade with dibenzyline was studied in a number of subjects with pathologic sodium retention and in other subjects who were receiving sodium-retaining steroids. Both forms of blockade facilitated escape from sodium retention or decrease in the quantity of edema; beta blockade was somewhat more effective in this regard than alpha blockade. Whereas these results again suggest a function of the adrenergic nervous system in renal handling of sodium, they further suggest that the role of alpha and of beta receptors in this regard will require reevaluation.

The mechanism of such actions was further explored with direct measurements of intrarenal hemodynamics as measured by Xenon-^{133}. Dogs were prepared with exteriorized bladders and individual renal arterial cannulas and prepared for gamma ray counting above the kidney. Xenon-^{133} was then injected rapidly intra-arterially and the rate of decay of renal radioactivity measured as a function of time. The resultant curves were analyzed by computer and shown to fit well to a sum of four exponentials. The effects of caval constriction on intrarenal blood flow were studied by this means and by the direct measurement of inulin and para animo hippurate clearances. The results suggest an effect of caval constriction upon intrarenal distribution of blood. The mechanism for the redistribution and the effects of various vasoactive drugs on renal circulation are under investigation.

Studies with cystinuria were greatly extended. The effects of penicillamine in systinuria were extended and compared with those of n-acetyl-penicillamine. Clinically, n-acetyl-penicillamine appears to induce less sensitivity reactions than penicillamine and to have less action as an anti-pyridoxine agent. Direct measurements of cystine excretion show that it has quantitatively as great or almost as great an effect in reducing urinary free cystine. Previous unexplained results of penicillamine in decreasing total urinary half-cystine were concerned for n-acetyl-penicillamine and were explored by (1) measurement of total excretion of cystine after institution of therapy, and (2) measurement of renal clearance of cystine, before and after treatment with the penicillamines. In this way, it was shown that the decrease in total cystine excretion with the penicillamines does not depend upon a previous increase in removal of the cystine by the urinary route and that the penicillamines consistently decrease serum cystine and have little effect on cystine clearance. Accordingly, the results establish a second role for penicillamine in cystinuria independent of that on the renal

tubules and on the sulfhydryl exchange in the renal tubules and in the urine. This effect manifested by a clear decrease in serum cystine with no change or decrease in renal clearance of cystine is under further investigation. Studies in cystinosis with penicillamine have been instituted. The extension of these studies to more patients has been deferred pending the development of a reliable method of measuring cystine pool.

The effects of adrenal factors on free water clearance have been studied. It was found that whereas small doses of sodium-retaining steroids have been shown to increase free water clearance while producing a corresponding decrease in sodium and osmolar clearance, very large doses may decrease free water clearance while decreasing osmolar and sodium clearance even more markedly. These results could be explained only as an effect of sodium-retaining steroids on proximal tubular sodium reabsorption. This effect was further explored by measuring the effects of aldosterone antagonists in subjects under the influence of sodium-retaining steroids while maximally hydrated. In such subjects, (both adrenalectomized dogs and patients with Addison's disease) blockade of the effect of sodium-retaining steroids with aldosterone antagonists produced increases of free water clearance, the result which confirms a proximal site for some of the sodium retention induced by the steroid.

Studies of Neuroendocrine Relationships

The excretion of free and bound epinephrine and norepinephrine was measured in normal subjects as a function of the time of day. The effect of changes of sodium balance and of sodium intake on catecholamine excretion was also measured. The results show a clear circadian pattern for catecholamine excretion in the normal with lowest values during the hours immediately after midnight. Acute changes in sodium balance induced by sodium loading or sodium deprivation did not produce concomitant changes in urinary bound or free epinephrine or norepinephrine. These results differ from those reported elsewhere in which less specific methods for analysis were utilized. It is confirmed that epinephrine excretion may be normal in patients with adrenal insufficiency following adrenalectomy and that excretion of norepinephrine and bound norepinephrine may be markedly increased in Addison's disease.

The effects of steroid hormones on nervous system activity were further studied. Adrenal insufficiency was found to produce marked augmentation of sensitivity of hearing which was represented approximately equally at all frequencies tested. Hearing was returned to normal in these patients with administration of carbohydrate active steroids. It was not affected by sodium-retaining steroids or by changes in sodium balance.

The central nervous system of normal and of adrenalextomized cats was analyzed for the presence of corticosteroids. It was found that in the normal animals, corticosterone and cortisol were found in brain, spinal cord, and pituitary in concentrations at least ten times those in peripheral blood. Steroids in all these tissues were significantly less two weeks after adrenalectomy, but were still significantly higher than the concentrations in peripheral blood. Results indicate concentration of steroid by nervous tissue.

The function of taste and smell was explored in patients with a wide variety of endocrine and non-endocrine disorders. In patients with extensive surgical resection of mandibular, maxillary and palatal areas, residual taste was measured and the results serve to extend existing knowledge of the areas for normal taste of the various modalities. A number of patients with hypogonadism were studied and found to have hyposmia and an inability to taste sour and bitter. Patients in this group were chromosomally of the XO type. A new syndrome was found in which facial hypoplasia, and retardation of gonadal and bone age were associated with a decrease of recognition sensitivity for taste, for stereognosis of the mouth and for smell.

LABORATORY OF METABOLISM

The work of the Laboratory of Metabolism will be summarized here by Section. Members of the three Sections continue to collaborate productively on a number of problems, but the

bulk of the work in each group stands independently and is so reviewed.

Section on Metabolism

Mobilization and Utilization of Free Fatty Acids

METABOLISM OF ADIPOSE TISSUE STUDIED IN VITRO.—An *in vitro* effect of thyroid hormone on adipose tissue has been demonstrated for the first time. Incubation of epididymal fat pads with triiodothyronine (1.25×10^{-5} M) significantly enhanced the response of the tissue to epinephrine, as measured by release of glycerol and free fatty acids (FFA). As little as 1 hour of incubation with hormone elicited a significant effect. Tissues from hypothyroid rats required longer exposure to the hormone, but after 2 hours the responsiveness to epinephrine, which is reduced in tissues of hypothyroid animals, was restored to normal. The thyroid hormone did not appear to influence the basal rate of glycerol release, but enhanced the responsiveness to epinephrine and also to ACTH and TSH. The responsiveness of the phosphorylase system to epinephrine was also enhanced in tissues incubated with triiodthyronine (T_3). These findings suggest that the effect of T_3 on adipose tissue is to "sensitize" the system that forms cyclic-3′5′-AMP.

Previous studies in this laboratory established for the first time that prostaglandins inhibit the lipolytic action of epinephrine, ACTH and several other lipid-mobilizing hormones. Studies completed during the past year show that direct addition of cyclic-3′5′-AMP to the medium at high concentration can stimulate lipolysis, compatible with the proposed key role of this compound in leading to activation of adipose tissue lipase. PGE_1 did not counteract the effect of added cyclic-3′5′-AMP. In addition it was shown that even high concentrations of PGE_1 do not counteract the effects of high concentrations of theophyllin. The latter compound inhibits a phosphodiesterase that normally breaks down cyclic-AMP.These results are interpreted to mean that under conditions in which the rate of cyclic-AMP formation is limiting (i.e., the low concentrations of theophyllin) PGE_1 can inhibit lipolysis, but that under conditions in which the further rate of formation is *not* limiting (i.e., at high theophyllin concentrations) PGE_1 becomes ineffective since it does not counteract the effects of cyclic-AMP once formed.

Work in other laboratories has shown that insulin inhibits the lipolytic action of epinephrine, ACTH and other lipid-mobilizing hormones, and that it acts by inhibiting production of cyclic-AMP. Thus, the effects of PGE_1 are closely analogous to those of insulin. For these reasons, the effects of PGE_1 on glucose metabolism were studied. It has been shown that PGE_1 stimulates glucose uptake by adipose tissue and that it enhances incorporations of glucose-C^{14} into fatty acids. PGE_1-217, a dehydrated derivative of PGE_1, does not inhibit epinephrine-induced lipolysis, and it did not stimulate glucose uptake. In contrast to its effects of glucose utilization by adipose tissue, PGE_1 did not affect glucose uptake by the rat diaphragm. Thus, PGE_1 is "insulin-like" in only a limited way. On the other hand, these findings are important in that they may clarify the fundamental basis of the action of insulin which may be related in some way to the adenyl cyclase system. The qualitative and quantitative responsiveness of this system in different tissues may vary, but the basic enzymatic processes in the membrane may be closely related.

Utilization of FFA inVivo and inVitro

FFA METABOLISM IN CELL SUSPENSION OF EHRLICH ASCITES TUMOR.—The convenient isolated cell suspensions obtainable by in vivo culure of Ehrlich ascites tumors in the peritoneal cavity of the mouse continue to be valuable in studies of basic aspects of FFA uptake and utilization. It has been shown that under appropriate conditions ascites tumor cells can effect net release of FFA to the medium.To demonsrate release it is necessary to use very small concentrations of FFA in the medium (i.e. to treat the commercial albumin preparations exhaustively to remove the FFA normally attached to them). Isotopic studies show that this release actually occurs at all times, but under most conditions there is a net uptake even

while endogenous FFA are being released into the medium. This is the first example of *net* release of FFA from a tissue other than adipose tissue. The results suggest that there need not be any unique mechanism in adipose tissue for release of FFA. Instead release may be so striking in adipose tissue only because the activity" of FFA builds up to sufficiently high levels to permit net release in the face of physiologic FFA: albumin ratios in medium or plasma. The results, if they can be extrapolated, also may help to explain some paradoxical findings in isotopic studies of FFA metabolism. For example, the apparently greater uptake of C^{14}-almitate over that of C^{12}-palmitate in perfused heart preparations may simply reflect exchange of intracellular unlabeled FFA for medium labeled FFA. The results also suggest that the intracellular FFA pools, low in terms microequivalents per gram, but highly significant in the total body mass, may influence the composition of plasma FFA and the behavior of labeled FFA introduced into the plasma.

The utilization of endogenous fatty acid esters was studied by first introducing labeled FFA into the cells and then incubating in the cells in the absence of labeled FFA. Under these conditions, the cells break down endogenous lipids and a net decrease in stored lipid esters was demonstrated. By far the largest fraction utilized was drawn from stored phospholipids. Unexpectedly it was found that the presence of a high concentration of FFA in the medium did not inhibit the breakdown of labeled lipid esters. This continued at about the same rate as in the absence of medium FFA, a large fraction of the label being released into the medium as FFA. However, in the presence of glucose plus FFA, depletion of endogenous lipid was prevented and there was a net increase. Fractionation of the cell lipid into sub-classes showed that lecithin was the major source of fatty acids used in the absence of exogenous substrate. Furthermore it appears that the stored phospholipids do not constitute a kinetically homogenous pool since radioactivity decreased more rapidly than total phospholipid concentration.

The presence of high FFA concentrations in the medium did not inhibit glucose utilization by ascites tumor cells. In this tissue there is no evidence to support the Randle hypothesis regarding competition between these major substrates.

Studies on the Consequences of Rapid FFA Mobilization.—Previous studies from this laboratory implicate high FFA plasma concentrations as relevant to a number of metabolic events: deposition of fat in the liver, increase of plasma lipoprotein concentrations, increase in plasma ketone body concentrations, and increases in metabolic rate of certain tissues. It has not been possible, however, to test the effects of high FFA concentration in isolation; i.e., it has been necessary to use hormone treatment or other devices to induce high FFA concentrations.

A method has been developed by which FFA can be infused in large amounts without the hemolytic and thrombotic implications ordinarily encountered. Arterial blood from a dog is continuously introduced into a newly designed centrifuge (developed by IBM under contract with the National Cancer Institute). The centrifuge separates cells and plasma and allows each to be withdrawn separately from the head. This separation allows introduction of FFA at high concentration into the plasma line leading from the centrifuge. This allows the FFA to become bound to the albumin *before* plasma and cells are recombined, thus avoiding hemolysis. With this method it has been possible to give as much as 5 grams of sodium oleate per hour without ill effects. Plasma FFA concentrations were increased 2–3 fold. With the development of this technique, it should now be possible to answer directly a number of interesting questions concerning effects of FFA concentration on other aspects of body metabolism.

Factors Controlling Plasma Lipoprotein Concentration

Previous work has led to the hypothesis that rapid uptake of FFA by the liver in some way stimulated lipoprotein formation and secretion. This problem has been further studied

using intravenous infusions of triglyceride emulsions in rabbits. It has been shown that after a 5-hour infusion of olive oil (emulsified in a dilute albumin solution) during which a total of 2 g/kg was injected, there was a marked increase in the plasma concentration of very low density lipoproteins. That this represented a true increase in lipoprotein concentration was established by preparative ultracentrifugation and analysis of fractions and also by paper electrophoresis. It had been previously postulated by other investigators that triglyceride emulsions induced changes in plasma lipid concentrations by "dissolving" lipids from tissue stores. If this were the mechanism it should not be accompanied by increases in lipoprotein-protein but rather by a simple increase in the lipid content of the emulsion in the blood stream. The present studies suggest that this is not the mechanism. The mechanism involved remains to be elucidated and could represent either an inhibition of the removal of lipoproteins or a stimulation of their secretion into the plasma. The latter interpretation would be consonant with the earlier studies mentioned above, but further work is needed before reaching a final conclusion.

Metabolic Studies in Refsum's Syndrome, a new lipid storage disease

Last year's progress report summarized preliminary clinical studies in an unusual neurologic disease, heredopathia atactica polyneuritiformis (Refsum's syndrome), in which there is marked accumulation of a branched-chain fatty acid, phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). Continuing clinical studies and animal studies (on the metabolism of phytanic acid and related branched-chain compounds) have led to the following tentative conclusions:

1. Patients with Refsum's disease do not synthesize phytanic acid endogenously at any significant rate. There is no significant synthesis of phytanic acid in normal rats either from acetate or mevalonate.

2. The metabolic error in Refsum's disease involves a degradative pathway for the oxidation and elimination of phytanic acid. However, it appears that the block is incomplete and there is some turnover of phytanic acid.

3. Animal studies show that both dietary phytol and phytanic acid, absorbed primarily by the lymphatic route, are potential precursors of body phytanic acid and lead to accumulation of the latter when fed at high doses. Both phytanic acid (3,7,11,15-tetramethylhexadec-2-enoic acid) and dihytrophytol (3,7,11,-15-tetramethylhexadecanol) can be converted to phytanic acid in the rat, showing that there are two potential pathways for the conversion of phytol to phytanic acid.

4. Modification of the diet of two patients with Refsum's disease so as to reduce the intake of these potential precursors (phytol, in chlorophyll, and phytanic acid, in butter fat and other ruminant fats) has led to a delayed but highly significant fall in plasma phytanic acid concentration. After one year on the diet, concentrations have decreased by more than 75%. In one patient there was an increase in nerve conduction velocity and some increase in muscle strength, but a final determination as to whether this dietary treatment is curative cannot be made at present. These studies taken together establish the nature of the metabolic error in this new lipid storage disease. Like phenylketonuria, the disease appears to be one in which the accumulation of a metabolite hinges on the nature of substrates presented in the diet. It will be important to elucidate the pathway of degradation and determine whether phytanic acid itself or one of its metabolites induces the nervous system dysfunction. The results already obtained on a restricted diet strongly suggest that any patients showing abnormal accumulation of phytanic acid deserve a trial with such dietary restriction.

Clinical Studies.—In the absence of identified case material in this country, collaboration has continued with Professor Sigvald Refsum, Professor Lorentz Eldjarn and their coworkers in Oslo. Normal control subjects have been studied in the Clinical Center, and two patients with Refsum's disease have been studied in Oslo. Labeled substrates prepared here are shipped to Oslo, and biological samples are collected and returned to us for analysis.

(*a*) When these studies were begun, it seemed highly likely that the phytanic acid accumulating in Refsum's disease might be endogenously synthesized. The basic branched-chain skeleton could be derived by extension of farnesyl pyrophosphate, a normal intermediate in cholesterol synthesis, by addition of a fourth isoprene unit. This reaction occurs normally in plants on the pathway for carotene biosynthesis. As reported last year, when mevalonic acid-2-C^{14} was injected intravenously into a patient with Refsum's disease, there was a normal rate of incorporation into plasma cholesterol, but virtually none into plasma phytanic acid. In order to rule out the possibility that biosynthesis might be going on, but at a very slow rate, this patient has been restudied using D_2O as precursor. A constant level of D_2O in body water was maintained for a period of four months. Incorporation of deuterium into cholesterol increased progressively, but incorporation into plasma phytanic acid was minimal, at the limits of detectability, and showed no tendency to increase with time. The small incorporation observed corresponds to replacement of at most 2 of the 40 hydrogen atoms in phytanic acid, and mass spectrometric results show that this incorporation is limited to the carboxyl end of the molecule, compatible with non-enzymatic exchange at positions adjacent to the carboxyl carbon. On biogenetic grounds, one would expect replacement of 14 hydrogen atoms if biosynthesis occurred from acetate. The high-resolution mass spectrometer was invaluable in connection with these studies.

(*b*) Studies in which phytol-U-C^{14} was given orally to six normal subjects and to two subjects with Refsum's disease demonstrate a marked reduction in the capacity of the latter to oxidize it. Labeled phytanic acid was demonstrated in the plasma of both controls and patients during the first day. However, in the normal subjects, this had virtually disappeared by 24–48 hours, whereas labeled phytanic acid persisted in the plasma of the patients and could still be demonstrated as late as 90 days after administration of the dose. The tracer dose was well absorbed (60–80%) and there was no difference between controls and patients in this regard.

In collaboration with Dr. Herbert Kayden at New York University, a patient with clinical manifestations virtually indistinguishable from those of Refsum's syndrome, but showing no phytanic acid in plasma or liver, was similarly studied. Results in this patient were comparable to those in normal control subjects. The possibility that this patient has a definite metabolic error, but one that involves a closely related or even identical pathway, is under investigation.

The conversion of tracer doses of phytol-C^{14} to $C^{14}O_2$ by the two patients with Refsum's disease was only 10–20% that of normal subjects. Since only tracer doses were given, these results may underestimate the severity of the block. In fact, the prolonged persistence of labeled phytanic acid in the plasma would suggest that degradative capacity may be more severly limited. For these reasons, studies have been started using a "loading" dose of 1 gram of phytol. Normal subjects convert approximately the same fraction of this 1-gram dose to CO_2 as they do of a tracer dose. Studies in patients with Refsum's disease using the loading dose are now in progress.

(*c*) Although routine analysis of human serum lipids by gas liquid chromatography does not reveal the presence of phytanic acid, it has now been possible to demonstrate that it is present normally, but at extremely low concentrations (0.2 mg/100 ml). A number of closely related branched-chain compounds have also been demonstrated to be present in normal human serum at these trace levels. In view of the large capacity of the normal subject to metabolize phytol and phytanic acid, one would not expect significant concentrations since the intake must be very low. Analysis of the Clinical Center diet shows that daily intake of phytanic acid may be about 60 mg and of phytol probably less than 5 mg.

ANIMAL STUDIES.—As previously reported, phytol is readily converted to phytanic acid by the rat. Similar results have now been obtained in the mouse, rabbit and chinchilla. In vitro conversion of phytol to phytanic acid has been demonstrated in liver homogenates.

Radioactive dihydrophytol and phytenic acid (the 2,3-unsaturated form of phytanic acid) have been prepared, and it has been shown that both can be converted to phytanic acid. Animals fed phytol chronically show very significant tissue concentrations of phytenic acid, but little or no dihydrophytol. After a single oral dose of phytol, phytenic acid could be demonstrated in the lymph, but no dihydrophytol could be found. These results suggest that the major pathway may involve, first, oxidation of phytol to phytenic acid, and then, reduction of the double bond, but until kinetic studies can be done, this conclusion remains tentative.

Intravenously injected C^{14}-phytanic acid (in rats) was converted to CO_2 as rapidly as intravenously injected C^{14}-phytol, indicating that phytol oxidation may involve phytanic acid as an obligatory intermediate. The pathway for degradation of phytanic acid is being investigated in rats in vivo and in vitro. Preliminary results suggest that the first step in breakdown is a decarboxylation reaction yielding pristanic acid. Seubert has studied the pathway for degradation of farnesoic acid in microorganisms. Farnesoic acid has a 15-carbon branched-chain skeleton analogous to that of phytanic acid but includes three double bonds. His studies show that degradation involves a carboxylation reaction which is biotin-dependent. Rats were maintained on a biotin-deficient diet for extended periods, but their capacity to oxidize phytanic acid remained unimpaired. We have shown that the same microorganism used by Seubert can grow on phytanic acid as the sole carbon source, and studies are in progress to compare the degradation of phytanic acid with that of farnesoic acid.

In an attempt to induce changes in the nervous system like those seen in Refsum's disease, rats were maintained on phytol diets, which induce accumulation of phytanic acid in the blood and tissues. However, high dosages arrest growth and cause a high mortality within the first month or two; low dosages are tolerated but as associated with only relatively small increases in tissue phytanic acid concentration. Rats have been maintained for up to a year on diets containing 0.5 by weight of phytol. In none of the animals thus far have there been changes in the retina, peripheral nerves, or central nervous system analogous to those seen in the clinical disease. However, because it is not possible to maintain high dose concentrations for extended periods of time, these negative results cannot be taken to rule out a cause-and-effect relationship between accumulation of phytanic acid and nervous system changes in Refsum's disease.

Lymphatic Absorption of Lipids

1. The absorption of phytol, phytanic acid and other related substrates has been studied in rats in which the thoracic lymph duct was cannulated. It was shown that phytol and phytanic acid are both readily absorbed largely by way of the lymph. Phytol appears in the lymph mostly in combined form, only a small fraction being present as free phytol. Most of the phytol was present in a fraction shown to be esters of phytol and long-chain fatty acids. It was shown that during the course of absorption as much as 15% of the absorbed phytol was converted to phytanic acid. Phytenic acid was also demonstrated in the lymph, but no demonstrable dihydrophytol or any aldehyde derivatives were present. The capacity for phytol absorption in the rat is large; after a 300-mg dose, as much as 60 mg was absorbed in 24 hours. Under similar conditions, phytanic acid was absorbed somewhat more rapidly, and the total absorption exceeded 100 mg.

Since a major source of phytol in the diet is that which is esterified to the porphyrin nucleus of chlorophyll, it was of interest to determine to what extent phytol is available for absorption. C^{14}-labeled pheophytin *a* was prepared from tobacco leaves grown in $C^{14}O_2$. This was administered to rats by stomach tube and radioactivity determined in collected lymph, urine, CO_2 and body tissues. Less than 5% of the administered radioactivity was recovered in these fractions. Of the small amount of radioactivity present in the lymph, less than half migrated in TLC with phytol or phytanic acid. If these results can be extrapolated to man, it would appear that die-

tary chlorophyll is not likely to be a major source of phytol, a finding of great relevance in designing appropriate diets for patients with Refsum's disease.

2. The origin of endogenous lipid in thoracic duct lymph: Even on diets devoid of fat, the lymph continues to show significant concentrations of lipid, but the origin of this remains unclear. It has now been shown that the fatty acid composition of thoracic duct lipid is quite different with respect to long-chain fatty acids from that of the lipid in the bile. An outstanding difference was the presence in lymph of a 20-carbon mono-unsaturated fatty acid which was not present in biliary lipid. The structure of this fatty acid has been established as docoso-13-enoic acid. This acid was also present in intestinal mucosa and in the intestinal contents of germ-free rats. It thus appears that the bile is not a major source of endogenous lymph lipids. Further studies are in progress to determine the origin of endogenous lymph lipids.

Section on Molecular Diseases

Studies of Hyperlipoproteinemia

In 1965 the Section introduced a system for identification of familial disorders of plasma lipid concentrations based on comparison of lipoprotein patterns with clinical and genetic information. Major effort was devoted during the past year to further development of this method and its application to study of more kindreds. The major accomplishments have been as follows:

1. An analytical sequence has been determined that begins with a minimum examination of the plasma by paper electrophoresis in albuminated buffer which segregates nearly all "normals" from "abnormals" and groups the latter in five types (I–V). When necessary to achieve firmer segregation of the abnormal types, cholesterol or glyceride determinations, or a simplified quantitation of alpha and beta lipoproteins can be added *seriatim.*

2. The rational base for this analytical sequence has been thoroughly tested by comparisons with other pertinent methods of separating lipoproteins including use of immunochemical methods for an absolute definition of lipoprotein content. The information provided by this approach is superior to that obtained by lipid determinations alone, is feasible and economical for adaptation by most clinical laboratories, epidemiologists and geneticists, and cannot be obtained by any other available single method for lipoprotein separations, with the possible exception of the new computer-adapted analytical ultracentrifuge. (A comparison is now in progress in collaboration with the Donner Laboratory.)

3. Electrophoretograms have now been obtained in more than 1500 subjects; the full analytical sequence has been performed in over 700 subjects. "Normal limits" of distribution for four key variables have been set using 450 normals, and about 3000 inpatient days have been devoted to intensive metabolic study of subjects with ramilial disease for correlation of clinical findings with abnormal lipoprotein patterns.

4. Through study of 120 affected individuals from 45 kindreds, "familial hypercholesterolemia" has been decisively split into at least two genotypes, which as now redefined are: Type II, the more common form of hyperbetalipoproteinemia, and Type III, a rare recessive syndrome with a peculiar anomaly in density of beta lipoprotein. Type III, or "broad beta disease", had never before been clearly segregated from among hypercholesterolemic subjects. It is associated with severe atherosclerosis and is more responsive to therapy than Type II.

5. Increasing experience with Type IV (endogenous hyperlipemia) and Type V (mixed hyperlipemia) strongly suggests the presence of more than one mutation, some of fairly high frequency in Americans. For the first time we have detected these heritable abnormalities in children and have laid groundwork for better means of segregating the defects, including the establishment of normal limits for "carbohydrate induction" in terms of glyceride responses to standard dietary loads.

6. Cooperative projects, including the analysis in Bethesda of ten samples per week from the "prediabetic" population (defined by maximum genetic hazard for diabetes) from the

Joslin Clinic and all subjects undergoing coronary angiography at the Peter Bent Brigham Hospital, have been established. Training in the techniques or other assistance has been provided to representatives of nearly 50 groups in America and abroad desiring to set up the same system. Offering, as it does, better standardization of nomenclature and enhanced recognition of familial or sporadic disorders having a significant association with atherosclerosis and diabetes, this project is being adjusted to long-range goals.

Studies of the Structure and Function of Lipoproteins

The chemical definition of lipoproteins and the functional interrelationships are pertinent to the use of lipoprotein patterns to define clinical disorders of fat transport. The study of heritable deficiencies in either beta lipoprotein (abetalipoproteinemia) or alpha lipoproteins (Tangier disease) has continued to be valuable in this regard. Six patients with abetalipoproteinemia were shown conclusively to have less than 1/100,000 of the normal amount of plasma beta lipoprotein (probably none at all), and four were shown to be unable to mobilize endogenous glyceride as well as from chylomincrons. It was concluded that beta lipoprotein is essential for moving glyceride out of cells. We have previously shown that patients lacking normal alpha lipoprotein can transport glycerides, assigning another function, yet unknown, to these lipoproteins.

Using immunochemical methods, it has now been demonstrated in seven patients with Tangier disease (from four different unrelated American families) that alpha lipoprotein antigenically different from the normal alpha lipoprotein is present. Parents of these patients have both normal and "Tangier" alpha lipoprotein, suggesting that this disease is due to a mutant structural gene. The homozygous abnormal is thus able to elaborate only a small amount of aberrant lipoprotein that fails to prevent cholesterol ester deposition in tissues. The latter was shown this year to involve even the skin of these patients.

Collaborative studies with Drs. Windmueller of NIAMD have provided strong evidence that the action of orotic acid in depressing blood lipids, an effect which he had shown previously, is due to specific inhibition of the elaboration of beta lipoprotein by the liver.

Studies of Tissue Lipidoses

The discovery by Drs. Brady and Kanfer (NINDB) of a sphingomyelin-cleaving enzyme was extended to tissues from five patients with Niemann-Pick disease. In all the enzyme was drastically reduced below control levels, strongly suggesting that this is the heritable defect in this disease. With the collaboration of Dr. Uhlendorf in DBS, 24 cell lines in tissue culture from homozygous abnormals and an equal number from heterogyzotes are now ready for enzyme studies. It has been previously shown here that the chemical defect is perpetuated in culture.

Protein Structure and Function

1. Efforts to determine the chemical properties, relationship of structure to biological and immunological function, and mechanism of action of parathyroid hormone progressed significantly during the year.

The tryptic peptides of parathyroid hormone were prepared and isolated and their amino acid composition determined. Polypeptide fragments from the hormone produced by other methods of cleavage, permitted the alignment of tryptic peptides in order, thereby providing a working model of the covalent structure of the hormonal polypeptide. By a variety of chemical and enzymic methods, limited cleavage of the polypeptide and selective modification of individual amino acid residues were achieved; this permitted identification of an active fragment of the hormone representing one-fourth of the total sequence containing a minimum structure requisite for both biological and immunological activity.

The radioimmunoassay technique has been extended to measurements of parathyroid hormone in the plasma of cows, goats, and sheep after stimulation or suppression of hormone production by changes in the concentration of plasma calcium and other ions. The rates of disappearance were estimated by the radioimmunoassay technique, for endogenous hormone

and for administered ^{131}I-labeled or unlabeled parathyroid hormone. These studies have permitted the calculation of the daily secretion rate of parathyroid hormone and the rate of synthesis necessary to maintain constant production of the hormone. It has been shown that plasma calcium controls parathyroid hormone secretion within narrow limits, hormone concentration changing rapidly and markedly (changes of 10-15 fold) in response to physiologically or pharmacologically induced changes in plasma calcium. It was determined that parathyroid hormone secretion varies inversely with serum calcium. From the calculated regression line and other observations it is clear that hormone is secreted continuously at a basal level, with further increments in secretion caused by progressive degrees of hypocalcemia. It was shown that plasma phosphate had no direct effect on parathyroid hormone secretion.

Efforts have continued to develop the radioimmunoassay technique for routine measurement of parathyroid hormone concentration in human plasma. Although the technique is not yet satisfactory for this purpose a number of clinical applications have been possible. It has been shown that the parathyroid hormone is produced by nonparathyroid tumors, explaining the syndrome of "ectopic hyperparathyroidism" in patients with certain types of nonparathyroid malignancy. Further, the development of antibody causing resistance to the biological actions of the parathyroid hormone has been demonstrated in patients treated for prolonged periods of time with commercial parathyroid extract. As a possible aid in development of the assay for work in human subjects, human parathyroid hormone has been extensively purified after extraction from parathyroid adenoma tissue. It has been found that human parathyroid hormone is closely similar to bovine hormone in its chemical and biological properties. With highly purified human preparations available, the immunological reactivity of human and beef hormone can be compared. Use of the human hormone might improve the assay for work in human subjects through proper selection of antisera or use of human PTH as tracer.

Studies have continued with evaluation of the effects of parathyroid hormone on ion transport in mitochondria. Although initially these studies suggested that the effect of parathyroid hormone on mitochondria was highly selective and perhaps reflected the true mode of action of the hormone, more recent studies suggest that these effects may not be specific. In view of the great potential interest in the effects of parathyroid hormone on ion translocation, studies are continuing to correlate the biological activity of derivatives of parathyroid hormone *in vivo* with effects on the *in vitro* system. This should conclusively establish the significance of the *in vitro* assay systems.

2. Studies of the conformation and immunological properties of ribonuclease have been continued in further efforts to understand the role of protein conformation in enzymic and antigenic activity. Derivatives of ribonuclease with minor changes in covalent structure have been shown to have widely divergent conformational stability detectable by marked changes in thermally induced denaturation and antigenic reactivity.

3. Collaborative studies of the treatment of cystinuria by use of penicillamine have been extended. Recent studies have further defined the mechanisms of action of the drug. Lack of change in clearance of cystine during treatment with the penicillamines and the appearance of the mixed disulfide in plasma indicate that the principle site of action is in plasma or gut, rather than in the kidney. The agent N-acetyl-penicillamine has been shown to be an even more promising therapeutic agent. The N-acetyl form of the drug appears to have a reduced potential for toxic reactions in long term use.

Section on Chemistry

The Section on Chemistry is mainly concerned with structural analysis, synthesis and biosynthesis of compounds of biological origin. The Section also undertakes the development of new analytical techniques for compounds of biological interest. At the present, much of the activity of the group is centered around

the mass spectrometer which appears to be generating a great deal of interest among biochemical investigators outside the Section. After some early difficulties, it can now be considered a reliable instrument around which biochemical experiments (e.g. isotope labeling) may be designed. Also, its utility in structural analysis is enormous (see below). In July the addition of a high-speed scanning system and tape recorder will add greatly to its value as a tool for structural analysis.

Alone, or in collaboration with other groups, members of the Section have this year:

1. Continued synthesis of compounds related to phytol for studies of Refsum's disease, (phytanic acid, phytenic acid, pristane and pristanic acid).
2. Identified pristane and phytane in rat liver.
3. Identified the metabolites of 2 fluorobenzoic acid.
4. Studied the enzymatic conversion of GTP to 2′-deoxy-GTP and located the label incorporated.
5. Identified an unusual case of hydrogen bonding in a 10-membered ring and studied the conformation of the molecule (dihydrotazettine methine alcohol).
6. Demonstrated the structure of the flavone milletine B from an Ethiopian fish poison.
7. Related the structure of a new alkaloid astrocasidine to astrocasine.
8. Proved the structure and absolute stereochemistry of astrophylline and synthesized a related compound.
9. Resolved plasma kininogen (kallidinogen) into two immunologically identical forms, I and II, differentiated by chromatographic and chemical behavior. Structural work on these compounds is in progress.
10. Developed an antiserum to kininogen I to assist in its purification and to answer the question whether kininogens I and II are present as such in human serum.
11. Developed a new method of long-range peak matching for mass measurement in the mass spectrometer.
12. Developed a solvent shift method for counting methoxyl groups using nmr spectroscopy.
13. Investigated the hydrogen bonding of ortho substituted benzoic acids.
14. Decided an important structural question regarding ureasterone.
15. Solved the structure and synthesized a homologue of the uropygiols.
16. Run the highest molecular weight compound ever analyzed by mass spectrometry, C_{72} H_{24} O_8 F_{28} N_4 P_4 (3628).
17. Developed a new blocking group for ketones.
18. Elucidated the structure of the "Salmonella Resistance Factor".
19. Completed a mass spectrometric survey of estrogen trimethylsilyl ethers.
20. Elucidated the biosynthesis of a series of methylenebisphloroglucinols and found an *in vitro* enzyme system capable of bringing about the critical step.

LABORATORY OF BIOCHEMICAL GENETICS

Nucleotide Sequences of RNA Codons

During the past year nucleotide sequences of 17 RNA codons were determined and were assigned to amino acids or special functions. The sequences of virtually all codons have now been determined in this laboratory. The following generalizations can be made concerning the nature of the code. (A) Amino acids, which are structurally or metabolically related correspond to structurally related RNA codons. (B) The code is logically degenerate. Recognition of the 3′-terminal base in a trinucleotide is most variable and fit several synonym patterns. Alternate 3′-terminal bases of synonyms are as follows: U=C; G=A; U; G; U=C=A; and A=G (U). Degeneracy patterns were also found at the 5′-terminal position of trinucleotides. For example, U=C in the case of leucine and tryptophan, and U=C=A=(G) in the case of N-formyl-methionine sRNA.

Mechanism of Codon Recognition

The patterns of synonym codons apparently define the characteristics of general codon recognition mechanisms. Purified sRNA fractions were used to gain further insight into mechanisms of codon recognition. Yeast Ala-sRNA of known base sequence and estimated to be greater than 95% pure was obtained from Dr. Robert Holley and was found to recognize the following synonym Ala-codons, GCU, GCC, GCA, and possibly also GCG. These results demonstrate that one molecule of sRNA can recognize 3, possibly 4, synonym codons, and suggests that recognition occurs by antiparallel, alternate base pairing between codons in mRNA and an IGC anticodon sequence in Ala-sRNA.

Cells often contain multiple species sRNA for the same amino acid. Multiple species of *E. coli*, Val-, Ala-, and Met-sRNA were separated by counter-current distribution techniques and the response of each fraction to synonym codons was determined. The major peak of Val-sRNA recognized GUA, GUG, and GUU. This peak may represent a composite of two sRNA species, one responding to GUA, GUG and GUU, the other only to GUG. However, a minor Val-sRNA species recognized only GUC and GUU. Ala-$sRNA_1$ responded preferentially to GCA and GCG; whereas Ala-$sRNA_2$ responded to CGU, GCC, GCA, and GCG.

Met-$sRNA_1$ accepted formyl groups and responded to UUG, CUG, AUG, and less well to GUG. Met-$sRNA_2$ did not accept formyl groups and responded principally to AUG. N-formyl-Met-sRNA apparently serves as an initiator of protein synthesis; selecting the first word to be read and phasing subsequent reading

All of the available evidence suggests that the third (occasionally the first) base of a triplet can hydrogen bond with alternate bases in sRNA. The degeneracy patterns noted above result from alternate base pairing.

Characteristics of Synonym RNA Codons

Relative template activities and specificities of synonym codons were investigated by assaying the formation of AA-sRNA-ribosome-codon complexes in reactions containing different concentrations of Mg^{++} and also in the presence of putrescine and spermidine. Synonym codons were found to differ markedly in both template activity and specificity. Also shifts in Mg^{++} concentrations had a greater effect upon the template activity of some trinucleotides than others corresponding to the same amino acids. The biological consequences of these corresponding to the same amino acids. The biological consequences of these findings remain to be assessed. Some synonym codons may play special roles in protein synthesis, such as specifying the beginning or end of the message; others may be necessary for the synthesis of certain proteins or may selectively influence the rate of protein synthesis.

Codon Recognition on 30 S Ribosomes

During protein synthesis on 70 S ribosomes AA-sRNA may attach to both 30 S and 50 S ribosomal subunits. However, codon recognition can occur on 30 S subunits only. Two binding sites for sRNA were found on 30 S subunits. AA–sRNA binding to one riobosmal site was dependent on K^+, binding to the other ribosomal site did not require K^+. The characteristics of each site were determined and compared to sRNA binding sites on 70 S ribosomes

Attachment of mRNA to Ribosomes

^{3}H-oligonucleotide templates were synthesized and the interaction between such templates and ribosomes were studied. The binding of ^{3}H-oligonucleotides of chain length 3 to 9 was dependent upon sRNA. The use of labeled oligonucleotides provides a highly sensitive technique for detecting codon recognition by deacylated sRNA or by a special function sRNA.

Template Activty of Modified RNA Codons

RNA and DNA contain three classes of codons differing in structure; 5′-terminal, 3′-terminal and internal codons. We previously

showed that modification of terminal codons markedly affect template activity. Since such mechanisms may also regulate the rate of protein synthesis *in vivo*, we have continued to explore the relation between codon modification and template activity.

Trinucleoside diphosphate analogs were prepared to assess the effects of such modifications upon codon recognition. Substituting 5′=, 2′=, 3′— terminal or 2′— internal ribose hydroxyls of oligonucleotides markedly affected their template activity in directing the binding of AA-sRNA to ribosomes. The relative template activity of oligo U preparations was as follows: p=5′= UpUpU > UpUpU > CH_3O=p=5′-UpUpU > UpUpU-3′-p > UpUpU-3′-p-OCH_3 > UpUpU=2′, 3′=cyclic phosphate. Trimers with (2′-5′) phosphodiester linkages, (2′-5′)-UpUpU and also (2′=5′)-ApApA did not serve as templates for phenylalanine- or lysine-sRNA, respectively. The relative template efficiency of oligo A preparations was as follows: p-5′-ApApA > ApApA >ApApA-3′-p > ApApA-2′-p.

The hexamers, UpUpUpUpUpU and ApAp-ApApApA were considerably more active as templates than the corresponding pentamers. These data indicate that two adjacent triplets are recognized by two AA-sRNA molecules bound to nearby ribosomal sites.

A doublet with 5′-terminal phosphate, pUpC served as a template for serine-sRNA whereas a doublet without terminal phosphate, UpC, did not. Al-through the template efficiency of pUpC was lower than that of the triplet. UpCpU the data show that serine-sRNA can recognize pUpC.

Universality of the Code

Base sequences of codons recognized by AA–sRNA from amphibian and mammalian liver (*Xenopus laevis* and guinea pig liver, respectively) were almost identical to those recognized by corresponding *E. coli* AA–sRNA preparations. Thus, synonym codon groups are largely universal. However, the following species-dependent differences in relative activity of synonym codons were detected:

Codon		sRNA		
		Bacterial (E. coli)	*Amphibian (X. laevis liver)*	*Mammalian (Guinea Pig liver)*
ARG	AGG	—	++++	+++
	CGG	+	++++	++++
ALA	GCC	+	+++	+++
	GCG	++++	—	++
ILE	AUA	—	++	++
LYS	AAG	+	++++	++++
SER	UCG	++++	+	+++
	AGU	+	++++	++++
	AGC	+	++++	++++
THR	ACG	++++	++	++

No differences found for additional synonyms corresponding to above amino acids and for all codons for: ASP, CYS, GLU, HIS, PHE, PRO, TYR, and VAL. It is possible that some species-dependent differences in codon recognition may selectively influence the rate of translation of messenger RNA.

Regulatory Mechanisms Dependent on Viral Infection

Infection of *E. coli* by T-2 bacteriophage results, within one minute, in the synthesis of a protein which apparently modifies one Leu-sRNA species present in the *E. coli* host. Simultaneously, host protein synthesis is inhibited. In collaboration with N. and K. Sueoka, who reported these phenomena, Leu-sRNA was prepared from phage infected and control *E. coli* cells and the response of each preparation to codons was determined. The modified Leu-sRNA produced after phage infection attached to ribosomes in response to poly UG but not to any triplet containing U or G, or to any other Leu-codon. The following hypothesis was advanced: The modified Leu-codon. The following hypothesis was advanced: The mcdified Leu-sRNA fraction inhibits *E. coli*, but not T–2 phage protien synthesis either by preventing the initiation of protein synthesis or by blocking two adjacent sRNA binding sites on ribosomes, and hence preventing further attachment of AA-sRNA to ribosomes.

Studies are in progress to define possible consequences of selective modification of com-

ponents required for codon recognition. Particular attention is being focused upon mechanisms which may selectively control the rate of protein synthesis during viral infection and embryonic differentiation.

LABORATORY OF CHEMICAL PHARMACOLOGY

The Adrenergic Neurochemical Transducer

Application of Steady State Kinetics

It has not been generally appreciated that monoamines appear to diffuse from nerve endings continuously at a rate proportional to the amine concentration. This is not readily apparent since the amine level is maintained constant by continuous synthesis. Rates of synthesis and efflux are equal, hence

$$K \text{ (rate of synthesis)} = k\, C_0$$

where C_0 is the amine concentration and k is the rate constant of edux.

Proof that efflux of NE is proportional to NE concentration of the normal steady state is obtained from the decline in level after blockade of synthesis with α-methyltyrosine. In this case,

$$\frac{d\,[NE]}{dt} = -k[NE] \text{ and } [NE] = [NE]_0 \exp(-kt) \quad (1)$$

Since synthesis is continuous (and zero order) then

$$\frac{d\,[NE]}{dt} = K - k[NE]$$

and the general expression for NE level becomes

$$[NE] = \frac{K}{k} - \left(\frac{K}{k} - [NE]_0\right)\exp(-kt) \quad (2)$$

After blockade of storage, the amine level does not decline to zero but to a new steady state concentration. The latter can be defined by the equation

$$[NE] = \frac{K}{k}$$

provided k is assigned a value larger than that for normal organs.

The application of kinetics to the drug-induced release of monoamines provides a valuable tool in disclosing new facets of the behavior of nerve endings and the nature of drug action. In this approach, it is assumed that a change in amine level can result only from change in one of the parameters in equation (3), i.e., the rate either of synthesis or efflux is increased. With a releasing drug, the action is best considered as an increased rate constant of efflux. Furthermore, the pharmacologic effects of a number of drugs bear a closer relationship to the increase in rate constant of amine efflux than to the final steady state level.

Turnover Rates and Times of Calecholamines and 5 HT

At the steady state, $K = k\, C_0$ (equation 3). To calculate the rate of synthesis, the rate constant k must be determined. For NE, this may be determined: (1) From decline in label after tracer doses of H^3-NE; (2) From decline in [NE] after blockade of synthesis by α-methyltyrosine; (3) Rate of refilling NE stores after depletion by tyramine; (4) From decline in H^3-NE after tracer doses of H^3-DOPA. The methods all give similar results.

Synthesis of brain 5HT is not readily blocked nor can 5HT be readily labeled. However, at the steady state

$$\text{Rate of synthesis } (K) \longrightarrow 5HT \xrightarrow{k_1} 5HIAA \xrightarrow{k_2} \text{loss of 5HIAA}$$

hence

$$K = k_1\,[5HT]_0 = k_2\,[5HIAA]_0 \ .$$

k_2, the rate constant of 5HIAA efflux id determined from the decline of its concentration after blockade of MAO and from the rise after blockade (with probenecid) of the process that transports it from brain. These methods, which yield results similar to those calculated from rise in [5HT] after blockade of monoamine oxidase, have the advantage that turnover rates can be determined after 5HT stores are depleted by reserpine, i.e.,

$$K = k_r\,[5HIAA]_r$$

where k_r is rate constant of 5HIAA efflux, and $[5HIAA]_r$ is the level of acid after reserpine

In a simplified procedure for measuring 5HT

turnover, each animal is used as its own control by taking advantage of relationship

$$\frac{[5HIAA]_0}{[5HT]_0} = \frac{k_1}{k_2}$$

Provided k_2 is unchanged, an increase in this ratio (normally about 1) signifies an increased 5HT turnover rate.

Results of Studies of Turnover Rates

The turnover time of brain 5HT is about 1 hr, showing that all the amine in granules is rapidly replaced without use of drugs. In contrast, the turnover time of brain NE is about 8 hr. The turnover time of NE (as well as 5HT) is similar in various brain regions despite differences in steady state levels of these amines in different parts of the brain. This implies that regional differences in concentration arise mainly from differences in the concentration of neurons that are essentially alike. The turnover times of NE in peripheral tissues are also similar despite differing levels.

In studies of the origin of NE nerve ending granules, the turnover time of NE in cervical sympathetic ganglion was found to be 6 times that in corresponding nerve endings. This might mean that granules in the nerve body are deficient in a binding component, perhaps ATP.

The levels of biogenic amines are controlled in part by product inhibition. Thus after reserpine, the formation of brain 5HT in rats is increased by about 30% as shown by increased steady state levels of 5HIAA. After blockade of MAO, when 5HT and NE levels rise to a plateau, rates of synthesis are almost zero. In view of these observations, it may be necessary to apply corrections to the values for K in the kinetic equations above.

A problem arises in explaining why NE in brain of rat, rabbit and cat have about the same turnover time; yet after blockade of MAO, NE in the rat rises rapidly, in the rabbit slowly, and in the cat not at all.

Kinetics of Storage

Releasable and resistant pools. Our observations suggest that the common view that NE is stored in tyramine-releasable and tyramine-resistant pools is not valid. When the tyramine level is maintained about 3 μg/g, NE stores in heart are depleted exponentially to levels too low to measure (T ½–51 min) as though the amine were confined to a single compartment. Since the dissociation of NE from its complex in granules is not a rate limiting step in NE release (see later), the tyramine presumably acts on the presynaptic membrane.

Our results with tyramine are inconsistent with our previous studies with H^3-NE from which it was concluded that the transfer of NE between granules and cytoplasm is the rate limiting step. From this it would be inferred that tyramine would deplete NE stores by 50% only after several hr. Improved techniques of H^3 counting made it possible to assay tissue H^3-NE, after the injection of 100 ng/kg. The level of 1-H^3-NE in heart now declines as a single exponental (T ½–13 hr), over a period from 2 min to 40 hr. On increasing the dose of H^3-NE 10-fold, a diphasic curve is once more obtained. In support of these results, H^3-NE formed in heart and brain after injection of H^3-DOPA declines as a single exponential during 1 to 40 hr. Thus, H^3-NE given in truly tracer amounts causes rapid and uniform labeling of endogenous NE. It may be concluded that NE rapidly equilibrates between granules and cytoplasm and that the loss of NE from the free pool is rapidly replaced by dissociation of amine from NE complex.

The diphasic decline, after more than tracer amounts of H^3-NE, is associated with an increased uptake of the label. The rapid half-life, about 2 hr, of this "excess" NE suggests that it gains access to some part of cytoplasm from which its disappearance is relatively rapid.

The Recapture Mechanism

Sympathetic stimulation (10/sec) of the colon from cats treated with phenoxybenzamine causes a 6- to 8-fold increase in NE overflow in the venous effluent. Similar results without phenoxybenzamine are achieved by stimulating at 30/sec. These results imply that the NE released on depolarization of the nerve terminal normally forms a complex with receptor sites, which acts as a brake to diffusion into the general circulation. When the integrity of

the axonal membrane is restored by repolarization, NE on receptors is recaptured through action of the pump. If receptor sites are occupied by phenoxybenzamine, they no longer hinder the diffusion of NE, which escapes into the circulation during depolarization, and is no longer available on subsequent repolarization. Thus, the blocking agent augments overflow simply because it interferes with shuttling of NE between receptor and nerve ending.

The venous overflow of NE after nerve stimulation in presence of phenoxybenzamine presumably represents the amount of transmitter actually released and bound to receptors, and normally re-incorporated into storage sites. Our results show that each nerve impulse releases about 70 pg of NE/g of colon. At this rate, amine stores would be exhausted within 30 min, this suggests that the important function of the re-uptake process is to insure that NE stores are not depleted.

Mechanism of NE Release by Nerve Stimulation

The distinctive action of phenoxybenzamine on the re-uptake process makes it a valuable tool in studies of the mechanism that liberates NE from nerve endings. The overflow of NE declines on repetitive sympathetic simulation (10/sec), and ceases within 15 min, though amine stores are reduced by less than 10%. Moreover, most of the release occurs within the first 3 min. A rest period of 1 hr is adequate to restore the response to nerve stimulation.

Since depletion of NE stores by drugs (tyramine and reserpine) can be rapid and complete, depletion by nerve stimuli must be limited by some step not involved in drug release. This raises the possibility that release of NE by nerve stimulation is limited to vesicles in the vicinity of the synaptic cleft. It is possible that nerve stimulation, which causes influx of free Ca^{++}, causes granules to fuse with the neuronal membrane. By this view, nerve excitation at a relatively high frequency would restrict vesicles to vicinity of membrane. In the presence of phenoxybenzamine, these vesicles would be depleted through constant overflow. When stimulation is stopped, the empty vesicles at the membrane could gradually be replaced by Brownian movement of fully loaded ones.

Electrolyte Requirements for NE Storage and Uptake

Our studies show that Na^+ is an absolute requirement for NE storage. Thus the efflux of H^3-NE, when heart slices from rats treated with H^3-NE are incubated with Krebs' Ringer, similar to that of heart *in vivo* (T ½–13 hr). Storage is markedly decreased in Na^+ free media (isotonic sucrose, Li^+ or choline) as shown by the rapid efflux of H^3-NE. The outflow is markedly decreased when sucrose is replaced by various amounts of Na^+, approaching an asymptote in media containing 40 mM of Na^+, as though some Na^+ dependent system were reaching a maximum rate. These results indicate that Na^+ is an absolute requirement in storage of NE.

K^+ inhibits the effects of Na^+. Thus efflux of NE is rapid in isotonic K^+ and is reduced by replacing K^+ by Na^+. However, much greater amounts of Na^+ are required to reduce the efflux in the presence of K^+, than of sucrose; Thus about 100 mM of Na^+ in the presence of 45 mM of K^+ are needed before the rate of efflux is reduced to an asymptote. K^+ appears competitively to inhibit the process stimulated by Na^+.

Ca^{++} is also an absolute requirement for NE storage since the omission of this cation ($^+$ EDTA) results in a rapid rate of efflux.

Preliminary studies show that heart slices fail to concentrate H^3-NE in Na^+-free media, containing isotonic K^+ or Li^+. These results are of potential importance: (1) They suggest that the active transport of NE is possible because the carrier mechanism has an enhanced affinity for NE in the presence of Na^+ and reduced affinity in the presence of K^+. This view would explain how NE is transported from an outside medium high in Na^+ to an inside one, high in K^+. (2) Many of the proposed effects of electrolytes on release or synthesis of neurohormones, might well be effects on storage.

Kinetics of Reserpine-Induced Release of Biogenic Amines

The action of reserpine on monoamines is closely tied up with the problem of their storage. Current opinion proposes two specialized storage mechanisms; one in granules is impaired by reserpine, the second a membrane pump, is insensitive to reserpine but inhibited by cocaine. As a result nerve ending models proposed by other workers generally include the following assumptions: (1) NE formation is normally controlled by the rate of utilization; (2) NE is stored in releasable and nonreleasable compartments; (3) NE in granules is stored by a specialized energy-requiring process which is impaired by reserpine; (4) NE is still taken up by nerve endings and held for some time after depletion of stores by reserpine; (5) NE is also taken up by a membrane pump that is blocked by cocaine, which however does not release the amine; (6) Tyramine, metaraminol and other polar phenylethylamines release NE by stoichiometric displacement from granules.

A kinetic analysis of amine release by reserpine seemed essential. In studies supporting the view that reserpine acts directly on granules, the drug added to a granule suspension prevents the uptake of H^3-NE, but does not release NE. To explain why the amine is not released from granules, the drug is postulated to act by blocking the uptake of dopamine into granules. Depletion of NE is then attributed to failure of synthesis to replenish the physiologic release of the amine.

We have shown this veiw to be untenable by showing that the maximal rate at which heart NE is released by reserpine is about 40 times greater than that of synthesis.

In applying kinetics to reserpine-induced release of monoamines, we have treated the membrane carrier mechanism as a barrier to free flow of amine. After blockade of NE synthesis, even without reserpine, the amine levels decline exponentially to zero; reserpine merely increases the rate constant of efflux and increases the speed at which the level declines to zero.

When synthesis of NE is not blocked, reserpine decreases the amine level, not to zero, but to the new steady state value defined by

$$[NE] = kC$$

where k describes the slope of the exponential by which the amine declines to the new steady state value. The rate constant is maximal after a large dose of reserpine (5 mg/kg i.v.); the amine stores then decline exponentially almost to zero. The maximum efflux rates are rapid. Half lives are about 16 min for heart NE and about 7 min for brain 5HT, NE and dopamine. Calculation of free amine at nerve endings after complete blockade of storage is 2 to 3% of normal for brain NE and DA and about 12% for 5HT. The higher level of 5HT reflects the greater rate of synthesis of 5HT. The high level of 5HT represents that available to receptors after reserpine administration.

The fact that reserpine, given in a maximally effective dose, depletes monoamines at a maximum rate provides a common frame of reference in comparing the effects of reserpine. Thus if a dose of reserpine releases heart NE at a rate less than that corresponding to a half life of 16 min, it may be concluded that storage mechanisms are incompletely inhibited.

The extent to which reserpine blocks the monoamine pump may be calculated from the ration of the observed rate of amine efflux to that after a maximally effective dose of reserpine.

In testing whether or not reserpine blocks the membrane pump, the drug must be given in maximally effective doses. Few if any studies have shown evidence of this. Our results demonstrate that when H^3-NE is injected into rats 4 hr after a maximally effective dose of reserpine, little if any label is taken up and retained by adrenergic neurons. Kinetic studies provide a more rigid proof that reserpine completely blocks the membrane pump. H^3-NE is given to rats shortly after a maximally effective dose of reserpine followed by a MAO inhibitor. A few minutes later, the traces of H^3-NE in heart disappear with a half-life that is much shorter than that of endogenous NE still formed in nerve endings, showing that the H^3-NE was not taken up by nerve endings.

Relationship of Pharmacologic Effects of Reserpine to Effects on 5HT Storage

Since the effect of reserpine on amine efflux is a measure of the blockade of the storage process, the pharmacologic effects of the drug may be compared with the inhibition of storage. Since previous studies indicate that blockade of NE storage does not account for the central effects of reserpine, we compared these effects with 5HT storage. The results show that the pharmacologic effects (including sedation, blepharospasm, etc.) are closely related to the rate constant of 5 HT efflux and that the most profound effects are elicited by doses of reserpine that alicit a maximum release. Doses higher than this do not increase the rate of efflux or the intensity of drug action. Thus 0.9 and 5 mg/kg of drug both deplete 5HT by about 90%. The higher dose reduces the 5HT level by 90% in about 20 min; the decline after 0.9 mg/kg is much less steep and [5HT] levels off in 1 hr at 75% of normal. In 4 hr however, the loss increases to 90%.

False Adrenergic Transmitters

A number of phenylethylamine analogues including metaraminol, 1-methyl NE, and octopamine are taken up and "stored" by adrenergic neurons by a saturable process and are released by nerve stimuli and by reserpine. A current misconception holds that these compounds release NE by simple physical displacement. Our results show that two separate processes are involved. Initially the drug is taken up and retained by neurons, where it shares occupancy with NE; the drug then enhances permeability of presynaptic terminals, possibly by eliciting persistent depolarization. Our results indicate that tyramine and guanethidine are also false transmitters.

Studies of the effects of these agents, both *in vivo*, and on heart slices have provided further insight into their action: (1) Guanethidine, tyramine, octopamine and metaraminol all release NE at the same maximum rate, about one-third that elicited by reserpine. (2) Desmethylimipramine prevents the release of NE by these substances (*in vivo* experiments).

In exploring the possibility that these drugs produce presynaptic depolarization, the effects of guanethidine and K^+ have been compared. High levels of K^+, produce an efflux of NE almost as rapid as that produced by guanethidine. The effects of both substances are blocked by bretylium and by excess Ca^{++}.

Kinetics may also be applied to the release of NE by the false transmitters. In this case the false transmitter may well increase the rate constant of efflux by increasing the porosity of the neuronal membrane. The rate would then increase with dose, approaching a maximum as the uptake process becomes saturated. At the new steady state

$$[NE] = \frac{K}{k}$$

In other words, these agents need not reduce the NE level to zero. Even after a maximally effective dose of bretylium or guanethidine, the steady state level is 3 times that after reserpine.

Relationship of Pharmacologic Effects of False Transmitters to Rate of NE Efflux

In previous studies we showed that the rate of NE efflux is directly proportional to the uptake of guanethidine by adrenergic neurons. From these results it was suggested that the rate of efflux and the sympatholytic effects were both related to the extent of presynaptic polarization. Preliminary results suggest that the rate of NE efflux is also proportional to uptake of metaraminol. Failure of this drug to lower blood pressure may be explained by the direct stimulatory effect of the compound on adrenergic receptors.

Effects of Desmethylimipramine (DMI) on Adrenergic Neurons

A number of substances, of which DMI is typical, exert little action of their own but are potent antidepressants, block the uptake of catecholamines, and potentiate the actions of NE and amphetamine. Last year we reported that DMI, like cocaine, has a selective action in changing the permeability of adrenergic neurons to NE. Furthermore it prevents the various false transmitters from releasing NE. In continuation of studies of the effects of DMI

on neuronal membranes, we have shown that large doses of NE displace H^3-NE taken up by nerve endings in rats. If animals are first given DMI, the NE is still taken up by nerve endings but fails to displace H^3-NE. These results suggest that DMI acts largely on granule membranes and interferes with the exchange of the amine between granules and cytoplasm (or neuronal membrane).

DMI given to rats whose NE stores are labeled, reduces the spontaneous efflux of H^3-NE without lowering the endogenous level. These results suggest that DMI reduces the synthesis of NE. We postulate that DMI by reducing the efflux of NE from granules (which contain little or no free amine) results in accumulation of free NE which then blocks its own synthesis.

A possible clue to the antidepressant action of DMI is the marked reduction in the maximum rate of efflux after reserpine administration. Finally DMI added in high concentration to heart slices *in vitro* causes the release of NE. This supports the view that DMI has changed the conformation of the neuronal membrane.

The Serotonergic Transducers

Role of Serotonin (5HT)

Precise definition of the role of 5HT in brain still eludes us though 5HT and NE transducers can be described in almost the same terms. Few drugs have a selective action on the 5HT transducer. Although our results strongly suggest that the action of reserpine is mediated through the continuous release of unbound 5HT, clear proof is still lacking.

Attempts are being made to find a role for brain 5HT by measuring the turnover rate of the amine in animals subjected to various physiologic states in the hope that this might disclose whether serotonergic pathways are involved. When rats are exposed to a temperature of 38° C, brain 5HT formation is increased by 75%. This suggests that 5HT neurons in brain may be involved in control of temperature by dissipation of heat.

Investigators at Pfizer & Co. have shown that p-chlorophenylalanine inhibits the synthesis of 5HT without eliciting pharmacologic effects nor counteracting the effects of reserpine. In our hands, large repeated doses of the drug (methyl ester) depletes brain 5HT of rats by 80% and brain NE by 30%. The decline in 5HT is definitely related to blockade of synthesis since the 5HIAA level declines and that of 5HT fails to rise after blockade of MAO. The animals are hyperactive but it is not known whether this is correlated with the loss of 5HT.

Attempts were made to establish the existence of 5HT stores in peripheral tissues from the uptake of H^3-5HT in thrombocytopenic rats. Preliminary results suggest that there may be small depots of endogenous 5HT in thyroid, heart, and other tissues.

Sympathetic Target Sites

Adipose Tissue Transducer System

The organism makes constant adjustments to the environment by a unique type of adaptation in which the nervous system causes the almost instantaneous activation of enzyme systems. In this regard, adipose tissue cells may be considered as transducer systems in which the input is the sympathetic transmitter and the output is FFA. With these cells, we are particularly concerned with the way in which a physiological signal—a catecholamine—is converted to a biochemical trigger, e.g., cyclic AMP inside the cell.

Last year, theophylline, which protects cyclic AMP from inactivation, was shown to be a powerful tool for studies of events in fat cells since its maximum lipolytic effect is 3 times that of NE. These studies showed that the activation of lipase by NE is normally limited by a ceiling in the steady state level of cyclic AMP and that theophylline, by blocking phosphodiesterase, raise this ceiling causing cyclic AMP to accumulate to a concentration that produces complete activation of lipase. In support of this view, the accumulation of cyclic AMP caused by theophylline was found to be closely associated with the lipolytic response and the blockade of phosphodiesterase.

Before concluding that cyclic AMP is responsible for the mobilization of FFA, it must

be shown that the nucleotide itself can actually increase lipolysis. Although the incubation of fat cells with cyclic AMP elicits only a slight lipolytic activity, the addition of theophylline in amounts having negligible activity by themselves, causes lipolysis equal to that obtained by excess amounts of theophylline.

Our studies indicate that cyclic AMP in adipose tissue is formed continuously, at a slow rate, in sympathectomized (SX) and adrenalectomized (ADX) rats. This indicates: (1) that NE is not necessary for adenyl cyclase activity but merely increases it, (2) that adenalectomy does not affect adenyl cyclase, but only the process that activates it.

Interaction of Sympathetic and Hormonal Systems

An important accomplishment has been the development of a precise method for the assay of adenyl cyclase. With this method, changes in the actual amount of adenyl cyclase may be distinguished from activation of the enzyme produced by NE or ACTH. The enzyme is measured by the rate of production of H^3-cyclic AMP^3 from H^3-APT. Cyclic AMP is separated from labeled contaminants by anion exchange chromatography and by treatment with a $BaSO_4.Zn(OH)_2$ gel. The formation of cyclic AMP was shown to be proportional to time of incubation and to enzyme concentration. The nucleotide AMP was identified by isotope dilution of the product itself and of the H^3-5′-AMP formed by hydrolysis with purified phosphodiesterase.

Adipose tissue from hyperthyroid rats is hyperresponsive to NE and the maximal response to the amine is more than doubled. This is related to the fact that the amount of adenyl cyclase is more than doubled. In contrast, adipose tissue from thyroidectomized rats is poorly responsive to NE; after treatment of rats with triiodothyronine, the response of the adipose tissue is restored. These results indicate that the link between the metabolic effects of the sympathetic and thyroid systems is through adenyl cyclase. Since adipose tissue from euthyroid, hyperthyroid and hypothyroid rats give the same maximal response to theophylline, it is concluded that lipase itself is not affected by throid hormone.

Adipose tissue from rats treated with cortisone is also hyperresponsive to NE due to an increase in adenyl cyclase. Again the amount of lipase is unaffected. However, the cortisone does not add to the maximum effect produced by thyroxin. The relationship between the permissive and induction action of cortisone is not clear from these studies.

After fasting for 48 hr, the adenyl cyclase is again increased without a corresponding increase in lipase.

Cortisone, thyroxine, cold-exposure and fasting do not enhance phosphodiesterase, in fact the activity of this enzyme is increased by cold-exposure.

Adrenergic Blocking Agents

Our studies with adrenergic blocking agents *in vitro* have clarified some of the confusion in classifying receptor sites in adipose tissue. Dose-response relationships indicate that DCI, a beta blocking agent, competitively blocks NE-induced lipolysis but has little activity on theophylline-induced activity. Phentolamine, an alpha blocking agent, acts at a different site since it blocks the effects of NE and theophylline to the same extent. Studies of adipose tissue homogenates indicate that this site is the lipase system itself.

Electrolyte Requirements

It is difficult to describe the effects of NE on fat cells in classical terms such as depolarization, since NE elicits almost as much lipolysis when the fat cells are incubated in isotonic sucrose as in Krebs' Ringer solution. The lipolytic activity of NE in isotonic sucrose is enhanced by the action of small amounts of K^+. On increasing the $[K^+]$ the lipolytic activity of NE is progressively diminished, and is absent at a $[K^+]$ of 100 to 150 mM. This inhibitory effect of K^+ is not counteracted by Na^+. The absence of Ca^{++} reduces NE lipolysis by only about 50%.

In considering the component parts of the adipose tissue transducer system, drugs may act on adipose tissue in a number of ways: (1) Displacement of NE from receptor sites,

(DCI); (2) Interference with membrane depolarization of cell by NE (adrenalectomy ?); (3) Inhibition of adenyl cyclase (no drug yet demonstrated); (4) Direct activation of adenyl cyclase (catecholamines); (5) Induction of adenyl cyclase (thyroid, cortisone); (6) Inhibition of cyclic AMP action; (7) Inhibition of phosphodiesterase (theophylline); (8) Activation of phosphodiesterase (nicotinic acid); (9) Inhibition of lipase system (phentolamine; (10) Shifts in K^+—insulin.

Studies on Chemical-Induced Shock

Previous reports from this laboratory have shown that ADX rats and rats whose adrenergic function is blocked fail to respond to external stimuli that require an increased expenditure of energy. The failure of epinephrine to elicit sympathetic responses in ADX animals indicates that their incapacity to withstand cold or strenuous work results from failure of sympathetic target organs to respond to transmitted messages. Communications are re-established by aldosterone as well as glucocorticoids, suggesting that the inexcitability of adrenergic receptors after adrenalectomy is related to changes in electrolytes.

We are now studying substances, categorically classified by Selye as stressors, and known to be much more toxic in ADX than in normal animals. We hope that the study of shock, induced by drugs whose mechanism of action is known, might lead to a better understanding of clinical shock. We started this study with the limited objective of establishing whether the toxicity of these stressor agents is enhanced in SX animals as well as in ADX animals. Our results show histamine and endotoxin, in doses that are not lethal to intact rats, are almost 100% lethal to both ADX and SX rats.

Treatment of ADX rats with a glucocorticoid or with epinephrine-in-oil provides partial protection against the lethal effects of histamine and endotoxin; complete protection is provided by giving both substances. Treatment of SX rats with epinephrine, alone, provides complete protection against the lethal effects of histamine and endotoxin.

Both histamine and endotoxin produce a blood pressure drop presumably by dilating the small blood vessels. The lethal effects of these substances in ADX and SX animals might stem from their action on the microcirculation. In normal animals, protection against hypotension is provided by signals sent to the brain via the baroreceptors, and the resultant adrenocortical discharge. In SX animals, there are no catecholamines to be discharged; in ADX animals, the catecholamines have little effect on the sympathetic system in the absence of glucocorticoids.

The lethality of formalin and tourniquet trauma is also greatly increased in ADX rats. Thus doses of formalin or a degree of tourniquet trauma that cause no deaths in control animals are 100% lethal in ADX rats but the toxicity is not enhanced in SX rats. Moreover, treatment of ADX rats with a glucocorticoid provides complete protection against the lethal effects of formalin and trauma. DOCA also affords considerable protection. Thus the lethal effects of formalin and tourniquet trauma are not mediated by the circulation. The results are consistent with the view that formalin and tourniquet trauma produce a toxic agent which is responsible for the toxic effects. Glucocorticoids would act by preventing the formation of this agent rather than by overcoming its effects. In support of this view, is the well-known fact that the lethal effects of tourniquet trauma occur only after removal of the tourniquet.

The Nonmast Cell Histamine Transducer

All tissues contain considerable amounts of histamine not in mast cells; in fact in some species including rabbit, cat (except for skin) and man, mast cell stores comprise only a small fraction of total histamine in the body. Last year we reported results which have led us to the provisional conclusion that nonmast cell histamine mediates exocrine secretions.

Selective Labeling of Nonmast Cell Histamine

The view that parenterally administered H^3-histamine selectively labels nonmast cell stores has now been fortified by studies showing that the specific activities of histamine in rat gastric mucosa and cat salivary gland are

almost indentical with those of amine released by cholinergic agents into saliva and gastric juice. From these results it may be inferred that the decline in radioactivity accurately reflects the endogenous turnover of histamine and that the release of histamine can be calculated from the release of radioactivity.

The assay of H^3-histamine by isotopic dilution has shown that the second part of the diphasic disappearance of radioactivity is artifactual and results from tritiated water present as an impurity in the injected H^3-histamine as well as from that formed by exchange in the body. Corrected values now reveal that turnover of H^3-histamine in various exocrine glands corresponds to a half-life of about 1 hr and in skeletal muscle of about 4 hr.

Distribution and Fate of Injected H^3-Histamine

In rats, the highly polar metabolite present in various tissues has been identified as a conjugate, presumably the riboside, of histamine itself and not of the acid as reported by others. In the cat, the uptake of H^3-histamine is also high in exocrine glands and occurs to some extent in all tissues. In exocrine organs H^3-histamine is rapidly converted to methylhistamine which is also retained by tissues. H^3-histamine in the cat is excreted in 2 phases; at first the urine contains considerable amounts of free histamine and methylhistamine. The rapid decline in the excretion of these amines coincides with their disappearance from plasma. At this time, the urinary excretion of the acid metabolites remains high. Since studies with tissue slices show that deamination occurs largely in kidney and liver, these results suggest that the bases are released into blood largely unchanged and are then deaminated by kidney and liver.

Methylhistamine injected into the submaxillary gland, intra-arterially, elicits some salivation; placed in lumen of cat stomach it elicits gastric secretion. These findings raised the question whether methylation of endogenous histamine leads to its inactivation or whether the substance is normally stored and has a physiological role.

Various cat organs contain considerable amounts of a substance that, like methylhistamine, is chloroform extratcable, deaminated by diamine oxidase, and reacts with dinitrofluorobenzene to form a derivative with a similar R_f value. However, mass spectrography (Dr. Highet, Lab. of Metabolism) of the substance indicates that it is not methylhistamine. The distribution of the "apparent" methylhistamine is of interest since it is high in brain stem and certain exocrine glands but absent from cerebellum and skeletal muscle.

The specificity of the histamine uptake mechanism was studied by measuring the uptake of H-methylhistamine in rats. The pattern of uptake and disappearance of methylhistamine closely resembles that of H^3-histamine. From current evidence we favor the view that methylhistamine is not normally formed and retained, and that exogenous methylhistamine may act like a "false transmitter".

Release of H^3-Histamine

Histamine appears to have an important role in exocrine glands. Thus stimulation of parasympathetic nerves or administration of acetylcholine or its analogues cause the release of histamine into the secretions of submaxillary gland, gastric mucosa, bile and pancreas. Pilocarpine also causes a marked efflux of H^3-histamine into these secretions. Gastrin and a factor in saliva cause the efflux of H^3-histamine into gastric juice. Secretin induces the efflux of H^3-histamine into bile and pancreatic secretions.

Finally preliminary results indicate that stimulation of skeletal muscle is associated with the release of H^3-histamine.

Synthesis of Histamine

Last year we reported evidence that the synthesis of histamine in the salivary gland was regulated by utilization. Additional studies show that after ligation of the esophagus to deprive the rat stomach of the stimulatory effects of saliva, gastric secretion is reduced by about 90% and the turnover of histamine by about 75%.

Factors That Affect Drug Action

Passage of Substances Across Membranes

Central nervous system. Since the pharmacologic effects of polar drugs are often studied after intraventricular injection, it is important to understand the nature of the boundary between ventricles and brain substance. After intraventricular injection, C^{14} labeled inulin, sucrose and mannitol enter brain tissue at rates roughly proportional to their diffusion coefficients in agar gel of infinite thickness. Such studies might make it possible to estimate the fraction of the ependymal surface permeable to large lipid-insoluble molecules.

Further studies have been made of the active transport system in rat brain which transfers 5HIAA directly from brain to blood. Last year it was reported that this process is completely blocked by probenecid. The transport system is also blocked by dinitrophenol given in hyperthermic doses. In addition it is markedly impaired when rats are exposed to a temperature of 38° C for 16 hr. It is possible that such impairment is a sensitive indicator of brain damage.

Intestinal absorption. Highly liposoluble substances like DDT and dieldrin are absorbed in part via the lymphatic circulation of the intestine. The substances are absorbed by the fat transport process of the intestinal epithelium, presumably due to their association with lipid micelles.

Biliary excretion of drugs. Carboxylic acids, sulfonic acids and chlorothiazide are secreted into bile by the same transport mechanism. Probenecid, a carboxylic acid, which markedly inhibits the biliary secretion of organic acids, is itself excreted in bile and appears to act competitively. The choleretic activities of probenecid and chlorothiazide are indirect effects stemming from the large quantities of water they take into bile by their osmotic effects. Ouabain, an active inhibitor of many transport mechanisms, is itself secreted into bile by a process that differs from those which secrete organic acids and bases. In addition, ouabain is also taken up by liver slices by an active transport process.

Thus the liver transports organic compounds by at least three general processes; one for acids, one for quarternary and perhaps tertiary amines, and one for ouabain and presumably other glycosides.

Enzymatic Mechanism of Membrane Transport

Research is directed toward a molecular-physiologic explanation of processes carried out by cell membranes. Two sorts of processes await explanation: (1) the active transport mechanisms which utilize energy to move cations, glucose, amino acids, catecholamines and other substances across various cell membranes; (2) processes by which nerve stimuli induce transient changes in conducting membranes and neurohormones produce transient permeability changes at receptor sites.

It is hoped that studies of the Na^+, K^+, ATPase from beef brain will provide a clue to the mechanism by which ATP energy is used for cation transport. Attention has been shifted to the active center of this enzyme. Previous work has identified as an acylphosphate, a phosphorylated intermediate whose formation is catalyzed by Na^+. We have attempted to determine the concentration of transport sites in the membrane by "trapping" the acylphosphate as a stable hydroxamate. The results suggest that Na^+ might cause two effects: (1) a reaction with ATP to yield the protein-bound acylphosphate and (2) a conformational change that protects the acyl derivative from reacting with hydroxylamine.

Attention is now focused mainly on the K^+-stimulated phosphatase step as the point where most of the ATP energy is utilized for the transolocation of ions. We have sought artificial substrates which interact specifically with the K^+-sensitive step. p-NPPase which catalyzes the K^+-stimulated hydrolysis of p-nitrophenylphosphatase resembles ATPase in certain respects; after adrenalectomy the activities of both enzymes in kidney are decreased and are restored by corticosterone. Differences in ouabain inhibition, however, show that the two enzymes are different. p-NPPase may provide a model for the study of K^+-activated systems; more recent studies suggest that acetylphosphate may serve as a

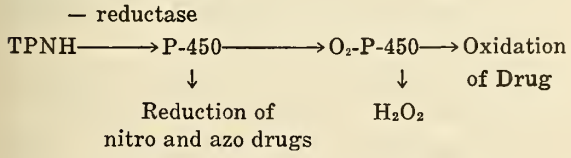
substrate for the K^+-sensitive step of the genuine transport ATPase.

Studies have shown that aldosterone does not act by changing the synthesis of transport ATPase. Thus aldosterone exerts drug effects on transport in the toad bladder but has no effect on ATPase or on enzymes involved in ATP synthesis. It is possible that aldosterone induces the synthesis of a membrane component controlling the diffusibility of Na^+.

An attempt is underway to identify adrenergic receptors by labeling with covalently fixed blocking agents. Experiments in connection with these studies have shown that reserpine produces a 5-fold increase in submaxillary glycoprotein in the denervated as well as in the innervated gland. The possibility that this action is mediated by the pituitary-adrenal system is under investigation.

Enzymatic Mechanisms of Drug Metabolism

Enzyme processes. Microsomes contain a cytochrome which in its reduced form combines with CO to form a complex; the cytochrome is called P–450 because the complex with CO has an absorption maximum at this wavelength. P–450 in microsomes is reduced by TPNH and then reacts with O_2 to form an "active O_2" complex which transfers O_2 to various drugs. In the absence of drug substrate, this complex breaks down to H_2O_2. P–450 also participates in the anerobic reduction of nitro and azo compounds. These general reactions are illustrated below



P–450 is a component of the TPNH-dependent enzymes in microsomes that are responsible for the hydroxylation, dealkylation, deamination and desulfuration of drugs and for at least one enzyme that catalyzes sulfoxidation.

TPNH cytochrome c reductase is now implicated as a component of P–450 reductase by studies showing that the reactions which utilize drugs and TPNH-cytochrome c redictase have practically the same Km's with respect to TPNH. In addition, 2′-adenosine phosphate inhibits the reductase and the drug reactions to the same degree.

Small but reproducible changes in the absorption spectrum of liver microsomes are produced by substrates and inhibitors of drug-metabolizing enzymes, even in the absence of TPNH. These changes are of two types. In presence of substrates, the dissociation constants for these changes closely agree with Km's (substrate) of the oxidation reactions, suggesting that they represent the formation of enzyme-substrate complexes. Substances that inhibit drug oxidation appear to act either by displacing substrate from the enzyme or by interfering with the flow of electrons between TPHN cytochrome c reductase and P–450.

Induction of microsomal phospholipid by phenobarbital. The increased oxidation and reduction of drugs produced by phenobarbital and other foreign compounds is associated with increased amounts of protein and phospholipid in liver microsomes. The increased content of protein can be explained by an increased rate of protein synthesis. However, studies of the incorporation of P–32 suggest that the increase in phospholipid is caused by a decrease in phospholipid catagolism.

Mechanism of Teratogenesis by Thalidomide

The mechanism of thalidomide-induced teratogenesis in laboratory animals, is still unknown. We have now shown that about 3% of the thalidomide in rabbit fetuses is bound to tissues irreversibly, mainly with nuclear RNA and DNA. This incorporation may be a characteristic of rapidly developing tissues, for 2 to 3 times as much labeled thalidomide is incorporated into nuclear RNA and DNA of rapidly growing rat liver (after partial hepatectomy) as in the liver of normal rats. These findings make it likely that thalidomide causes teratogenicity by acylation of RNA and DNA or its precursors and suggest that alkylating agents and thalidomide cause teratogenic effects through similar mechanisms.

Such a reaction would explain our findings that differences in the embryo toxicity of thalidomide in the rabbit and the rat are minimized when the drug is administered intravenously since the effects are likely to be related to a high level of drug action over a short time. By giving the drug intravenously, reproducible effects are obtained in rabbits with doses ranging from 2.5 to 10 mg/kg and in rats with doses of about 10 mg/kg.

Pharmacogenetics

The problem of individual variability in response to pharmacologic agents is receiving much attention because increasingly large populations are being exposed to drugs. Since each of the multiple enzymes that control drug metabolism is genetically controlled, mutations may lead to toxicity through accumulation of the drug (acatalasia, atypical cholinesterase and slow isoniazid inactivation).

Our studies are focused on the basic question whether inbred animals respond more uniformly to a drug than outbred animals (F_1 generation hybrids). The total variability of the strain is reduced, providing that genetic components of variation, such as color and antigenic variation predominate over environmental effects. Reports on responses to barbiturates are conflicting. Some claim that the response of inbred mice is more variable than that of outbred mice, others that it is less.

Our results show that hexobarbital elicits a sleeping time that, in some inbred strains of mice, is more variable than in outbred mice, whereas in others it is equal and in still others it is less. These variations are attributable largely to differences in rate of drug metabolism. On recovery of righting reflex, the brain hexobarbital level is similar in all strains, indicating that the CNS sensitivity to the drug is remarkably uniform.

Strain differences in hexobarbital metabolism may be related to participation of multiple forms or the isozymes of a given enzyme. Lactate dehydrogenase (LDH) which exists as 5 isozymes in most body cells, provides us with a model of how these forms result when 2 dissimilar subunits combine randomly to form active polymers. The synthesis of the subunits may be controlled by gene activity, but in addition the catabolism of enzymes may be an important variable in determining distribution. The relative stabilities of Pure LDH 1 and 5 were studied as possible variables in their biological regulation. Stability to heat was differentially altered by pH, ionic strength, NADH, oxaloacetate, malate, and fructose-1-6 diphosphate. The results show that substances may be fixed to allosteric sites thereby protecting the molecule from inactivation or accelerating heat denaturation. Three distinct allosteric sites have been identified on LDH 1 and 5.

Clinical Studies with Desmethylimipramine

An NIH-sponsored collaborative study with the Karolinska Institute is providing a partial answer to an important question: Are individual differences in metabolism of liposoluble drugs used in treatment of mental diseases large enough to account for individual differences in side effects and efficacy. In this study, plasma levels of the antidepressant drug desmethylimipramine (DMI) are determined by the isotope derivative technique dveloped in this laboratory (see methods) by Dr. W. Hammer, a participant in this project. The results indicate:

1. Plasma levels of patients on the same dosage regimen differ by as much as a factor of 20.
2. Thus far, side-effects are seen only in those patients who metabolize the drug slowly and who have the highest plasma level.
3. In general therapeutic effects on depression are associated with the concentration of DMI in the plasma.
4. Patients shortly after treatment with barbiturates have very low levels of DMI.
5. The side-effects in two patients were characterized as "extreme anxiety". No detectable DMI was found in the plasma of these subjects and subsequent studies have shown extreme anxiety to be a re-
6. The psychiatrists collaborating in this research now insist on plasma levels of flection of lack of treatment.

DMI; for the first time they feel that they can control therapy and toxicity.

Development of New Methods of Analysis

The ever increasing potency of modern drugs, especially those used in the treatment of mental disease, has created the need for more methods of higher sensitivity for the assay of organic compounds.

The isotope derivative technique, described in last year's report has been applied to the routine assay of desmethylimipramine and has yielded particularly rewarding results. This method is based on the extraction from plasma of desmethylimipramine and its subsequent acetylation with H^3-acetic anhydride of high specific activity. The method has been modified so that as little as 5 ng/ml of drug can be accurately measured.

A potentially simpler approach for the routine assay of drugs in ng amounts is the use of gas liquid chromatography with an electron capture detector. This sensitive device responds to organic compounds containing halogen or nitro groups at levels of about 10^{-15} M. If a drug does not contain these groups they may be introduced by forming a derivative of the compound after its extraction from plasma.

A method for assay of amphetamine, theoretically applicable to other aliphatic primary amines, involves condensation with Dansyl chloride (the demethylamino analogue of naphthalenesulfonychloride) to yield a highly fluorescent derivative.

A method has been developed for the assay of methylhistamine in biological tissues. In this procedure methylhistamine is assayed as the dinitro-fluorobenzene derivative.

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Introduction

The year began with Dr. Leon Jacobs continuing as Acting Scientific Director. In late September Dr. John R. Seal joined NIAID as Associate Director for Intramural Research. Dr. Seal had retired from the United States Navy on September 1, 1965, after serving in various capacities in the Navy medical research program during the major part of his military career. Dr. Jacobs remained as a consultant to the Director, NIAID, until January 1, 1966, when he retired from the Commissioned Corps of the U.S. Public Health Service and joined the Division of Biologics Standards NIH. These transitions had little effect on the momentum of the broad-based intramural research program. More serious in prospect was the retirement or resignation of other program leaders, and as the year closed the Institute was recruiting for a new Clinical Director with the need to restaff the Laboratory of Clinical Investigations to a large degree, facing needs to restructure and redirect its research effort related to tropical medicine involving changes in the programs in tropical virology and parasitology, and seeking leadership for the medical mycology research program.

Despite these unsettling influences, the year was a remarkably productive one with a continuing output of scientific publications reflecting the leadership role the NIAID Intramural Research Program holds in many aspects of infectious diseases and immunology.

Dr. Karl Habel was selected to give the Annual Dyer Lecture at the National Institutes of Health. Drs. Habel, Leon Jacobs, Robert Huebner, and Robert Coatney were awarded Distinguished Service Medals by the United States Public Health Service for their scientific contributions. Dr. Maurice Landy received the DHEW Superior Service Award. Many other honors were afforded the scientific staff as are detailed in the reports of the individual laboratories.

A new parameter was added to the responsibilities of the NIAID Intramural Research Program with the assignment of Chairmanship of the NIH Cholera Advisory Committee to Dr. Seal. This responsibility includes the scientific management of the SEATO Cholera Research Program and the Pakistan-SEATO Cholera Research Laboratory in Dacca, East Pakistan. The SEATO Cholera Research Program was developed as a result of the spread of cholers in Southeast Asia, the Pacific, and westward from its traditional endemic focus beginning in 1958, and includes the sponsorship of scientific symposia for exchange of information, grants, training contracts, and research in cholera in SEATO member countries with basic funding by the Agency for International Development (AID) and scientific management by NIH as a result of several AID-DHEW, AID-NIH interagency agreements. The Pakistan-SEATO Cholera Research Laboratory in East Pakistan is sponsored under the umbrella of the Program and a government of Pakistan-AID agreement but receives additional funding and support through a NIH–PL 480 research grant and from several SEATO member countries for conduct of cholera research. Among its more notable contributions during this year have been information on the high degree of effectiveness of United States manufactured cholera vaccine in the prevention of cholera, the long duration of vaccine induced immunity, particularly in

adults in cholera endemic areas, added data on the value of tetracycline in the therapy of cholera along with comparative data on the lesser effectiveness of several other antibiotics, new information on non-cholera diarrheas and on a malabsorption syndrome which occurs in Americans subsisting on a local diet, and recognition of hypoglycemia as a complication of cholera in infants.

The Board of Scientific Counselors of NIAID was asked to undertake a review of the entire viral research program. Departing from the usual custom, three meetings were held during the year, one at the Middle America Research Unit (MARU), Canal Zone, Republic of Panama, and another meeting featuring a review of the "slow" or chronic virus disease research sponsored by the NINDB, the Extramural Research Program of NIAID, and the Rocky Mountain Laboratory. One important area in which the advice of the Board had been sought was that of arbovirus research. The Board emphasized the National needs for NIAI intramural research to continue a strong program in arbovirus research and recommended that the excellent small program at the Middle America Research Unit (MARU) be strengthened and extended both by the assignment of a more broadly based mission and staff to MARU and by more active scientific collaboration with the Gorgas Memorial Laboratory and university-based research groups. The Board also recognized the leading role of the Rocky Mountain Laboratory in research on animal models of slowly developing, progressive viral infections causing neuropathies, pulmonary, vascular, and renal disease, the probable relevance of the work to certain human diseases, and the probable inability of academic institutions to play any prominent role in this type of research during the next decade. It recommended strengthening of the viral research competence of the Rocky Mountain Laboratory and installation of new facilities to permit inoculation of material of human origin, particularly brain specimens from humans dying of "Kuru," into the animal species being studied.

The importance of the Intramural Research Program to the Collaborative Research Program of the NIAID was more evident as the demands on the intramural scientific staff increased. The Laboratories of Infectious Diseases and Tropical Virology undertook the testing of many of the reagents being developed in the Research Reference Reagents Branch, provided seed stocks of viruses, developed new methodology and trained some contractor personnel in their use, site-visited contractors' installations, and provided informed members to advisory committees. The Laboratory of Infectious Diseases was especially involved in the Vaccine Development Branch with several of the staff making important contributions as project officers and participants in the field testing of new vaccines such as the oral adenovirus type 4 vaccine and parainfluenza vaccines, research for further definition of the oncogenic potential of various adenoviruses, initial testing of vaccine against *mycoplasma pneumoniae*, developments in methodology, and advisory services to the Vaccine Development Committee. The Laboratory of Immunology provided very similar support to the Transplantation Immunology Branch.

The variety and scope of the Intramural Research Program is illustrated in the selected examples of research which are summarized in the following pages.

LABORATORY OF CLINICAL INVESTIGATIONS

A major emphasis of the laboratory has been in the further study of respiratory virus dynamics in the infection of human volunteers derived from the Federal prison system. Other research on the mechanisms of fever and host responses in various syndromes, in immunogenetics, in parasitology, and in leprosy has continued.

Transmission Dynamics in Respiratory Infections

Using Coxsackie A–21 virus as a model, extensive studies were made in human volunteers of transmission dynamics. Quantitative studies were carried out on the presence of virus in respiratory tract secretions, the role of breathing, speaking, coughing and sneezing

in release of viral particles to room air, the effect of particle size in creation of viral aerosols which remain in suspension in room air for long periods of time and on the transmission of infections via the airborne route to other volunteers. Coughing was shown to be the most important source of airborne viral particles and human-to-human transmission via the airborne route conclusively demonstrated. Airborne transmission of Coxsackie A–21 virus infections between artificially infected volunteers and other non-immune volunteers under dormitory conditions was also demonstrated. Virus was recovered from the dormitory air by use of large volume air sampling techniques during these trials.

Remarkably small doses of virus were infective when aerosolized in small particles and this was particularly true in adenovirus infections. The human infectious dose (HID_{50}) was about 30 $TCID_{50}$ for Coxsackie A–21, 1 $TCID_{50}$ for adenovirus type 4, and 0.68 $TCID_{50}$ for rhinovirus. Even smaller doses given by nasal drops caused similar illness in the case of Coxsackie virus and rhinovirus. A larger dose of adenovirus by nasal drops was necessary to infect and only the aerosol route regularly produced typical illness.

Antibody in Nasal Secretions and Serum

By challenge experiments in volunteers the apparent greater importance of 9S–14S antibody in resistance to rhinovirus infection than 7S or 19S antibody was demonstrated. 11–14S antibody was shown to appear in nasal secretions, tears, and saliva after its first appearance in sera and was associated with only γA immunoglobulin in these secretions. This evidence strengthened the hypothesis that γA antibody in secretions results from an active selective transport mechanism between serum and secretion but does not rule out a possible local production of antibody.

Chilling and Respiratory Infection

Controlled experiments were made on the effects of chilling with and without an actual decrease in body temperature on the course of rhinovirus infections. No evidence could be obtained that chilling before, during, or subsequent to the clinical disease in any way altered its course.

Adenovirus Soluble Antigens

Purified hexon and fiber antigens from adenoviruses types 1 and 4 were administered to volunteers with good neutralizing antibody response in a high percentage of subjects. The 7S antibody response was similar in degree and duration to that observed in human subjects given infectious virus.

Influenza

Influenza was produced in volunteers with three $TCID_{50}$ doses of A2 virus when given as an aerosol. Low levels of homologous neutralizing antibody did not protect against infection with this small dose nor did heterologous antibody in higher titers. A/Equi 2 virus caused a high incidence of infection but infrequent illness in human volunteers. Serial passage of equine influenza virus in man did not increase its pathogenicity for man or decrease its pathogenicity for equines. Their findings suggested that A/Equi 2 virus is rather host specific and that horse-to-man transfer under natural conditions is probably a rare event.

Mechanisms of Fever and Host Responses

Extensive studies are in progress relating to the pathogenesis of fever, reticuloendothelial function and immunological reactivity of humans and experimental animals. Investigations are being carried out in patients with familial Mediterranean fever, recurrent fever of unknown etiology, leprosy, a variety of neoplasms and normal volunteers.

Through utilization of a specific and sensitive assay for etiocholanoalone, it has been shown that increased plasma levels of this steroid are not correlated with febrile episodes in a variety of patients. The administration of this pyrogenic steroid is proving useful as a tool for studies of bone marrow reserve, granulocyte kinetics and fever.

A bentonite flocculation test was developed for the demonstration of circulating anticryp-

tococcal antibodies in patients with cryptococcosis.

Patients with Hodgkin's disease have an enhanced phagocytic capacity. Likewise, patients with lepromatous leprosy have an increased reticuloendothelial clearing capacity in addition to a variety of immunological defects.

It was also shown that passively transferred endotoxin tolerance persists for at least 3 weeks despite rapid decay of passively transferred antibody.

Systemic Fungus Infection

Attempts to improve chemotherapy of systemic mycoses met with mixed success. *In vitro* tests of Nocardia suggested two potentially useful antibiotics: ampicillin and capreomycin. A combination of amphotericin B and hydroxystilbamidine appears promising in the treatment of mice infected with North American blastomycosis. Intraventricular administration of amphotericin B by use of a new prosthesis has not been encouraging. Hamycin, a new antifungal antibiotic, was dropped from clinical trials because of poor chemotherapeutic effect.

Factors affecting the cure of cryptococcosis with amphocericin have been the subject of continuing study. The role of immunologic paralysis, the occurrence of delayed hypersensitivity, and the means of adaptation by Cryptococcus to chemotherapy have been evaluated with respect to their effect on prognosis.

Leprosy

The leprosy program continued with the evaluation of rimino compound B.663, in the treatment of lepromatous leprosy. The patient under study is of particular interest because he had received no prior or concurrent sulfone therapy. Increasingly precise bacteriologic techniques have confirmed the antibiotic potency of B.663 against the leprosy bacillus.

In addition, experience suggests that B.663 possesses an anti-inflammatory action against erythema nodosum leprosum reactions. These reactions constitute the most objectionable feature of sulfone therapy, which is currently standard, and B.663 may prove to have wide application used in combination with the sulfones.

Laboratory of Infectious Diseases

The efforts of this laboratory continue to be focused in several major programs:

1. Cancer and leukemia virus studies.
2. The viral and mycoplasma causes of acute respiratory diseases.
3. Rubella.
4. Epidemiology of picoranaviruses and eosinophilia meningitis in Oceania.
5. Bacterial metabolism and physiology.
6. Medical mycology.

The virus research groups work in close relationship with the National Cancer Institute on the problems of oncogenic viruses and with the National Institute of Neurological Diseases and Blindness on Rubella and related problems.

Adenoviruses as Oncogenic Agents

Eight of 31 human adenoviruses have been shown to cause cancers in hamsters (types 3, 7, 12, 14, 16, 21, and 31). The first and chief indication that the tumors induced by adenovirus inoculation were actually directed by the adenovirus genes was the discovery of nonvirion complement fixing (CF) antigens in the virus-free tumor cells. Similar nonvirion antigens, now called T antigens or neoantigens occur in cells infected with adenoviruses. These antigens were shown to be new proteins which were synthesized by the input virus before new viral DNA appeared. The T antigens therefore represent a revolutionary new category of virus induced antigens. Many different lines of research here and in other laboratories confirmed these findings not only for adenoviruses but for other DNA viruses, such as SV40 and polyoma.

Tumor and T Antigens as Possible Determinants of Adenovirus Oncogenesis

The presence of virus-specific but non-virion T antigens in cells infected and/or transformed by human adenoviruses were confirmed for oncogenic strains of simian, canine and

bovine adenoviruses. Thus, the regular presence of T antigens in adenovirus tumors suggested that such antigens may be determinants of oncogenesis, a hypothesis that was supported by remarkable correlations between groupings of the adenoviruses according to oncogenic potentials, Green's G+C DNA base compositions, Rosen's hemagglutinins (HA), and T antigens.

Adenovrus-SV40 Hybrids

Exciting new discoveries on the transfer of SV40 and adenovirus genetic information from the adenovirus type 7–SV40 hybrid to other adenoviruses were made. Not only was the SV40 genome transferred from type 7 to type 2, but the genome of type 7 responsible for stimulating production of the virus specific T antigen was transferred as well. These observations proved without question that viral DNA was responsible for the synthesis of the non-virion T antigens.

Adenovirus-Associated Viruses (AAV's)

A new defective virus, dependent entirely on adenoviruses acting as helpers for replication, was discovered almost simultaneously by workers at Baylor University, The University of Pittsburgh and the Laboratory of Infectious Diseases. The AAV virus was found to be the smallest of the known viruses, measuring no more than 15 mμ in diameter. Its DNA was shown to be double stranded and to have a base composition much different from that of the adenoviruses from which they were derived.

Also, recently discovered were two additional serologically distinct AAV's. The AAV's are quite unique among mammalian viruses; however, similar wholly dependent "satellite" viruses are found among the plant viruses.

Leukemia

Extensive studies with several animal models continued. It was shown that infectious virus was obtained when hamster tumor cells resulting from Bryan Rous Sarcoma Virus (defective virus) were grown in mixed culture with chick embryo fibroblasts. When such mixed cultures were injected into RIF-free chicks, noninfectious sarcomas resulted which had the karotype of chicken cells and CF antigens of the Rous Sarcoma cells. Tissue cultures derived from these sarcomas remained free of infectious virus until an avian leucosis virus was added, following which Rous Sarcoma Virus appeared. In other studies, a quantitative test for murine leukemia viruses was developed as was an *in vitro* assay system for mouse (Moloney) Sarcoma Virus.

Vaccine Against Mycoplasma Pneumoniae

A protective effect of a formalin inactivated *M. pneumoniae* vaccine was demonstrated in volunteers. Men who developed antibody following inoculation of the vaccine resisted challenge with a virulent suspension of the organism, whereas illness occurred in 10 of 13 in unvaccinated controls.

Mycoplasma Epidemiology

Considerable advances were made in understanding the epidemiology of *Mycoplasma* behavior of this organism a longitudinal study of seven training platoons was made at the Parris Island, S.C., Marine Recruit Training Center. Fifty to 74% of recruits possessed *M. pneumoniae* growth-inhibition antibody at the start of training. Among the antibody negative (i.e., susceptible) recruits the infection rate for the 14-week training period was quite high—60 to 80%. Growth-inhibition antibody correlated well with resistance to infection, but it was not completely protective since approximately 20% of recruits with pre-existing anibody became infected during training. The latter finding provides the first evidence that reinfection with M. pneumoniae occurs under natural conditions.

Mycoplasma—Fundamental Studies

Three major advances were made in understanding these organisms. First, the hemolysin of *M. pneumoniae* was found to be a peroxide. This finding has implications in the pathogenesis of mycoplasma-induced disease as well as in autoimmune phenomena induced by mycoplasma. Second, the DNA homology technique

revealed the genetic distinctness of the mycoplasmas from bacteria. This finding clearly established the mycoplasmas as a separate group of microorganisms, a group with its own identity. Third, the protective antigen of *M. pneumoniae* was defined as a lipoprotein. The lipid hapten of this complex was further defined as a low molecular weight phospholipid containing glycerol, four amino acids and several fatty acids. The chemical structure of this protective antigen is nearing definition.

Host Resistance to Respiratory Disease

In volunteers challenged with type I parainfluenza virus, serum antibody did not correlate well with resistance to infection. In contrast, antibody in nasal secretions did correlate well with resistance to infection. Such antibody proved to be the decisive factor in determining the outcome of experimental challenge with type I parainfluenza virus. An inactivated type I virus vaccine stimulated serum but not nasal secretion antibody. Such vaccinated individuals did not resist experimental challenge with the virus. However, antibodies developed in the nasal secretions of volunteers following infection and such individuals resisted rechallenge.

Studies in Oceania

Picornaviruses have been found to be frequent causes of infection among adults and children in Honolulu. The exact nature of the problem remains to be defined since the Coxsackie Group A related viruses recovered are not pathogenic for suckling mice and neutralizing antibody cannot be studied.

An outbreak of Dengue in French Polynesia druing 1964–65 has been further analyzed and indicates that this was the first introduction of an arthropodborne virus into the island in the life span of individuals studied.

More evidence has been obtained on the relationship between *A. cantonensis* and eosinophilic meningitis in studies in Thailand and Oceania. The probable source through ingestion of raw shellfish, fish and vegetables has been indicated. Recent outbreaks in French Polynesia apparently resulted from contaminated lettuce.

Rubella Virus

These studies are supervised by Dr. John Sever of NINDB and Dr. Huebner of NIAID. In collaboration with Drs. A. Fabiyi, G. Gitnick, L. White, R. J. Hildebrandt, and D. A. Fuccillo, they are studying the role and behavior of rubella and other viruses as causes of perinatal disease and defects.

Rubella virus given to pregnant ferrets early in gestation produced runting and abortion of the newborn. Neutralizing antibodies developed in all inoculated animals, showing that active infection had been produced. Newborn ferrets given rubella virus develop a chronic infection lasting 6 to 8 weeks. The newborn and pregnant ferret represents the first and as yet only animal study system available for rubella virus other than primates. Should the runting and abortion prove to be due to infection of fetal tissues, this system may prove extremely useful in the study of birth defects.

Rubella virus syndrome is also being studied clinically, serologically and epidemiologically. Preliminary data derived from the NINDB Perinatal Study groups have provided a profile of the immune status to rubella among women of childbearing age. Considerable geographical variations were observed; antibodies were found in 85% of the women in the eastern part of the U.S., in 70% on the west coast and in less than 50% in Hawaii.

A new rubella vaccine development program was initiated in collaboration with NIAID's Vaccine Development Branch with Dr. John Sever, NINDB, as the major project officer on a number of contracts. This program is only in the beginning stage; however, several types of vaccines produced on commercial contracts should be available for field testing early in FY 1967. Reference virus pools and antisera needed for evaluation of vaccines have been produced in this laboratory and distributed to the various contractors.

Various methods for increasing the titer of rubella virus have been successful, and these have been utilized for producing complement fixation test antigens and also seed stocks for candidate rubella vaccines.

Bacterial Metabolism and Physiology

Hydrogenomonas eutropia has been utilized in model system study of electron transport mechanisms because of its ability to utilize molecular hydrogen. Further evidence was obtained on the role of cytochome c but the exact enzyme which mediates transfer of electrons has not as yet been defined. Solution of this problem will enable studies on the role of organic nutrients in metabolism in autotrophic and heterotrophic cells.

Studies on the role of iron and siderophilin in the growth and metabolism of *Staphylococcus aureus* continue. Marked physiological differences exist between iron-deficient and iron-rich cells of *S. aureus* and these are additionally altered by growth of such cells in the presence or absence of glucose. Iron-deficient cells vary in the following characteritsics: poor growth, imparied respiration, increased glycolytic activity, decreased catalase activity, a reduction in cytochromes and cytochrome oxidase, and decreased sensitivity to cyanide inhibition. Iron seems to act as an inductor of electron transport and respiratory enzyme formation. There have also been further studies on the enzyme which releases teichoic acid from *S. aureus* cell walls. This is produced in varying amounts by nine different strains and has been purified 10-fold in one preparation. Na^+, Mg^+ + and cobalt are required for activity. If further purification can be achieved and the linkage point of attack identified, removal of teichoic acid from the cell wall may permit identification of the role of this acid in cell-wall replication and in the binding of certain antiobiotics.

Studies on the structure, replication and genetics of streptococci also continued. These include characteristics of L-form colonies. By electron microscopy, small intravescular masses of elementary bodies in the order of 0.05μ are seen but filtration studies have failed to demonstrate colony forming capacities of units smaller than 0.22μ in size. The surface receptor site of Group C streptococci has been identified but the phage receptor site in Group A streptococci remains unidentified despite intensive efforts to identify its location and nature. The coexistence of two M antigens in the cell wall of Group A streptococcus has been confirmed and the occurrence of a type 12 M protein in a Group C streptococcus observed. Efforts to achieve laboratory transduction of Group A streptococci have not as yet been successful but continue since this apparently occurs in nature and the ability to manipulate the organism in the laboratory will open pathways to the genetic study of virulence and antigenic variability.

Histoplasmosis

Various lines of study have been pursued. Several nitrogenous suppdements to sterile soil enhanced the multiplication of *H. capsulatum* but in nonsterile soil inhibited growth. These studies along with a number of analyses of soils from other sites suggest that no single nutritional factor determines wheter H. capsulatum can grow at a particular site. Rather, there is indication that the distribution of bacterial antagonists is the limiting factor.

At the Middle America Research Unit, the role of bats in dissemination of histoplasmosis was further identified. An outbreak in persons following a visit to a bat cave was intensively studied with development of both clinical and serologic evidence that previous disease was relatively protective. The disease was also identified for the first time in Western Texas when found in free-tail bats.

LABORATORY OF BIOLOGY OF VIRUSES

Previously established experimental studies on the structure of viral nucleic acids and the molecular events involved in virus replication have continued during this fiscal year, but, in addition, there has been an increased effort aimed at determining the functional aspects of these nucleic acids. As in the recent past, these basic biochemical investigations have not been limited to phenomena occurring in virus-infected cells but of necessity have tested similar parameters in normal control cells.

Viral Structure

Findings on virus structure have varied from electron microscopic demonstration that polyoma virus may be made up of complex lamination of protein layers with a DNA core to the characterization of the physiochemical configuration of viral RNA and DNA molecules. Three projects have looked at the structure of the nucleic acid from DNA viruses and two from RNA viruses. In both types of viruses the studies have included single and double-stranded forms of the two kinds of nucleic acids. In fact, in the case of the rat virus this work represents the first definitive demonstration that there exists a mammalian virus containing single-stranded DNA. A very interesting "satellite" virus which is a small agent unable to replicate by itself and found multiplying only in the same cell with adenoviruses has been shown to be a DNA virus whose nucleic acid is double-stranded. Further information on the structure of the DNA of SV 40 virus—a tumor inducing agent—has been obtained through studying the effect of radiation on its physical properties.

Cell Response to Viral Infection

The examination of the biochemical events taking place in the virus infected cell are inseparable from study of nucleic acid functions of both the cell and the virus. Since messenger RNA is required for the production of all specific proteins and this function occurs in a physical unit made of ribosomes, one of the staff has been developing methods for isolating and purifying these cellular elements. The importance of the cellular polyribosomes for translating the virus information into viral protein products has been demonstrated in the vaccinia (DNA), Reo virus (RNA) and poliovirus (RNA) systems. Furthermore, in basic studies on the mode of action of the imporant antiviral substance, interferon, evidence has been obtained that the effect may be localized at the level of association of viral RNA with ribosomes.

Viral Oncogenesis

Polyoma virus DNA synthesis in lytic infection has been found to be preceded by the early appearance of specific "tumor" antigen and an increased activity of enzymes required for DNA synthesis. Of special significance is the simultaneous increase in cell DNA synthesis since this may be a requirement for integration of viral genome into cell genome during oncogenic transformation. Another experimental finding having significance concerning the relationship of tumor virus DNA to cell DNA is the demonstration that a single cell can be transformed by two different DNA tumor viruses—SV 40 and polyoma. Such doubly transformed cells contain two types of specific tumor antigens induced by both viruses and appear to have an increased growth potential. This finding raises the possibility that more than one site for integration of viral genome is available on the genome of the cell.

Genetic Relatedness

Several of the laboratory's projects have been aimed at nucleic acid functions in the normal mammalian cell. Previous results had shown a degree of common structure in the DNAs of various animal species varying according to their evolutionary relatedness. Now by study of pieces of the cell genome, the commonness has been shown to reside chiefly in those areas of the DNA highest in G–C base content. By manipulation of conditions during comparison of, for instance, the DNAs of man and chimpanzees, previously undemonstrable differences can now be shown. Another significant finding is evidence pointing to the existence of repeating sequences of bases in cell DNA and the separation of these stretches of the genome into classes according to their frequency of occurrence. Preliminary experiments have been initiated on the separation and characterization of chromosomes from mammalian cells. Already techniques have been developed for obtaining large, workable, amounts of partially purified human chromosomes which can now be tested for a variety of biochemical functions.

LABORATORY OF TROPICAL VIROLOGY

Major efforts continued to be related to the study of the properties of South American

hemorrhagic fever viruses. Amapari virus, a new member of the Tacaribe group, was shown to be more adaptable to suckling hamsters than to suckling mice. The advantages of tissue culture cell lines derived from newborn rabbits MA III) and green monkey kidney (Vero) were investigated for propagation of Tacaribe, Amapari and Junin viruses. Each cell line had definite advantages and disadvantages. Efforts to obtain cytopathogenic effects with Machupo virus in human embryonic kidney cell lines were essentially unrewarding.

MIDDLE AMERICA RESEARCH UNIT (MARU) PANAMA CANAL ZONE

Efforts were concentrated toward the further definition of South American hemorrhagic fever viruses and on the prevalence of arboviruses in Central America. More than 11,000 sera have been placed on file for screening for antibody to arboviruses. The bulk of these sera were obtained in collaborative studies between MARU and INCAP in Costa Rica during ICNND sponsored nutrition surveys throughout Central American countries. IBM data processing has been utilized for data recording, retrieval, and analysis of the information which is accumulating on arbovirus infections.

Hemorrhagic Fever

Continued surveillance in San Joaquin resulted in diagnosis of 18 sporadic cases of dis- where trapping results indicated residual popease during calendar year 1965. Most of these were concentrated in two areas of the town ulations of *Calomys callosus.*

Field studies in Bolivia, Peru, Paraguay, and Brazil were successfully completed. Testing of human sera to date reveals no evidence of past HF infection. At least 5 candidate HF agents have been recovered from *Calomys* captured in Brazil and San Ignacio, Bolivia.

Studies in experimental animals were extended. Adult *Rattus rattus, Proechimysguyannensis* and possibly *Mus musculus* do not develop chronic infections with viruria following Machupo virus inoculation. Other rodent species are being tested.

Asymptomatic infection was induced in newborn *Calomys* by Junin, Tacaribe, and Amapari viruses. Antibodies were produced by all viruses. Analysis of animal tissues for evidence of chronic infection is in progress.

Cross challenge tests in marmosets indicate that infection with Junin or Tacaribe viruses does not protect against subsequent lethal Machupo infection. Attempts to attenuate Machupo virus for this host are under way.

Arbovirus Studies

Continuous sentinel monitoring of virus activity was maintained at Gamboa in the Canal Zone. Eighteen strains of VEE virus, seven of EEE and one of Bussaquara were recovered between June and December 1965. As in previous years activity declined below detectable levels from January through April.

A study of dynamics of infection by VEE and related Pixuna and Mucambo viruses in reservoir rodents *Proechimys semispinosus* and *Sigmodon hispidus* was completed. Viremias ranged from 2–5 days duration, most animals survived, and virus was recovered from throat swabs, but not feces or urine. Animals developed anitbodies to all three viruses following infection by any one of them. VEE infection conferred complete protection against Pixuna and Macambo viruses, but Pixuna infection failed to protect completely against subsequent VEE challenge.

A study of antigenic variation among VEE strains was begun. It appears that donor host is not a significant variable, and that clear cut geographic differences are recognizable.

LABORATORY OF GERMFREE ANIMAL RESEARCH

The laboratory continues to take advantage of the germfree animal to study certain infectious processes and immune reactions under conditions where previous infection and immune responses have not altered the host's reactivity. The germ free animal has seemed a particularly promising model for the study of autoimmune phenomena.

Immunoglobulins in Germfree Mice

It has been shown that germfree mice usually synthesize and contain in their sera only $7S\gamma_1$and IgM globulins. These proteins are synthesized by spleen and lymph nodes. In conventional mice similar tissues form all immunoglobulins. The ileum of conventional mice forms principally IgA globulin while that of germfree mice usually forms none. Levels of $7S\gamma_1$ and IgM globulins can be increased by immunization with ferritin and the somatic lipopolysaccharide of *Escherichia coli*. A single high dose of endotoxin and two appropriately spaced, but not a single injection, of ferritin result in the synthesis of $7S\gamma_2$ globulin by the spleen. Formation of this protein shows a high correlation with the appearance of germinal centers in the spleen.The response to endotoxin is characterized by a prolonged increase in IgM globulin to levels between 1.5 and 2 times control levels, and this response is indepdendent of dose between 0.5 and 50 μg. A single injection of ferritin produces a transient increase of IgM globulin to 0.2–0.2 mg/ml, about double the control levels, followed by a 30 percent increase in $7S\gamma_1$ globulin. The secondary response to ferritin shows an increase in IgM globulin of a magnitude similar to that seen in the primary response, but the elevation is much more prolonged. In addition, the serum concentration of $7S\gamma_1$ globulin approximately doubles. Much of the relatively large increases produced by these immunizations cannot be accounted for as specific antibody.

Genetic Factors May Regulate Immune Tissue Destruction

The incidence of autoallergic thyroiditis in two strains of guinea pigs was compared. The Hartley strain animal developed the disease more frequently and after lower antigenic immunizing doses than did the Strain 2 guinea pig. Nevertheless, the antithyroid antibody titer and delayed skin reactivity in the two strains of guinea pigs were identical. These strain differences, in the face of an apparently equal gross immune response, may be due to quantitative differences in the classes of immunoglobulins produced, to variations in cell structure in which one type may be more easily damaged than the other, or to different amounts of reactive intermediates which are involved in immune tissue destruction.

Clostridium Perfringens Lethal to Germfree Guinea Pigs

A high mortality rate is associated with the transfer of germfree guinea pigs to an open animal room where they come into contact with a variety of bacterial species. In studies designed to investigate the nature of this infectious process, a strain of *Clostridium perfringens* was isolated from the intestinal contents of moribund ex-germfree guinea pigs. *In vitro* cultivation of this organism led to the production of heat-labile exotoxin(s) which on intraperitoneal injection into mice produred death. Oral inoculations of germfree guinea pigs with this *Clostridium* produced death within 12–18 hours. Other investigations have shown that conventional guinea pigs often harbor this organism in the intestinal tract yet are tolerant to heavy oral doses. These studies have provided new knowledge as to the nature of this increased susceptibility of germfree guinea pigs to bacterial exotoxins.

Severity of Amoebic Infections Influenced by Associated Bacteria

Conventional guinea pigs harboring a variety of intestinal bacterial species often develop fatal amoebiasis after intracecal inoculation with *Endamoeba histolytica* whereas germfree guinea pigs do not develop severe disease. By monocontaminating groups of germfree animals with known species of bacteria and infecting each group with the same strain of *E. histolytica*, differences in severity of the infections have been observed depending upon the bacterial species present in the intestine. When *Bacillus subtilis* was used as the bacterial associate, all animals succumbed to the infection. However, the infection of guinea pigs with amoebae and a species of *Micrococcus* resulted in no mortality. Various gradations of severity were observed with other known species of bacteria and, in addition, differences

were observed in the intestinal lesions produced. These preliminary observations suggest that the severity of experimental intestinal amoebiasis in guinea pigs may be influenced by the species of associated bacteria.

Low Serum Lysozyme Levels in Germfree and Ex-Germfree Rats

Levels of serum lysozyme in germfree and ex-germfree Fisher and Sprague-Dawley rats have been found to be significantly lower than in conventional animals of the same strains. However, no significant difference in enzyme free rats. Large amounts of lysozyme are present in leukocytes and a close correlation is known to exist between enzyme levels and total granulocyte counts. In the three animal groups studied, this granulocyte-lysozyme association has confirmed, i.e., the lowest levels of lysozyme and the lowest leukocyte counts being found in the germfree rats.

Immunoglobulins Quantitated in Human Malaria

The importance of the increases of the various immunoglobulin classes formed in response to infection with the malaria plasmodia has been emphazied by recent studies. Serial quantitation of the three immunoglobulins IgM, IgG and IgA during primary attacks of vivax and cynomolgi malaria have shown that a close association exists between increased immunoglobulin levels and the formation of specific malarial antibody. Large amounts of IgM and IgG globulin were formed during the course of the primary attack in all volunteers —the increase in IgA globulin was less striking. Volunteers with the milder cynomolgi infections of lower parasitemia synthesized less of the three immunoglobulins than did those with vivax malaria. No significant increases were observed in IgD globulin during the primary malarial infection. Fractionation of sera taken from a single vivax volunteer during the primary attack demonstrated that both the 19S and 7S fractions had specific antibody against the infecting strain. However, during the secondary response, when the patient relapsed, there was no specific malarial antibody activity in the 19S fraction and antibody activity appeared to be confined to the 7S fraction.

Laboratory of Parasitic Diseases

A relatively broad approach to the problems of parasitism has been the primary concern of the laboratory, emphasizing basic research, coupled with field studies in various areas of the world.

Five field projects have been conducted outside the U.S. Dr. Allen W. Cheever has continued his studies on schistosomiasis pathology in Salvador, Bahia, Brazil. Two other programs on schistosomiasis will reach completion soon; one supported by PL–480 funds in Egypt on mass treatment with sodium antimony dimercapto succinate (Astiban); the other in Puerto Rico on continuous drug infusion. Two additional PL–480 programs, one concerned with amebiasis in India, the other with toxoplasmosis in Israel, have continued at a productive level.

Schistosomiasis

The release of cercariae from the snail host is not influenced by an innate circadian rhythm. Light can stimulate their release in the absence of temperature change. If the light intensity is at least 60 foot candle power, even a few seconds of light can stimulate cercariae release. In the absence of light, a temperature rise of 4° C. causes their release, but a 2° rise does not.

Low temperatures inactivate cercariae; their sedimentation rate at 1–3° C. is 4.95± 0.09 cm per minute. At 11 to 13° C. cercariae become sufficiently inactive so that they cannot maintain their positional level in the water; these data suggest that human infection will not occur in water below this temperature range.

The determination of cercarial concentrations in natural bodies of water is fundamental to an understanding of their population dynamics and to the transmission of the infection. A simple continuous flow centrifuge has been developed for the quantitative collection of cercariae. Seventy to ninety percent of the

organisms were consistently recovered from one-liter water samples and over 50 percent from larger samples. The technique appears promising for field use.

Laboratory studies indicate that miracidia infected snails 10 meters from their point of release, and were infective up to 8 hours after they hatched. These and other data suggest that miracidia have a capacity to infect snails in the field at considerable distances from their point of release from the schistosome egg.

Highly important findings were made concerning the pathogenesis of huma schistosome infection based on quantitative data obtained from perfusion of cases autopsied at the Hospital Professor Edgard Santos in Bahia, Brazil. The study has revealed a number of parasitologic differences between patients with and without disease, i.e., with or without Symmer's fibrosis. Patients with Symmer's fibrosis often have heavy infections, and many of those with lower worm burdens have definite or presumptive evidence of previous treatment. Egg distribution is distinctive in cases of Symmer's fibrosis. The majority are found in the small intestine, and rectal tissues contain very few eggs. The opposite is true in cases without evident anatomic disease. In Symmer's cases, eggs in the liver are concentrated in the portal areas, whereas in the other cases they are distributed randomly within the liver. Approximately 8% of "asymptomatic" cases have worm burdens comparable to those of the more heavily infected Symmer's cases. The factors determining the presence or absence of disease are not clear.

Collaborative studies on the effect of single monthly injections of Astiban to village populations in Egypt were continued. These studies have shown that urinary shedding of ova of *S. hematobium* ceased in about 90 percent of recipients under such therapy and had not resumed in any significant number of victims during a six month period after therapy was stopped. These observations indicate that the drug in this dosage has both chemoprophylactic and chemotherapeutic properties and the method would seem to be suitable for mass treatment of populations in hyperendemic areas.

In Puerto Rico it was shown that a slow continuous infusion or a series of closely spaced small injections of Stibophen was better tolerated by the patient and therapeutically more effective than were larger, more widely spaced injections. Very similar observations were made with Astiban (sodium antimony dimercapto succinate).

Amebiasis and Trichomoniasis

With the aid of a sensitive microfluorimeter and measuring techniques developed in this laboratory, it has been possible for the first time to demonstrate a spectrum of antigenicity in some 16 strains or lines of *Entamoeba histolytica* growing in association with a mixed bacterial flora. It has also been shown that antigenic differences exist between 3 strains of *Trichomonas*, two of them derived from a single source, but maintained under different conditions for 18 months.

Fluorescent antibody serology has confirmed a high incidence of amebic liver abcess in patients seen at the hospital in Hyderabad, India. In addition, about 55% of cases with the vague symptomatology of chronic intestinal or hepatic amebiasis" are positive in the FA test for amebiasis, compared to 30% of patients without such clinical signs. There is considerable serologic evidence accumulating that the amebiasis picture in countries such as the United States cannot be compared in any realistic manner to that in an endemic area such as India. There has been no correlation between positive serologies and infection with *E. hartmanni*, so-called "small race *E. histolytica*," thus supporting the view that this is a separate species from *E. histolytica*.

Continued improvement has been made in the medium used for the axenic cultivation of *E. histolytica*. Considerable variation was encountered in the ability of different lots or horse serum and liver extract, respectively, to support growth. These axenic cultures have for the first time made available a system for the testing of drugs *directly* against amoebae. Prior tests conducted on amoebae grown in association with microbial flora were always inconclusive because of the possibility that the drug

acted on the microbes which served as food for the amoebae. Humatin (Paromomycin sulfate) killed axenic amoebae at levels of 8 and 16 micrograms/ml medium.

In vitro synthesis of polyamino acids was demonstrated using subcellular fractions of axenic *E. histolytica* under controlled conditions. Tetracycline, an antibiotic apparently without an effect on the mammalian protein syntehtic machinery, was markedly inhibitory. This provides the first direct evidence that the therapeutic action of tetracycline in amebiasis is specifically on the amebae and not on the associated bacteria, and affords a clue as to its mechanism of action. Polyribosomes from the amoebae, constitute the first identification of these biochemical structures in a protozoan cell.

Glucose and Glycerol Utilization in Parasites

Comparative studies of glucose and glycerol uptake by the larval and adult forms of *Taenia teaeniaeformis*, and by different species of trypanosomes under various conditions have revealed interesting similarities and differences. The larva absorbs more glucerol aerobically than anaerobically, a difference lacking in the adult worm. For the larva, an inverse relation exists between concentration of environmental glycerol and glucose leakage. In the absence of Na^+, marked inhibition of glucerol and glucose uptake occurs, the effect being more pronounced on the latter. Phloridzin completely inhibits glucose uptake, but has no effect on that of glycerol. The data suggest that at very low environmental glycerol concentrations, absorption involves active transport mechanisms, while at higher concentrations diffusion, probably facilitated diffusion, appears to prevail.

Of 8 species of trypanosomes studied, only *T. gambiense* and *T. rhodesiense* consumed large amounts of glucerol. When *T. gambiense* was kept in a mixture containing equal amounts of glycerol and glucose, definite mutual inhibition of absorption was observed. In addition, other differences between tapeworms and *T. gambiense* were found to exist in respect to extraneous influences.

Laboratory of Parasite Chemotherapy

The major research effort of the laboratory continued to be centered on problems in malaria. Resistance of strains of falciparum malaria to all commonly used synthetic antimalarial compounds remains a severe problem, particularly in Vietnam and other areas of Southeast Asia. The need for new and effective drug regimens to combat this problem has dictated continuation and expansion of this area of research. Further studies on the many host-parasite-vector combinations now available in the simian malarias have contributed much to our program of evaluation of antimalarial agents and to our understanding of the biology of the parasite in the primate host.

Malaria—Human

Studies continued in the clinical testing of antimalarial drugs in human volunteers, with emphasis on strains of parasites resistant to certain compounds and on possible methods for overcoming the resistance problem. Activity of cycloguanil pamoate (CI–501), the injectible long-acting antimalarial, and that of CI–564, a combination of CI–501 and DADDS (the acetylated diaminodiphenyl sulfone), was evaluated against strains of vivax and falciparum malarias resistant to chlorguanide, the parent compound of CI–501. While CI–564 was effective against the normal strain of *Plasmodium vivax*, when the strain had been made chlorguanide-resistant the drug had only minimal antimalarial activity. Trails of CI–564 against multiresistant strains of *P. falciparum* were encouraging. Although these strains were resistant to normal doses of chlorguanide or CI–501, in most cases volunteers were success fully protected from or cleared of infection when CI–564 was used as a suppressive or therapeutic agent.

Four additional strains of drug-resistant falciparum malaria were isolated and studied in volunteers. These strains were all from Southeast Asia and included one from Thailand (Thai II), one from Malaysia (Malayan IV) and two from South Vietnam (SV–1 and SV–II). The first three strains have been extensively evaluated and all possess a wide spec-

trum of resistance, being refractory to chloroquine, chloroguanide and mepacrine. The SV–I strain appeared to be sensitive to 50 mg. of pyrimethamine, but the others were also resistant to this compound. While quinine sulfate was curative when given as a 5-day course of treatment in cases of Thai II and Malayan IV, THE SV–I strain appears to be resistant to a 10-day course of quinine, requiring 14 days of this drug for a cure. The SV–II strain is a more recent isolate, and was derived from an infection which had recrudesced after extensive therapy with quinine (10 grains t.i.d. X 14 days, followed by 5 grains t.i.d. for 6 weeks). This strain is resistant to chloroquine and chlorguanide.

In line with the urgent need for alternative methods of treatment of the resistant strains, studies have been done on the usefulness of sulfonamides, either alone or in combination with pyrimethamine. Forty volunteers have participated in the evaluation of thee sulfonamides: sulfadiazine, sulfamethoxypyridazine (Midicel), and sulforthodimethoxine Fanasil). The Malayan III strain of *P. falciparum* was used for the initial studies; it is resistant to all synthetic antimalarials tested, but sensitive to the 5-day treatment with quinine sulfate. Sulfadiazine was given in a 5-day course of treatment, while the longer acting compounds. (Midicel and Franasil) were given as single doses. Results indicated varying degrees of antimalarial activity for all three of the sulfonamides when used alone, but that none was completely reliable for the termination of these malarias. When pyrimethamine (as a single dose) was given concurrently with the sulfonamides the curative effect was significantly enhanced. A pyrimethamine-Fanasil combination was evaluated against three additional resistant strains: Thai II, Malayan IV and South Vietnam I. In a total of 24 volunteers treated, this combination was successful in effecting a cure in 21.

Studies have continued on the West African strain of *P. ovale* to characterize the clinical and parasitological patterns of this infection in volunteers. Contrary to prior descriptions, ovale malaria appears to present moderately severe clinical manifestations. The strain responds readily to standard antimalarial regimens. A single dose of 10 grains of quinine sulfate successfully clears patent paraistemias in about four days. Observations on relapse patterns of quinine-treated, mosquito-inoculated cases indicate a wide range of intervals between initial and subsequent attacks. While relapse has been seen as early as 17 days after treatment of the primary attack, of particular interest are the extended latent periods sometimes seen. In one case the interval between primary attack and first relapse was 255 days.

Malaria—Simian

Studies on simian malaria as a possible zoonotic disease of man have been conducted on several fronts. A new strain of *Plasmodium brasilianum*, the South American quartan parasite of monkeys, has been transmitted to volunteers through the bites of infected mosquitoes. The infectionin man was extremely mild toes. The infection in man was extremely mild. man to man through subinoculation of infected blood. Similarly, the quartan parasite of monkeys of Southeast Asia, *P. inui*, has been successfully transmitted to volunteers through the bites of infected mosquitoes. Clinical manifestations were mild. The infections were self-limiting and antimalarial intervention not required. Similar exposure of volunteers to mosquitoes infected with *P. gonderi, P. Coatneyi, P. fieldi*, and several additional strains of *P. cynomolgi* have not resulted in detectable parasitemias.

Of great interest has been the isolation of a strain of *P. knowlesi* from a person recently returned from Malaya. This strain has been studied in 8 volunteers and in rhesus monkeys. The infection is characterized in man by moderately severe clinical manifestations. The fever pattern is quotidian, with fever as high as 104.8° F. The maximum parasite count has been about 20,000 per cmm of blood. The infection is generally self-limiting, but antimalarial intervention has been deemed advisable in three cases. It has been possible to infect monkeys through mosquitoes fed on human carriers. The course of infection in monkeys is generally very severe, almost in-

variably terminating fatally in the absence of antimalarial intervention. This is the first documented case of a human acquiring a monkey malaria under natural conditions and definitely establishes it as at least an occasional zoonosis. To follow this lead, studies were re-established in Malaysia, particularly in the area from which this case was known to have originated, to determine the extent to which this zoonosis might exist as a real problem. Results of this limited study are not yet complete; however, it seems evident that, although this zoonosis may occur occasionally, it is not a significant problem in the overall consideration of malaria in these areas at this point in time.

Extensive studies on the exoerythrocytic stages of the simian malarias have been initiated, primarily to provide a reliable system for the evaluation of antimalarial compounds against these cryptic forms, but also to expand our knowledge of the entire life cycle of the primate malarias. Techniques have been devised for the direct massive intrahepatic inoculation of sporozoites, which enhances the location and identification of these stages on subsequent biopsy. Using this method it has been possible to demonstrate developmental exoerythrocytic stages of *P. coatneyi, P. fieldi, P. knowlesie, P. brasilianum* and several strains each of *P. inui* and *P. cynomolgi*. Preliminary studies are under way to determine the activity of known antimalarial agents against these forms.

Immunological Studies

The availability of a large number of human, simian, avian and rodent malarias has made possible continuation of studies on the development and persistance of specific antibody, as determined by the fluorescent antibody (FA) method and methods of immunoelectrophoresis. Monkeys infected with various species of malaria normally develop antibody response to a very high level within three weeks after infection. These levels seldom increase, even though the parasitemia persists for extended periods.

FA tests have been conducted on several large series of serum samples from Nigeria, Upper Volta and Liberia (West Africa). These era were tested against five *Plasmodium* antigens and the responses, in general, indicate an increase in titer with age and higher titers in males than infemales. In all groups under 13 years of age, the highest response was to the *P. falciparum* antigen. Those over 13 years of age had equal mean titers to the *P. falciparum* and the *P. brasilianium* antigens, the latter species being an indicator of previous *P. malariae* infection.

Examination of sera from individuals infected more than 10 years previously with *P. vivax* (during the renowned Campfire Girls epidemic in California) indicated that, although the FA responses were weak, they could be distinguished in most cases from persons of the same age who had not experienced malaria.

Antigenic analyses of the simian malarias, are being done preliminary to use of parasiten or their components in experiments on immunization. Electrophoretic analysis reveals a minimum of four or five distinct components. Immunoglobulin fractions have been collected from 10 strains or species and are being used, along with whole parasite extracts, for immunization of animals.

LABORATORY OF IMMUNOLOGY

Autoimmunity

Immunofluorescence studies have established that serum immunoglobulins from patients with myasthenia gravis react *in vitro* with striated muscle of a wide variety of both vertebrate and non-vertebrate species. The occurrence of striated cells in the thymus of several vertebrate species has been confirmed; reciprocal absorption studies with striated muscle and thymus have suggested common antigenic determinants in these tissues. By electron microscopy it was established that there are thymic cells possessing the sarcomeric and myofilamentous structure of striated muscle. In collaboration with NINDB it was shown that the occurrence of these immunoglobulins in human sera is correlated with the occurrence of thymomas but that in the thymomas studied, there was no correlation with myasthenia gravis.

Thyroid gland transplants have been made into deep intramuscular sites in inbred Strain 13 guinea pigs. A significant number of the Strain 13 isografts of normal thyroids showed evidence of mononuclear cell infiltrates some weeks later. This partial rejection of normal thyroid isografts constitutes an indication that skin grafts may not be a wholly adequate indicator of histocompatibility of other tissues or organs. Some of the findings on lymph node transfers given in a previous annual report have also been consistent with such a possibility.

In view of the age and familial influences on certain human demyelinating diseases, and prior experiments on experimental autoimmune encephalomyelitis (EAE) in guinea pigs, studies on EAE were extended to primates. Guinea pig spinal cord in complete Freund's adjuvant was administered to Rhesus monkeys of various ages as a single injection. Animals ranging in age from laboratory-bred newborns, infants, and juveniles to fully mature adults proved susceptible to allergic encephalomyelitis. Of 22 animals tested, all developed marked neurological disorders and only two survived the disease. Severe inflammatory lesions were found in the central nervous system. Hemorrhagic retinopathy preceded or accompanied the clinical and neurological symptoms in nearly all cases. Since in neonates and prematures the onset of clinical manifestations was delayed and took a more prolonged course and the pattern and distribution of brain lesions differed between newborn and older animals, there is an age-dependent factor in this disease in monkeys.

Transplantation Immunology

Pretreatment of donor mice with any of wide variety of antigens apparently unrelated to transplantation antigens leads to markedly diminished capacity of donor lymphoid cells to evoke in recipient mice fatal graft-vs-host reactions across H–2 histocompatibility barriers. Alterations in donor spleen cells were not a reflection of coexistent lymphoid hyperplasia induced by some antigens, rather reduced donor cell immunocompetence appeared to be related to some form of antigenic competition. Pretreatment of donors with a single antigen by no means pre-empted the available population of competent cells. Instead, it appears to impose an overall reduction in their capacity to respond to a second unrelated antigenic stimulus. This kind of modification of donor immunocompetence represents a novel, unrestricted approach to the development of transplantation tolerance in higher mammals.

Hypersensitivity

The availability of inbred strains of guinea pigs, a species in which the different facets of the immune response are best known, has been utilized as a unique resource for analysis of the mechanisms and genetics of hypersensitivity. Use of chemically defined synthetic polypeptides for this purpose is advantageous in that the specificity of the resulting immune response is assured by the known chemical composition and the hereditarily determined capacity to respond against these structures. The presence of lysine residues in otherwise antigenic copolymer synthetic polypeptides consistently results in the lack of antigenicity for Strain 13 guinea pigs; immune response in Strain 2 pigs is normal. These findings open the way for investigation of issues such as whether a peptide need be antigenic in a given host in order to evoke tolerance and to affect the response to another antigen.

Mitogens

The plant mitogens from the red kidney bean *Phaseolus vulgaris* and from the root of the pokeweed *Phytolacca americanus*, each of which produce distinctive immunologic and biochemical transformation of human peripheral lymphocytes, have been progressively purified and partially characterized. Saline extracts of these source materials display hemagglutinating, leukagglutinating and mitogenic properties. Mitogenic activity was found by different analytical methods to be associated with a single protein component. Purified mitogens proved to be glycoproteins—but displaying different biological characteristics. Distinctive chromatographic and electrophoretic mobilities for the kidney bean and the pokeweed mitogens were indicative of separate and distinc-

tive structures. The transformation *in vitro* of peripheral blood lymphocytes by the pokeweed mitogens involves two transformed cell types: a large blast-like cell indistinguishable from that seen in *in vitro* transformation by the kidney bean mitogen and cell type at present seen in mitogenic transformation, with features resembling early antibody-forming cells. Histochemical and radioautographic studies on pokeweed-stimulated cultures revealed that 50–60 percent of the initial population of cells were transformed by the pokeweed mitogen and that prominent among these was a distinctive cell type with cytologic characteristics resembling plasma cells.

ROCKY MOUNTAIN LABORATORY HAMILTON, MONT.

Selected Zoonoses of Regional Importance

Rabies was isolated from five bats, four of which were collected in western Montana and one in Idaho. One isolate is particularly noteworthy since it was obtained from a sick bat collected in December.

The discovery that mice frequently survive after rabies infection prompted an inquiry into the role of maternal antibodies on the resistance of offspring of immune white mice. Placental transfer of antibodies could not be shown, but lacteal secretions were found to account for acquired resistance and colostrum was not more important than milk. Immunity, which increased with continued suckling, existed for more than 35 days after birth. In other studies on immunity against rabies attempts were made to characterize a rabies-virus-inhibiting substance which develops in brains of animals that have recovered from rabies. This virus-neutralizing factor did not appear after vaccination, even after live virus vaccination. As the titer of the inhibiting substance increased, the virus content of the brain tissue decreased. The ability to distinguish recovered animals from other immunes by demonstration of this inhibitory substance enables a study of the role of abortive rabies in the natural epidemiology of the disease.

Last year it was shown that an ether extract of *F. tularenis* was a reliable skin-test antigen for the detection of past infections with this organism. This test was applied to laboratory employees who had been vaccinated with Ft. Detrick live tularemia vaccine. All individuals developed agglutinins ranging in titer from 1:20 to 1:640 but mean titers were lower than those expected after natural infection. Most vaccinated individuals reacted positively to the skin-test antigen but the average size of reaction was smaller than that arising in people who have had the natural disease.

Rickettsial Diseases

Investigations on the role of domestic animals and wildlife and their ectoparasites on the epidemiology of typhus have been continued in Egypt and South America. In Egypt serologic evidence of infection of livestock was found again this year, but serum titers were not as high. Among wildlife tested, antibodies against *Rickettsia prowazekii* have been shown, with reasonable certainty, in only two specimens of *Rattus rattus*. So far, attempts to isolate typhus rickettsiae from 250 wild rodents and ticks collected from them or domestic animals have failed. In South American studies, supported in part by the Pan American Health Organization, serologic surveys were conducted in two known endemic areas in Chile. Typhus antibodies were not detected in serums taken from cattle, sheep, goats, llamas, or burros, but 12 of 80 human serums collected near La Quiaca were positive, three of which had dominant antibodies against epidemic typhus.

In contrast to observations in Egypt and Chile, findings in Ethiopia indicate that typhus is still prevalent. Of 99 serums submitted from patients thought to have had a rickettsial infection, none reacted with *R. conori*, but 62 were positive for Q fever and 43 were positive for typhus; 19 of the 43 reacted principally against *R. prowazekii*, 7 against *R. typhi* and the remaining 17 had equal titers against both antigens. This year Dr. Reiss-Gutfreund submitted three strains of typhus to RML for confirmation of their identity. These strains, isolated in the same area from which previous isolations were made from live-

stock and ticks, were identified as follows: *R. prowazekii* (JRS) isolated from *Hyalomma truncatum* taken from cattle; *R. typhi* (ZH97) from *H. truncatum* taken from cattle; and *R. typhi* (L308) isolated from human body lice.

A burro and two goats were inoculated with the ZRS strain of *R. prowazekii* to determine the susceptibility of these hosts and to evaluate the serologic response after infection. Attempts to isolate rickettsiae from the blood of these animals failed but all developed higher antibody titers against epidemic than against endemic typhus.

Transovarial passage of *R. rickettsi* in *Dermacentor andersoni* has been investigated in ticks fed on normal and spotted-fever-immune guinea pigs. For six generations, 12 lines of *D. andersoni* fed on normal nimals transmitted the virulent R type to 100 percent of their progeny. In the same number of generations, 8 of 19 lines of *D. andersoni* fed on immune animals lost rickettsial infection. In the remaining 11 lines, 100 percent transovarial passage occurred and virulence of the R type was not changed.

Further studies were made on the immunologic and serologic responses to Q fever vaccines. A single dose of phase I or II vaccine in guinea pigs was as effective as two doses, provided the same total quantity was given. Antibody of the 19S type was produced for the first 30 days, after which 7S appeared. A dimethylsulfoxide extract of phase I antigen, which is a polysaccharide-fatty-acid complex, protected guinea pigs and induced the production of only phase I antibody.

Field trials with human volunteers of phase I and phase II vaccines have been organized under the supervision of the AFEB Commission on Rickettsial Diseases. The results so far indicate that the administration of 30 μg of Henzerling phase I vaccine in a single dose provided complete protection against 3000 $GPID_{50}$ aerosol doses. Similar results were obtained when 165 μg on Henzerling phase II vaccine was employed, but a four-fold increase in phase I agglutinins in three of the six men challenged later suggests that limited multiplication of the challenge organisms may have occurred.

Reference examination of serums by the radioisotope precipitation (RIP) test is one of the services provided by this laboratory as WHO Regional Reference Center for Human Rickettsioses. Of all serologic tests for Q fever, the RIP test is considered to be most sensitive and specific. Among various groups tested, infected rates have varied from 2% to 66%, a rate found among veterinarians in large animal practice. Clinical Q fever has not been a factor in any of the groups studied. In other representative tests 4.3% of 208 patients in tuberculosis hospitals in the Midwest and 62% of 99 persons in Ethiopia, who were thought to have had typhus, were seropositive.

Transmission of Disease Agents by Vectors

In several collaborative efforts, chiefly with NAMRU–3 in Egypt, some major segments of classification of ticks from various parts of the world were completed. Some important taxonomic studies of the New World chiggers also were undertaken. During the year, 52 new species, of which six were assigned to new genera, were described in papers published or in press.

Investigations have been continued on the genetics of a paralytigenic factor in *Dermacentor andersoni*; the 7th generation of an inbred line from British Columbia has shown the same remarkable ability to cause tick paralysis as did previous generations. In view of the suspected relationship of *Wolbachia* in *Argas arboreus* to this tick's ability to paralyze pigeons, the causal relationship of *Wolbachia* recently isolated from *D. andersoni* to tick paralysis in mammals is being investigated.

Other research in this project has been quite diverse. Studies have been continued on three strains of the Hughes virus complex isolated from *Ornithodoros denmarki* and *O. capensis*. Eastern equine encephalitis (EEE) virus was shown to survive in *Ornithonyssus bacoti* for five to seven days. In other studies involving this virus, the common mealworm, *Tenebrio molitor*, was evaluated as a research tool. The larvae, which were found to be as

susceptible as mosquitoes, yielded virus titers as high as 10^6 when held at 98° F.

Encephalitides and Tick-Born Diseases

In view of the reported isolation of WEE from naturally infected snakes, studies were conducted during an acute outbreak of WEE in northeastern Montana to determine whether snakes may acquire virus from *C. tarsalis*-bird cycle. Since 7 or 10 pools of *C. tarsalis* collected during the outbreak contained WEE virus, and 64% of 85 chickens in flocks in the immediate area had developed antibodies, the outbreak was characterized by intense virus activity. Eighteen cases in man were confirmed serologically and an estimated 300 cases occurred in horses. More than 300 snakes of five species and 110 leopard frogs were collected during the post-epidemic period and tested under various conditions supposedly conducive for the isolation of virus, but none was recovered.

Studies were renewed on the immunologic response in human volunteers vaccinated with an experimental Colorado tick fever vaccine in 1961. In March 1965, 7 of 10 individuals still had neutralizing antibodies varying in index from 32 to as high as 4,470. After skin test with the vaccine all 10 persons developed a significant rise in titer, persumably from the small amount of antigen used in the skin test.

Psittacosis-Lymphogranuloma-Trachoma (PLT)

Research on the PLT group of agents has been conducted chiefly to develop and improve methods of isolation and purification, to develop methods whereby more luxuriant growth can be obtained, and to develop more sensitive and specific diagnostic reagents. Of two new cell lines established (mouse embryo and mouse placenta) the latter was more suitable for the propagation of PLT agents.

The radioisotope precipitation (RIP) test continues to show promise as a highly sensitive technique for detecting antibodies against the PLT group. Several aspects of the test, however, require further study before it can be used as a practical tool. Since P^{32} has a relatively short half life, a new batch of antigen has to be prepared about every two months. This difficulty has been partially overcome by labeling with C^{14}-tagged adenine, but, unfortunately, only a small part of the label was incorporated into the meningopneumonitis particles. Further studies with other C^{14} components are planned to find one with a higher rate of radioisotope incorporation.

Screen tests have been conducted on various groups of human serums. Significant titers were demonstrated on the serums of 15% of 415 Montana fur trappers, 25% of 29 Dubois sheep station personnel, and 23% of 88 Northern Cheyenne Indians. The RIP test confirmed CF findings in a group of serums from 14 patients with "chronic carditis," all of whom had PLT group CF antibodies.

The significance of these interesting results is as yet unknown. Five of six serums from clinical cases of lymphogranuloma venereum (LGV) had RIP antibodies. The RIP test appears to be more sensitive than the CF test in detecting prior PLT infection. These findings suggest that PLT group infections are far more prevalent than they generally were thought to be.

Further epidemiologic study of trachoma in two boarding schools on the Northern Cheyenne Indian Reservation were conducted during the year. In March 1965, 538 students were examined—82 were thought to have trachoma. A single isolation was made from a student with stage I trachoma. Serums obtained from these students were tested by both the CF test and the RIP test. By the CF test, 25% of serums from possible cases had CF activity in dilutions of 1:4 or greater. None of these serums reacted to trachoma type-specific cell-wall antigens. In contrast, 58% of these serums reacted positively in the RIP test. The results of the RIP test also correlated well with the clinical classification of eye disease. Serologic tests of serums from a few adults with stage II or IV trachoma revealed the presence of high RIP titers in the absence of any CF activity. This finding suggests that the two tests measure different type of antibody and that the RIP antibody response is more durable than the CF response.

Chronic Progressive Viral Disease

This year another animal disease, chronic interstitial pneumonitis of sheep, was shown to be caused by a slow-growing filterable agent. In the 15 months this project has been underway typical disease appeared in four of eight sheep inoculated intravenously with filtrates of a suspension of lungs from naturally infected sheep. Three of nine sheep which received similar material directly into the right lung also developed the disease. Sheep inoculated intracerebrally with similar material have remained healthy as have goats inoculated either intravenously or directly into the lung. The extent of lymphoreticular proliferation in the lungs of sheep that died or were killed was particularly striking.

Additional results of an experiment begun in August 1962, more clearly indicate the differences in the distribution of scrapie virus during advanced disease between goats inoculated intracerebrally and those inoculated subcutaneously. In the former, virus was virtually limited to the nervous system, whereas in those inoculated subcutaneously that became infected with scrapie 19 and 21 months after inoculation, virus was found in many extraneural sites, notably lymphocytic tissues. Such wide distribution outside the nervous system has not been found so far in a goat that did not develop disease until 33 months post inoculation. Results have again indicated the importance of lymphocytic tissues in the pathogenesis of scrapie. During the first 12 months after inoculation only one of 15 goats yielded virus, a small amount in the left prescapular lymph node that drained the site of inoculation. During nine months of the second year, virus was detected in six of nine goats examined. Of 36 tissues regularly tested, only lymphocytic tissues contained detectable amounts. Twenty-nine months after inoculation, signs of clinical disease have not yet appeared in 15 remaining goats. Results obtained in this experiment are interpreted as indicating a long latent period, during which virus cannot be detected, followed by slow replication and spread of virus primarily in lymphocytic tissues.

The agent which causes encephalopathy of mink was found to pass through both 100 mμ millipore filters and 100 mμ gradacol membranes. Thus, the size of the infectious agent is well below that of known non-viral pathogens. In Pastel mink severely affected with this disease the largest amount of virus was found in the brain, with lesser amounts in various other tissues, but it could not be recovered from blood or serum. When small amounts of virus were present in the inoculum, incubation periods of over 400 days were observed.

The viremia that occurs in Aleutian disease was studied in Sapphire (an Aleutian color phase) and Pastel (a non-Aleutian color phase) mink. In Sapphire mink the virus could be recovered at biweekly intervals until death which occurred about 12 to 22 weeks after inoculation. During this period the levels of serum gamma globulin rose by 40–45% without any apparent effect on the presence of virus in the blood. In Pastel mink virus was recovered at two weeks and 12 weeks post inoculation but not thereafter through 22 weeks. Pastel mink remained free of serum protein changes and are still healthy 32 weeks after inoculation. In another experiment virus was detected in the mesenteric lymph nodes of two apparently healthy Pastel mink 22 months after inoculation. These observations on viremia indicate a clear-cut difference in the mode of virus growth in the two color phases and further emphasizes the importance of genotype in the pathogenesis of the disease.

Allergy and Immunology

Further comparisons have been made of the Hartley strain guinea pigs and strain 13 guinea pigs regarding their suitability for research on delayed hypersensitivity. Previously, strain 13 guinea pigs were shown to produce antibody less actively than did the Hartley strain. Strain 13 guinea pigs developed pure delayed hypersensitivity to egg albumin when sensitized with a conjugate of paraaminobenzoic acid (PABA) and hen egg albumin (HEA), whereas Hartley strain guinea pigs developed an immediate type of hypersensitivity. When both strains were inoculated with live BCG, they developed hypersensitivity to tuberculin but the reaction in strain 13 guinea pigs were much weaker. When these guinea pigs were

sensitized with killed Q fever phase I rickettsiae and tested several weeks later with phase I cells, reactions were similar in both strains.

Immunoprophylaxis Against Tuberculosis

Previous studies have shown conclusively that heat-treated cell walls of *Mycobacterium tuberculosis* when coated with light mineral oil are highly immunogenic and protect mice against aerosol challenge with fully virulent tubercle bacilli. Over a two-year period numerous vaccines have been prepared from 12 different batches of cell walls and tested repeatedly. Except for a few formulated with an insufficient quantity of oil, all vaccines have been highly potent in repetitive tests. Mice immunized with these cell-wall vaccines have harbored up to five logs fewer virulent mycobacteria in their lungs after aerosol challenge than did unvaccinated control mice. In contrast, mice vaccinated with the standard Rosenthal's BCG vaccine had about two logs fewer when they were cultured after aerosol challenge. None of several vegetable oils, which are metabolizable, served to enhance immunogenicity of cell-wall preparations. Both heavy and light mineral oil were effective whereas kerosene was ineffective in enhancing potency. Drakeol 6VR, which has been approved as an adjuvant in human vaccines, was found to be as satisfactory as light mineral oil. The synthetic hydrocarbon, 7-n-hexyloctadecane, was slightly superior to light mineral oil. The 7-n-hexyloctadecane had one advantage not shared by other oils, because mixtures of this substance with Tween 80 and cell walls disperse instantly in saline to give stable emulsions.

Preliminary tests were undertaken to determine whether the cell-wall vaccines contained any endotoxic factor which would be harmful if this product were used in man. Mice survived intraperitoneal inoculations with doses as high as 10 mg and 11-day-old-chick embryos were not killed by intravenously injected doses of 400 μg, the maximum dose employed in contrast, 0.25 g of cell walls from several enteric Gram-negative bacteria constitutes as average median lethal dose for chick embryos. Likewise, 20 μg of Gram-negative cell walls will evoke a typical endotoxic febrile response in rabbits whereas doses of mycobacterial cell walls as high as 1 mg did not produce significant fever.

The potency of cell-wall vaccines as tested by the aerosol challenge method was shown to be dependent upon a specific cell-wall antigen which was shared in part by related species such as *M. kansasii*, *M. avium*, *M. aquae*, and *M. butyricum* but not by species such as *Salmonella*, *Brucella*, *Listeria*, and *Corynebacterium pseudotuberculosis*. The results of these experiments also gave preliminary evidence that cell walls from H37Ra conferred a higher degree of immunity than any other preparations so far tested.

Skin tests in rabbits and guinea pigs sensitized with living or killed whole cells or cell walls of *M. tuberculosis*, *M. balnei*, group I, II, III, or IV of the unclassified Mycobacteria were performed in continuing efforts to develop more specific antigens for use in detection and differentiation of mycobacterial infections. As previously reported for *M. tuberculosis*, *M. phlei*, and *M. butyricum*, protoplasms and cell walls of these additional Mycobacteria give rise to delayed reactions when injected intradermally into sensitized rabbits or guinea pigs. The specificity of the reactions produced by cell walls and protoplasm differed. Minimal amounts of cell walls produced lesions in animals sensitized with either homologous or heterologous material. However, weaker dilutions of protoplasm which give rise to reactions in animals sensitized with homologous antigens usually would not elicit reactions in those sensitized with heterologous material.

In collaboration with Dr. Leon Schmidt, safety tests of the standard cell-wall vaccine enhanced with Drakeol have been initiated at the National Center for Primate Biology, University of California at Davis, California. Because of reactions at the site of inoculation, which were entirely unexpected, future potency tests of this product in monkeys have been delayed until further attempts are made to eliminate the factor responsible from the cell-wall vaccine. The safety test has been redesigned to provide preliminary information on the protective properties of this vaccine for monkeys.

Bordetella Pertussis Antigens

Further studies on the histamine sensitizing factor (HSF) of *B. pertussis* were directed chiefly toward finding a practical method for isolating and purifying large quantities of this substance. Reasonably pure preparations have been obtained by magnesium sulfate precipitation of HSF from alkaline saline extracts of acetone-dried cells. HSF in Dupanol sensitized mice to histamine, to serotonin, to passively and actively induced anaphylaxis, and it increased antibody production by adjuvant action. This material was also found to induce hyperacute experimental allergic encephalomyelitis (EAE) in rats that had received guinea pig cord suspensions. Thus, these preparations of reasonably pure HSF have most of the activity found in whole *B. pertussis* cells and possibly a single substance is responsible for most of these biological activities.

B. pertussis strains are known to vary tremendously in their antigenic composition, as judged by agglutination and agglutination absorption tests. Six specific antigens for *B. pertussis* have been described. No significant differences in protective or histamine sensitizing ability were found among strains containing antigens 1, 3; 1, 2, 4; 1, 2, 3, 4; or 1, 2, 3, 4, 5. If immunity can be transferred passively with antigen-specific antiserums it would be possible to identify the antigen associated with histamine sensitizing production and immunity in mice.

Endotoxins

The native hapten extracted from protoplasm of *Escherichia coli* was studied further to determine whether this substance was indeed a precursor of endotoxin. This substance was found in nine of 11 strains of *E. coli* representative of four serotypes but none could be extracted from strains of *Citrobacter freundii, Salmonella enteritidis, Salmonella typhi,* and *Serratia marascens.* The molecular weights of native haptens isolated from these strains of *E. coli* averaged about 150,000; their physical dimensions averaged about 15 Å by 1200 Å. In spite of the close serologic and chemical identity (sugar composition) of native hapten with endotoxin, it is unlikely that this material is a direct precursor of endotoxin. The physical dimensions could not conceivably be those of a subunit of endotoxin, and it does not behave like subunits derived from bile salt-dissociated endotoxin.

The results of a study of the effect of surfactants on endotoxins have provided additional data in support of the theory of a micellar structure of endotoxin. When fully active endotoxin was treated with sodium desoxycholate, subunits formed, with a molecular weight of 20,000 and dimensions of 10.5 Å by 250 Å. The dissociated endotoxin was about 100-fold less potent in the pyrogenicity test in rabbits and it was no longer capable of stimulating an antibody response in rabbits. When the bile salts were removed by dialysis, the subunits reaggregated to form endotoxin particles with a molecular weight of 500,000 and dimensions of 40 Å and 1,000 Å. The biologic activity of the dialyzed material was essentially restored. These results were consonant with the theory that the elements of endotoxin are composed of micellar aggregates of chain-like subunits. Also, it seems that the varying physical structures of isolated endotoxins reflect only the end-to-end aggregation of basic elements produced by the extraction procedure or subsequent treatments.

Microbial Proteins and Nucleic Acids

Studies have been continued on the genetic relationships between various microorganisms as determined by the degree of polynucleotide similarity. Nucleotide base sequence homology between species was determined by mixing radioisotope-labeled and sheared DNA fragments of one species with agar-embedded DNA of another species.

Recently taxonomists have suggested that two new genera, *Francisella* and *Yersinia,* be added to the family Brucellaceae. *Pasteurella tularensis* and *P. novicida* would be placed in the genus *Francisella* and *P. pestis* and *P. pseudotuberculosis* in the genus *Yersinia. P. tularensis* and *P. novicida* were found to have a strong reciprocal cross relationship but to have no genetic relationship with *P. pestis, P. pseudotuberculosis,* or *P. multocida.*

Biology of Microbial Agents in Arthropods

This new project is concerned with the development of arthropod tissue cultures, their use for biologic studies of arthropod-transmitted pathogens, and with the development and behavior of pathogens in arthropod vectors. So far, attempts to develop a serially propagating line of tick cells from metamorphosing nymphal viscera have not been successful, but some explants have survived for 240 days. Tick hemocytes cultures survived for an average of three weeks, with some retaining living cells for four months. Colorado tick fever virus was successfully grown in primary cultures of nymphal *Dermacentor andersoni*. By 21–35 days after inoculation, the virus content of cell-free media had increased by as much as three to five logs, and in one instance virus was still detected for 126 days after inoculation.

Electron microscopic study of the subcellular structure of *Rickettsia rickettsi* and *R. prowazekii* confirmed findings of previously reported studies that their internal structure closely parallels that of many bacteria. In infected ticks *R. rickettsi* were found in tissues of the hind gut, salivary glands, genital system and Malpighian tubules. Each organism was surrounded by a "halo" thought to be created by a tissue-digesting enzyme.

LABORATORY OF BACTERIAL DISEASES

Mycoplasma

Mycoplasma organisms have been repeatedly isolated from the tissues of normal mice, particularly from the brain and lung and in one instance, from blood. All of these isolates proved to be *Mycoplasma neurolypticum*. Similarly, *M. neurolyticum* also has been recovered from brain, lung, liver and enlarged lymph nodes of mice of several leukemia bearing strains, and from mice with transmissible leukemia atuopsied in the terminal stages of the disease. The possible role of *M. neurolyticum* and other mycoplasma in the origin of leukemia, and the role they may play in producing confusion and misinterpretation of experimental results in leukemia research is being explored.

Work on murine and other animal mycoplasma has led to the first identification of swine mycoplasma as tissue culture contaminants. A mycoplasma isolated from murine leukemia cells in culture by a scientist at the Sloan-Kettering Institute was shown to be related to strains of *M. granularum*, a serotype previously recovered only from swine. These studies also confirmed the work of other investigators (LID) that some previously unclassified tissue culture contaminants were strains of *M. hyorhinis*, another serotype of swine origin.

In a collaborative study with Dr. Leon Smith, St. Michaels Hospital, Newark, N.J., over 60 blood specimens from normal postpartum patients have been cultured. Only one recovery of mycoplasma was made in this series. This patient developed a low grade fever 24 hours after delivery and *M. hominis*, type 1, was recovered from the blood. There was a concomitant rise in antibody against *M. hominis* in the patient's serum. This finding provides additional evidence of the pathogenicity of *M. hominis*, type 1, for humans, and indicates the necessity of a search for these organisms in fevers of unknown origin.

In germfree guinea pigs inoculated with *Mycoplasma pneumoniae*, the organisms were recovered from the nasal passages with regularity over the six-week test period. Results with a high passage laboratory strain and with a recent human isolate were similar. There was no clinical disease nor gross pathological findings. Only low serum antibody titers developed. The germfree guinea pig does not appear to be a suitable host for the study of *M. pneumoniae*.

In collaboration with the Perinatal Research Branch, NINDB, a study is under way to determine the normal mycoplasma flora of monkeys. About 80% of the monkeys cultured have yielded mycoplasma. To date at least four types of mycoplasma have been isolated. Preliminary data indicate that at least one of these types may be similar to, or identical with, a type isolated from man.

Brucellosis

As the program for eradication of bovine brucellosis has progressed, new and unexpected problems have arisen which threaten to impede progress toward total eradication of the disease. The question has arisen whether the use of *Brucella abortus*, Strain 19 vaccine may be responsible for foci of infection in certain problem herds, and whether the vaccine strain may actually be responsible for some infections. This Laboratory is collaborating in the study of unusual strains of *Brucella* isolated from wild and domestic sources in an attempt to clarify their ecological relationships.

Studies on DNA homology in the genus *Brucella* were extended and completed. By the technique employed, DNA derived from smooth cultures of the three classical species, and from *Br. neotomae*, and rough *Br. suis*, cannot be differentiated one from another. The genus is thus highly homogenous and the S to R change was not reflected in the homology studies.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

INTRODUCTION

The following reports give some indication of the work of the NIAMD in the past year. Several features may be noted. There is a continuing increase in interest and work in the field now known as Molecular Biology. This leitmotif of biological sciences in the 1960's has expanded to embrace work in clinical departments, pathology and biochemistry, and has also influenced the direction of work in the more physical and chemical sciences. In the latter case, the ready availability and the acknowledged biological importance of synthetic or purified polynucleotides and proteins (or their monomers) has stimulated quite basic studies of their physical and chemical properties. It seems reasonably certain that we are in the midst of a revolution in biology. Part and parcel of this revolution is the increasing interest in biological materials shown by chemists, physical chemists and theoretical chemists. And with this interest and activity, there has occurred an increasingly close collaboration between physical and biological scientists. The answers to many fundamental problems in biology can only be found in the details of the chemical and physical interactions of the complex molecules found in living systems. It seems evident, therefore, that at NIH a systematic effort must be made to provide facilities for a larger number of physical scientists, including those mainly concerned with theory.

Engineering efforts to find ad hoc solutions to specific diseases cannot, it would appear, play more than a minor role in the eventual understanding of biological processes and control of their disorders. The trend in the work of the scientists in NIAMD is clearly in the direction of investigation of biological materials and processes at their most fundamental level.

It is not always apparent how many of these highly detailed and complicated studies of what seems to be very minor problems may have practical and clinical applications. Bacteriophage, for example, may seem an unusual material for scientists to work upon, as these organisms only infect bacteria. Their extreme simplicity, however, (some strains have as few as a dozen genetic units compared to a hundred thousand or more in mammalian cells) permit accurate studies on the biochemical mechanisms of transfer of genetic information. This in turn serves as a model for virus infection of animal cells. Indeed bacteriophage infection of bacteria may well have significant implications in human disease as witnessed by the fact that diphtheria bacteria only produce diphtheria toxin when they are infected with a certain bacteriophage. Hence, work in NIAMD in circular forms of DNA whose present function is unknown may well have implications far beyond bacteriophage.

On another level, the extensive studies on induction of enzyme synthesis in bacteria in NIAMD may appear unrelated to human disease. However, unless we can understand this relatively simple biological system it seems clear that we will never understand how the human organism can adapt to the extraordinarily varied diets people live upon, nor the dramatic situations in which certain cells change their pattern of adaptation and become cancerous. In fact, the best hope for eventual control of cancer in man is much more likely to derive from studies of the forces involved in the interaction of nucleotides in DNA than from empiric testing of anti-tumor drugs.

Another major problem in human disease involves congenital abnormalities. There is some worry nowadays that, with improved methods of maintaining individuals possessing deleterious genes, we may eventually approach the situation in which the population is divided only into physicians and patients. A rational approach to this problem seems to involve two factors: (1) differentiation, since errors in this process may lead to non-genetic congenital malformations and (2) mechanisms of disease production from deleterious genes. The first aspect is currently under study under defined conditions as the differentiation of the mammary gland. The hormones required, some of the biochemical reactions involved and their morphologic expressions have been elucidated. The exact role of the hormones and their mode of action, if they can be uncovered, could lead to practical approaches for preventing errors in differentiation—namely congenital anomalies.

The mechanism of the production of disease from genetic errors has already been partially clarified in many cases. In sickle cell anemia, not only the nature of the amino acid substitution which is the primary defect, but also a sensible chemical rationale for how this substitution produces the clinical picture of sickling have been advanced—largely by scientists now at NIAMD. The precise biochemical lesions in galactosemia, xanthinuria, homocystinuria, cystinuria, cystathioninuria, and gout have been elucidated over the past few years at NIAMD and elsewhere. In most cases a sensible and effective therapy has been devised after the precise metabolic defect has been discovered. Just as importantly, tests have been devised for many of these inherited defects so that the healthy carrier can be identified. With this information there is hope that a positive eugenics type approach may eventually be able to reduce the incidence of these diseases.

Many times apparently esoteric chemical procedures are developed which appear to be of only theoretical significance. Recently, however, NIAMD scientists, by separating the optical isomers of certain morphine derivatives, were able to show that one of the isomers actually antagonized the dependency effects of morphine but retained analgesic activity.

The fund of basic knowledge in the biological sciences is still immeasurably small compared to the rich and marvelously complex organization of structures and compounds that result in living cells. We can therefore anticipate that rational therapy for the serious diseases that beset mankind will await a clearer knowledge of biological processes.

LABORATORY OF NUTRITION AND ENDOCRINOLOGY

Studies on Folic Acid

The catalytic and kinetic properties of the conversion of folic acid and dihydrofolic acid to the metabolically active tetrahydro-form have been investigated utilizing the highly purified dihydrofolic reductase from liver. At neutral pH values with TPNH as the reducing agent, the enzyme is completely specific for dihydrofolate, whereas folic acid and DPNH are inert as substrates. However, in the acid pH range, 4–5, both folic and dihydrofolic acid serve as substrates and DPNH may serve as the reducing agent.

Kinetic analysis of the reaction indicated that the apparent affinity of dihydrofolic reductase for dihydrofolate is remarkably high. In addition, the interaction of dihydrofolate with the enzyme does not follow the expected "normal" kinetic behavior with respect to the velocity of the reaction *vs.* dihydrofolate concentration. On the other hand, the interaction of folic acid with the enzyme exhibits "normal" kinetics and the analysis suggests that folic acid is bound to the enzyme some 50 times less strongly than dihydrofolic acid. The interaction of TPNH with the enzyme exhibits "normal" kinetic behavior at neutral pH when dihydrofolate is used as substrate. However, at acid pH values "abnormal" kinetics are noted using either dihydrofolate or folate. In addition, TPN was observed to be a potent inhibitor of the reductase reaction. Kinetic analysis demonstrated that the apparent affinity of the enzyme for TPN was almost the same as for TPNH. This is in line with the

observation that TPN as well as TPNH is an effective stabilizing agent for the enzyme.

Previously reported studies which described a marked activation of this enzyme by urea and organic mercurials suggested that dihydrofolic reductase possesses many of the properties of an "allosteric" enzyme. From this point of view, the "abnormal" behavior of the reaction with respect to dihydrofolate is not surprising since many of the "allosteric" enzymes exhibit this type of "abnormal" kinetics. Thus the "normal" kinetic behavior observed when folic acid is used as the substrate correlates with the lack of significant stimulation of the enzyme by both urea and the organic mercurials in the presence of the fully oxidized folate, pteroylglutamic acid.

The studies on the more complex and as yet unidentified forms of folic acid in tissues were directed to the stockpiling of a stable concentrate and the development of chromatographic techniques to be used in further purification procedures to yield several components as pure compounds. (Drs. B. T. Kaufman and J. C. Keresztesy)

Large-scale Processing of Biological Materials

The large-scale laboratory continues to assist NIH investigators in procedures which require processing and preparing materials in the volume too large for normal laboratory facilities. Fermentation operations were again this year in greatest demand. (D. L. Rogerson, Jr. and Dr. J. C. Keresztesy)

Studies in Experimental Nutrition

The studies on the development of liver necrosis in rats as influenced by various dietary components and state of "germfreeness" have been continued. It was found that many of the test animals may die without showing any, or very little, obvious damage to the liver cells. Other parameters for assessing liver damage are being explored.

The possible role of the intestinal flora in the prevention of blacktongue in dogs was investigated. The antibiotic, terramycin, was without effect. The administration of mixtures of antibiotics, together with the isolation techniques, are under study to influence intestinal flora in rats and dogs which will prove to be beneficial to the host. (Dr. F. S. Daft and E. G. McDaniel)

Study of Protein Hormones

Modifications of the structure of thyroid stimulating hormone (TSH), whether by enzymic or chemical means destroy both biological and immunological activity. Physicochemical studies with bovine growth hormone have shown that in alkaline solution a monomeric form exists which again dimerizes upon acidification. (Dr. P. G. Condliffe)

Diabetes and Fat Metabolism

The induction of diabetes in rats, by hormones from a transplantable pituitary tumor, was studied. Partial pancreatectomy was necessary to reduce the antidiabetic effect of endogeneous insulin from the host's pancreas. Based on this finding partially pancreatectomized rats have been used to quantitatively study the hormonal interactions of ACTH and growth hormone upon the induction of diabetes. A synergism was demonstrated. Immunoassays of insulin in blood showed that the hormonally induced diabetes is not due to a fall in insulin level for actually the insulin level increased. (Dr. R. W. Bates)

The metabolism of the diaphragm and fat tissue from rats after the induction of diabetes, by operative procedures, were investigated. The diaphragm muscle *in vitro* responded normally to insulin but the adipose tissue was more insensitive to insulin. This latter partially accounts for the *in vivo* peripheral resistance to insulin found in some diabetics. (Dr. S. S. Chernick)

Mechanism of Action on Isolated Fat Cells

Using individual fat cells, it was demonstrated that many of the effects of hormones on the fat cells are at the level of the cell membrane. The hormones modify the permeability of the wall either directly or indirectly, e.g., lipolytic hormones release fatty acids which will alter cell permeability. The cell membrane may be altered sufficiently so that intracellu-

lar enzymes are released to the medium. These effects are believed to be mediated by effects on the phospholipids in the cell wall. (Dr. M. Rodbell)

Metabolism and Function of Fat Soluble Vitamins and Related Substances

Lipid analyses of testes from eight species of animals including man showed characteristic fatty acid patterns, particularly in the polyunsaturated acids, for each species. These differences could not be explained soley by the varying diets. Human testis had the highest content of the most unsaturated fatty acid (22 carbons, 6 double bonds) found in tissues.

A comparison of the lipid changes which occur in rat testes damaged by deficiencies of vitamins A and E and also of zinc revealed that the most marked loss of phospholipid and alteration of the proportions of polyunsaturated fatty acids occurred in vitamin E deficiency. These results suggest a special involvement of vitamin E in lipid metabolism in the testis.

The biopotencies and metabolism of the amine and N-methyl amine analogs of tocopherols we studied in chicks. α-tocopheramine was equally as active as standard α-tocopherol whereas β-tocopheramine (like β-tocopherol) was about one-third as active. N-methyl-β-tocopheramine, however, was fully as active as β-tocopherol as was also N-methyl-γ-tocopheramine. The tocopheramines were recovered unchanged from the blood and liver and were not converted to their tocopherols or quinones. (Dr. J. C. Bieri)

Reversible Chemical Modifications of Cytochrome

Salicylaldehyde was found to react specifically with the lysine residues of cytochrome c to form a complex with reduced activity in the NADH reductase system and in the oxidation of ascorbate. Decrease of activity and also of solubility was proportional to the number of lysine groups involved (up to a total of 19 per molecule). Dialysis of the complex removed all salicylaldehyde and restored solubility and the ability to oxidize ascorbate but did not restore NADH-reductase activity until treatment with acid or alkali. The latter observation suggested the existence of polymers and chromatographic evidence for at least eight molecular species was obtained. (Dr. J. N. Williams)

Relationship Between Purine-Pyrimidine Balance and Hepatic Fat Metabolism

Perfusion studies comparing livers from normal and orotic acid-fed rats have shown that the normal liver synthesizes and secretes into the perfusate ample quantities of fatty acids. The liver from the orotic acid-fed rat has a reduced rate of fatty acid synthesis but more strikingly there is an almost complete block in lipid secretion into the perfusate. Further evidence that orotic acid interferes with lipoprotein formation was obtained by immuno- and paper electrophoretic analysis. β-lipoproteins were absent and α-lipoproteins significantly reduced in plasma from rats fed orotic acid. The disappearance of plasma β-lipoproteins was complete one week after administering orotic acid but the effect was reversed by feeding adenine for only one or two days. (Dr. H. G. Windmueller)

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Polysaccharide Metabolism

Bacterial Systems

Work on the 3-amino sugar isolated from *Xanthomonas campestris* has been completed. The crystalline preparation has been identified unequivocally as 3-acetamido-3,6-dideoxy-D-galactose. Studies directed toward localization of this compound in the bacteria have been similarly completed with the demonstration that most, if not all, of the amino sugar is covalently bound to a second rare sugar, 6-deoxy-D-mannose, and is recoverable from the cell wall lipopolysaccharide. In addition, a sensitive and highly specific color reaction has been developed which now permits the ready identification of N-acetylated-3-amino sugars in biological preparations. (Dr. G. Ashwell)

Current studies on the mechanism of bacterial polysaccharide biosynthesis have resulted

in the isolation of polysaccharide-deficient mutants. Work with these organisms has now provided the first preliminary evidence for the role of a lipid intermediate in exocellular polysaccharide formation. (Drs. J. Hickman and G. G. Ashwell)

Studies on the mechanism of deoxy sugar formation, utilizing tritiated TDP-glucose labeled specifically on carbon 3, have been completed. This work has afforded new insight into the complex rearrangement accompanying deoxy sugar biosynthesis with the surprising demonstration that tritium from carbon 3 migrates uniquely and quantitatively to carbons 2 and 6. Extension of this work is in progress whereby a new procedure is being developed for the chemical synthesis of tritiated glucose labeled specifically in carbon 4. (Dr. O. Gabriel)

Animal Systems

The conversion of glucose-6-P to inositol occurs in at least two steps. The first is a DPN-dependent cyclization of glucose-6-P to D-myo-inositol-1-P, and the second is a Mg^{++}-dependent dephosphorylation to inositol. D-myo-inositol-1-P, observed in nature for the first time during these studies, was isolated as a crystalline compound after incubation of a dialyzed extract of rat testis with glucose-6-P in the presence of DPN, but in the absence of Mg^{++}.

The two steps in the formation of inositol are catalyzed by different enzymes, a cyclase and a phosphatase, separable on the basis of the fact that the cyclase is more heat labile than the phosphatase. Phosphatase free from cyclase has been prepared, but cyclase free from phosphatase has not yet been prepared. The phosphatase is highly specific for inositol monophosphates with equatorial conformation. Since the enzyme will not cleave glucose-6-P it can serve as an assay reagent for cyclase activity measured in terms of liberation of P_i from D-myo-inositol-1-P.

Testis is 10–100 times as active as other tissues with respect to cyclase activity, while many tissues are at least as active as testis with respect to the phosphatase. (Dr. F. Eisenberg, Jr., and Mr. A. H. Bolden)

Galactogen, a polysaccharide present in the albumen gland of the snail, *Helix pomatia*, consists of D- and L-galactose units in a ratio of 6–7:1. GDP-L-galactose has been isolated from an ethanol extract of these glands and is presumed to be the precursor of L-galactose present in the polysaccharide. This nucleotide derivative of L-galactose is formed by the epimerization of GDP-D-mannose. (Drs. E. F. Neufeld and E. M. Goudsmit)

Glycoprotein Studies

Antibodies

The microsome-associated antibody that can be derived from the lymph nodes of immunized guinea pigs is being studied in order to determine the relationship of the microsome-antibody complex to antibody synthesis and secretion. Specifically, microsomes derived from guinea pigs immunized with dinitrophenyl-protein conjugates are complexed with antibodies, selective for the dinitrophenyl determinant, which can be specifically displaced by soluble normal gamma-globulins. Heavy chains derived from normal gamma-blobulins readily displaced microsomal-anti-dinitrophenyl antibody from the microsome while the light chains were essentially inactive. Since all the covalently linked carbohydrate of gamma-globulin is known to reside in the heavy chains the role of the oligosaccharide moiety was examined. Brief treatment of gamma-globulin with dilute sodium metaperiodate led to the isolation of a product that at ⅕ the concentration of untreated gamma-globulin was equally efficacious in the displacement assay. Thus far analysis has revealed that only the fucose and sialic acid are lost by periodate oxidation. A generalized nonspecific effect of periodate has been ruled out.

When rabbit gamma-globulins are digested with pepsin at pH 4.5 at least five subfractions can be chromatographically resolved. An unresolved digest of this kind was active in dissociating anti-dinitrophenyl-antibody from the microsome. The component(s) responsible is as yet unknown.

The nature of the microsomal binding site of the anti-dinitrophenyl-antibody is to be

analyzed by further studies of sub-fractions of normal gamma-globulins capable of displacing the specific antibody. (Drs. M. Kern and R. W. Swenson)

Studies on Liver, Oviduct and Mastocytoma Glycoproteins

Designed to study the mechanism by which sugar residues become attached to proteins. Many important secretory proteins contain covalently bound sugars. Among them are a number of hormones, most plasma proteins, blood group substances and epithelian secretions. The mode of glycosylation of these substances is of obvious importance.

The transfer of N-acetylneuraminic acid-C^{14} from its CMP derivative to an acceptor associated with liver microsomes was examined. The transfer product was solubilized by sonication and subjected to immunoelectrophoresis. The endogenous microsomal acceptor was found to give precipitin lines with antiserum prepared against rat serum, and is thereby tentatively identified as a mixture of precursors of plasma glycoproteins.

The transfer of xylose-C^{14} from UDP-xylose-C^{14} to endogenous acceptors was studied in particulate preparations from oviduct of laying hens and in soluble enzyme preparations from mouse ascitic mast cell tumor P–815. Xylose was transferred to serine residues of an endogenous protein acceptor by one of the enzyme fractions obtained from mastocytoma. This occurred in the absence of protein synthesis. Thus it is concluded that glycosylation of proteins begins after completion of the polypeptide. While this concept has generally been accepted for the addition of sugars far removed from the polypeptide, this is the first demonstration involving the sugar adjacent to an amino acid residue.

Besides catalyzing the formation of xylosyl-serine linkages, oviduct and mast cell tumor preparations transfer xylose to another, as yet unidentified, site, as evidenced by the formation of alkali-stable, protein-bound xylose. (Drs. E. F. Neufeld, E. E. Grebner, P. J. O'Brien, and Mrs. C. W. Hall)

Ribonucleic Acid

Studies on RNase II

The potassium-activated phosphodiesterase (RNase II) of *E. coli* was previously shown to be an exonuclease. The direction of hydrolysis of polyribonucleotides by the enzyme has been investigated. Exonucleolytic attack can begin at that end of the molecule bearing an unesterified C-3′-hydroxyl group, or, alternatively, at that end bearing a C-5′-phosphomonoester group. Furthermore, there are strong indications that the enzyme tends to hydrolyze a given chain more or less completely before going on to degrade another chain. That is to say, one chain is degraded at a time, rather than random hydrolysis of all the available chains. In order to study the possible role of RNase II in the degradation of messenger RNA, an *in vitro* protein synthesizing system that is free of detectable ribonucleases has been developed. The system uses purified aminoacyl-transfer RNA, a soluble fraction from *Lactobacillus arabinosus* as a source of transfer enzymes, and nuclease free *E. coli* ribosomes. In order to have ribosomes free of RNase I, a mutant devoid of that enzyme is used as a source of ribosomes. A technique to free these ribosomes of adsorbed RNase II was developed. This method depends on the sedimentation of ribosomes through a gradient of sucrose on a preparative scale. The ability of purified RNase II to degrade synthetic messenger RNA (i.e., poly U) when it is combined with ribosomes was then investigated. Little protection against degradation is afforded. In the course of these investigations a new method for the detection and preparation of poly U-ribosome complexes was discovered. The method depends on the fact that, under certain conditions, polyacrylamide gels bind-free poly U, while poly U bound to ribosomes passes through the column with the ribosomes. (Drs. M. F. Singer, N. G. Nossal, N. M. Thanassi, and D. M. Logan)

Studies on RNase I

Release of RNase I from *E. coli* occurs to the extent of 50% as a result of osmotic shock. This probably represents the membrane-bound portion of the nuclease. The same procedure

removes almost all of DNA endonuclease. Since these cells remain viable, they can be used for biological experiments. Encouraging results have been obtained on the infection of cells with isolated DNA. (Drs. N. G. Nossal, Y. Anraku, A. Weissbach, and L. A. Heppel)

Thionucleotide Studies

Biosynthesis of thionucleotides (M. N. Lipsett, A. Peterkofsky, and J. S. Norton) has been studied in a cell-free system. This pyridoxal-requiring enzyme system will form 4-thiouridine from uridine residues in a preformed sRNA chain by transferring sulfur from a cystine donor. At least two other thionucleotides are also produced. This reaction represents a new type of structural modification introduced at the sRNA level, comparable to methylation and the pseudouridine rearrangement.

A pure species of *E. coli* tyrosine sRNA has been isolated, with the help of Dr. B. P. Doctor, and shown to contain two 4-thiouridine residues per molecule. When this sRNA is converted to the disulfide state, the new disulfide bond has been shown to be intra- rather than inter-molecular. The amino acid accepting ability of this sRNA is not affected by disulfide formation. (Dr. M. N. Lipsett)

The sulfur moiety of 4-thiouridine in the sRNA molecule exhibits some but not all of the usual reactions of sulfhydryl groups. In the sRNA molecule, the hydrogen bonding of 4-thiouridine responds typically to denaturation with heat or urea. Mild oxidation of sRNA can form disulfide bonds between two such thionucleotides. These bonds are not denatured with heat or urea, but can be split with reducing agents. (Dr. M. N. Lipsett)

Biochemical Studies of Lysogeny

When the bacteriophage lambda, an inducible temperate phage, vegetatively multiplies in a host cell, it forms a new DNA exonuclease. This same type of nuclease has now been observed during the growth of the temperate phage 434 and this enzyme is being purified and studied. On the other hand, the virulent phage T4 does not produce the lambda type exonuclease. A study of other phages, such as noninducible temperate phage 299, the *B. megaterium* phages, etc., is under way to study their ability to produce new nucleases.

The linear duplex DNA of phage lambda, when it enters a lysogenic cell, is rapidly converted to a circular, nonended form. In our work, mutants of lambda unable to lysogenize a host cell, were also found to form circles after superinfection of either sensitive or lysogenic cells. Studies on the requirements for the syntheses of circular lambda DNA formed from superinfecting λ, have shown that the circular DNA can be formed from the linear form in the absence of any DNA synthesis. In addition, lysogenic induction of *E. coli* K12λ leads to the formation of a circular DNA species, which is synthesized *de novo* after induction and appears to be a nonended lambda DNA molecule. This latter DNA species comprises only 1–2% of the total DNA being synthesized.

Investigation of various mutants of phage lambda have shown that early mutants, blocked in the synthesis of lambda DNA, also cannot make circular DNA after lysogenic induction. Nevertheless, these mutants, when infecting a host cell, show a conversion of the linear lambda DNA molecule to a circular form. A survey of late mutants has shown that those which are capable of synthesizing linear lambda DNA after lysogenic induction will also make the circular DNA species. No mutant has yet been found which can only make circular DNA or linear DNA exclusively. (Drs. A. Weissbach, W. E. Pricer, Jr., A. Lipton, and L. A. Salzman)

Enzymes Near the Bacterial Surface

Surface Enzymes and their Removal by Osmotic Shock

A set of degradative enzymes is apparently located just under the cell wall and external to the protoplast membrane in *E. coli*. Evidence for this is: (1) histochemical studies (with Wetzel, Spicer) using the electron microscope; (2) the fact that enzyme activities can be measured with intact cells, although not completely; (3) the selective release of these enzymes by osmotic shock and spheroplast for-

mation while most of the proteins remain within the cell membrane. These surface enzymes occur not only in *E. coli*, but also in other gram-negative as well as in gram-positive bacteria.

Several of the released enzymes have been extensively purified from the shock fluid. These include alkaline phosphatase and a specific acid hexose phosphatase. Recently a new surface enzyme was discovered, which specifically cleaves uridine diphosphoglucose (a UDPGase). Also, a specific protein inhibitor for 5′-nucleotidase and UDPGase has been found; its role in cell regulation is being investigated. Both enzyme and inhibitor are being purified.

Osmotic shock involves exposing cells to 20% sucrose and ethylenediamine-tetraacetate (10^{-3} to 10 M). Next, the mixture is centrifuged and the cell pellet is quickly dispersed in cold water or 5×10^{-4} M $MgCl_2$. This procedure causes cells to lose 4% of their proteins as well as nucleotides and amino acid pools, but they remain viable and recover after a lag period. A group of enzymes, including phosphatases, RNase, DNase and UDPGase is selectively released. Shocked cells represent a useful biological tool. Experiments here and in other laboratories indicate temporary loss of ability to concentrate sugars. Thus, they can be used for study transport mechanisms and also the role of degradative enzymes. Further, the shocked cells have increased permeability to large molecules that ordinarily do not penetrate intact cells.

Hexose phosphatase is one of the surface enzymes released by osmotic shock. Its level in the cell was widely varied and even completely suppressed by different growth conditions. The factors that control the concentration of this enzyme are being studied. (Drs. R. W. Brockman, H. F. Dvorak, Y. Anraku, and L. A. Heppel)

Enzymatic Utilization of Model Compounds in Bacterial Systems

A group of enzymes involved in the utilization of L- and mesotartaric acid has been isolated and serves to outline the path of utilization of these compounds by strains of *Pseudomonas putida* and *Pseudomonas acidovorans*. By a series of pyridine neucleotide-linked reactions, the tartaric acids are oxidized to dihydroxyfumaric acid which, in turn, undergoes reductive decarboxylation to D-glycerate. Dihydroxyfumarate, which is in equilibrium with its keto form, a β-ketocarboxylic acid, is also readily decarboxylated spontaneously in aqueous solution to yield a mixture of tartronic semialdehyde and hydroxypyruvic acid. The last two compounds are enzymatically reduced to form D-glyceric acid. The enzymes involved have been crystallized and are being studied from the viewpoint of mechanism of catalysis. (Drs. L. D. Kohn and W. B. Jakoby)

The enzyme responsible for the formation of nicotinic acid mononucleotide (Drs. P. M. Packman and W. B. Jakoby) has been crystallized and has been shown to catalyze the following reaction, the mechanism of which is under investigation:

quinolinic acid + phosphorylpyrophosphate⟶
nicotinic mononucleotide + CO_2 + pyrophosphate.

By extracting precipitates of highly purified proteins with solutions of varying degrees of concentration, a wide variety of proteins has been crystallized. Included are proteins with a molecular weight ranging from 30,000 to 3,500,000, those with high and low polysaccharade content, proteins with a variety of catalytic function and even complexes of more than one protein. The method appears to be of general usefulness. One application of the technique (Dr. C. R. Steinman) is in the purification of an aldehyde dehydrogenase, a type of enzyme enjoying ubiquitous distribution which is being brought to the stage where it may be studied on the protein level.

Control of Phenotypic Expression of Genetic Information

The Role of Galactose in the Induction of β-galactosidase and Galactoside-permease in E. Coli

In contrast to its reported role as a catabolite repressor, low levels of galactose have been found to stimulate the induction of β-galactosidase in *E. coli* strains lacking galactokinase.

The stimulation does not occur in strains capable of rapidly metabolizing galactose. This action of galactose is due to the induction of a new permease for certain galactosides which is not induced by the usual inducers of the lac operon such as TMG, IPTG and lactose. The assay for permease has been found to be extremely sensitive to the osmolarity of the test solution; 10–20-fold increases in the sensitivity of the determination have been achieved under conditions of relatively high hypotonicity. (Dr. I. G. Leder and Mr. J. W. Perry)

Hormone-induced Differentiation of Mouse Mammary Gland in vitro

Mammary gland explants from mice in the middle of their first pregnancy were organ-cultured on synthetic media to which insulin, hydrocortisone and prolactin were added. These hormones stimulate the synthesis of casein phosphoproteins after 48 hours of culture to a rate several times greater than the initial rate of synthesis. Any of these hormones alone or in pairs elicited minimal or no stimulation. The effect is selective on the casein fraction of phosphoproteins.

The synthesis of α-lactalbumin and β-lactoglobulin is similarly dependent upon the presence of all three hormones. On the other hand, the synthesis of soluble cytoplasmic nonmilk protein derived from mammary epithelial cells is stimulated maximally by insulin alone and is believed to reflect cell proliferation. There is a coordinate increase in the synthesis of electrophoretically separated casein components throughout mammary gland development. The increase in α-lactalbumin and β-lactoglobulin synthesis is not coordinate throughout mammary gland development; as the lactational state is approached the α/β ratio increases. *In vitro* the 3-hormone combination effects stimulation of casein and whey protein synthesis of the same magnitude, with the same time course, and the ratio of whey proteins to casein synthesized corresponds well with the ratio found in mouse milk.

It has been concluded that a pool unphosphorylated or incompletely phosphorylated casein exists in mammary gland, and that phosphorylation of the polypeptide chains is a major mechanism for incorporation of phosphate into casein. Human placental lactogen has been found to have the same activity as prolactin in this system.

The selective biosynthetic responses to the hormones are parelleled by specific histological changes in the alveolar epithelium of the gland. Thus tissue cultured in the three hormone system is distinguished from tissue cultured in systems lacking one or more hormones by the characteristic orderly arrangement of epithelial cells of each alveolus, the very large nucleoli, and the accumulation of a great deal of stainable material in the alveolar lumina. The time course of all these histological and cytological changes proceeds in parallel with the synthesis of milk proteins.

Evidence has been obtained which indicates that the hormone-dependent induction of the synthesis of milk proteins requires the prior synthesis of DNA by the epithelial cells. (Drs. Y. J. Topper, F. E. Stockdale, R. W. Turkington, and D. H. Lockwood)

Reduction of Protein Disulfide Bonds by Liver Enzyme Systems

Mammalian liver is known to possess enzyme systems which catalyze the reduction of disulfide bonds in single peptides and proteins. The enzymes have been conventionally classified either as transhydrogenases (e.g., glutathione-insulin transhydrogenase, GIT) for which a small thiol may serve as primary reductant, or as reductases (e.g., glutathione reductase) for which some other small molecule (e.g., TPNH) can serve as hyrdogen donor. Studies of the action of GIT on insulin have been continued. Spectrophotometric evidence indicates that GIT can itself be reduced by GSH. The fact that such thiol-treated enzyme is strongly inhibited by sulfhydryl reagents, in contrast to untreated enzyme, supports the idea that the groups thus reduced are disulfide bonds. These observations have further suggested that a reduced, thio-containing form of the enzyme may serve as active intermediate in the action of this enzyme on insulin and other proteins. (Dr. F. Tietze)

LABORATORY OF CHEMISTRY

Section on Biochemicals Mechanisms

Electrolytic Cleavage of Tyrosyl-Peptide Bonds and the Mapping of Tertiary Structure

A novel technique has been developed for conducting electrolytic oxidation or reduction on sensitive or polyfunctional substances. A buffered solution of an enzyme is caused to flow continuously in a thin film over a rapidly rotating cathode or anode. By regulation of voltage and rate of flow, the degree of oxidation or reduction can be controlled at will. Thus, with ribonuclease at pH 5, cleavage of the peptide bonds following the three exposed tyrosines is effected specifically, while the three buried tyrosines remain intact. Similar studies on disulfide reduction are in progress. By combining the data from a series of such experiments, it should be possible to achieve partial mapping of the surface of an enzyme *in solution.* (L. A. Cohen, L. Farber, and S. Isoe)

Synthetic Models for Hydrolytic Enzymes

Whether amides acylate on nitrogen or oxygen is usually difficult to ascertain by spectroscopic methods. In the case of the tautomeric system of cycloserine, it has been virtually impossible to reach a decision; however, the problem has now been solved by means of nuclear magnetic resonance spectroscopy. The introduction of a double bond into the five-membered ring alters dihedral angles of the ring substituents to a degree sufficient to allow a distinction to be made. Thus, acetylation leads to the N-acetyl derivative, whereas tosylation occurs on oxygen. Such information is needed to help in assessing the role, if any, of the powerful nucleophile, hydroxylamine, in hydrolytic enzyme mechanisms. (L. A. Cohen, G. W. Milne, and C. R. Gunter)

Oxidation Mechanisms in Metabolic Processes

The catalytic roles of ubiquinone and Vitamin K in oxidative phosphorylation require that a pathway be available for the direct reductive cyclization of quinone to chromanols under mild conditions. It has been possible to demonstrate, *in vitro,* such a direct reduction by means of sulfhydryl groups. Dihydrolipoic acid was found to be a particularly effective reducing agent. Of special interest is the fact that this reaction is favored by non-polar media, as would exist in the lipid particles of mitochondria, the site of oxidative phosphorylation. (L. A. Cohen and M. A. Oxman)

Section on Carbohydrates

Studies Bearing on the Presumed Ester Linkage in Certain Glycoproteins

The lability toward alkali of the *N*° acetyl-*D*-galactosamine residues in ovine submaxillary gland mucoprotein has led to the suggestion that this sugar (and the sialic acid residue attached thereto) is attached to the nonpeptide-bonded carboxyl groups of glutamic and aspartic acids by an ester linkage involving C–1 of the amino sugar. Since no authentic 2-acetamido-1-O-cyl-2-deoxyhexoses were known, it became of interest to synthesize representatives of this class of substances and to examine their properties. Using techniques developed earlier by Dr. R. Harrison, Dr. T. D. Inch was able to prepare a variety of 2-acetamido-1-*O*-acyl-2-deoxyhexopyranose derivatives of the D-glucose and D-galactose series. The behavior of six 2-acetamido-1-*O*-acyl-3,4,6-tri-*O*-benzyl-2-deoxyhexopyranoses with alkali was studied. Under conditions which cleave the carbohydrate residues from ovine submaxillary gland mucoprotein at a measurable rate, these synthetic substances hydrolyze much too fast for measurement. Indeed, the substances autalyzed at a rate convenient for measurement at 50° in aqueous dioxane and in the absence of alkali. These results may be viewed as casting some doubt on the presence of ester linkages in glycoproteins.

Not only do the 2-acetamido-1-*O*-acyl-2-deoxyhexopyranoses readily hydrolyze in the absence of added catalyst but they also react with alcohols. Those having an acyloxy function at C-1 *cis* to the acetamido group at C-2 undergo simple transesterification to give the free 2-acetamido-2-deoxyhexose while those with a *trans* arrangement give a *trans* glycoside. This simple, stereospecific and remarkable reaction

has not been observed before; it is not shown by the fully acylated 2-acetamido-2-deoxyhexopyranoses. In a study of the solvolysis of 2,3,5-tri-*O*-benzyl-α-D-arabinofuranosyl chloride, 2,3-di-*O*-benzyle-5-*O*-*p* nitrobenzoyl-α-D-arabinofuranosyl chloride, and 2-*O*-benzyl-3,5-di-*O*-*p* nitrobenzoyl-α-D-arabinofuranosyl chloride, Dr. C. P. J. Glaudemans has shown that acyl groups far removed from C-1 may inhibit the solvolytic removal of groups from C-1. It is possible that the failure of fully acylated 2-acetamido-2-deoxyhexopyranoses to give glycosides when treated with alcohols may be attributed to the deactivating influence of the acyl groups at C-3, C-4, and C-6.

The readiness with which trans-2-acetamido-1-*O*-acyl-2-deoxyhexopyranoses react with alcohols suggests that analogous esters (of glutamic and aspartic acid) may serve as intermediates in the formation of the O-glycosides (of serine and threonine) which have been shown to be present in some gylcoproteins.

N,N-Diacylhexosamines and the Conformation of Amino Sugars

In the course of the above research (a new type of amino sugar derivative) the 2-diacylamino-2-deoxyhexopyranoses was discovered by Dr. Inch. An extensive investigation of the chemical behavior of *N,N*,-diacyl derivatives of the hexosamines was undertaken. Particular attention was devoted to 2-(*N*-acetylbenzamido)-2-deoxy-D-glucose. On heating in chloroform solution, this substance undergoes N— O acyl migration and gives at least three products: 1-*O*-acetyl-2-benzamido-2-deoxy-α-D-gluco-pyranose, 2-acetamido-1-*O*-benzoyl-2-deoxy-α-D-glycopyranose, and 2-acetamido-1-*O*-benzoyl-2-deoxy-α-D-glucofuranose. Benzoyl migration predominates over acetyl migration in this case.

The conformations of the ordinary acetylated forms of the common N-acelylhexosamines (the 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxyhexopyranoses of the D-glucose, D-galactose, and D-mannose series) are not apparent from the nuclear magnetic resonance spectra of these substances. In contrast, the n.m.r. spectra of the corresponding substances with two acyl groups attached to nitrogen are readily susceptible to conformational analysis. Drs. Inch and J. R. Plimmer have thereby 2-acetamido-2-deoxy-D-mannopyranose (*N*-acetyl-D-mannosamine) derivatives most probably do not exist in the C–1 conformation which is normal for 2-acetamido-2-deoxy-D-glucopyranose and D-galactopyranose.

Studies Toward the Synthesis of Polyvinyl Glycosides

In view of the fact that the serological properties of many natural substances depend upon the nature of the external carbohydrate residues rather than on the backbone structure to which they are attached, attention has been turned to the synthesis of glycosides of polyvinyl alcohol. Since glycosylation of polyvinyl alcohol is beset with numerous and apparently insuperable difficulties, the study of the synthesis of monomeric materials, the two vinyl D-glucopyranosides was undertaken. This work was initiated by Mr. T. D. Perrine in the Rocky Mountain Laboratory, Hamilton, Montana. Starting with the known 2-chlorethyl 2,-3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside, Mr. Perrine succeeded in synthesizing (by a considerable number of steps) the hitherto unknown vinyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside. Using a wholly different approach, Dr. C. P. J. Glaudemans was able to convert 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose into the two anomeric vinyl 2,3,4,6-tetra-*O*-benzyl-D-glucopyranosides and showed that these substances could be polymerized through the action of boron trifluoride. Dr. R. K. Ness was able to dibenzylate these two substituted glycosides and thus obtain, for the first time, the anomeric vinyl D-glucopyranosides. It is of some theoretical interest to note that, while the α anomer is comparatively stable to the action of alkali, the β anomer is converted into 1,6-anhydro-β-D-glucopyranose; thus the behavior of the vinyl D-glucopyranosides parallels that of the phenyl D-glucopyranosides.

Higher-Carbon Sugars

The second nonulose from the avocado, which had been identified earlier as D-*erythro*-L-*galacto*-nonulose through degradation experi-

ments, has now had its identity confirmed by two methods of synthesis. Both the natural and synthetic nonuloses were reduced to the same pair of nonitols and the structures of these were proved through oxidation with *Acetobacter suboxydans*. (H. H. Sephton)

Studies on the higher-carbon sugars and other carbohydrates in *Sedum* species and Pichi tops (*Fabriana imbricata*) were continued. (N. K. Richtmyer and E. W. Tracy)

Thio Sugars

D-Glucose diphenyl dithioacetal was prepared for the first time, and converted into phenyl l-thio-α-D-glucopyranoside and phenyl l-thio-α-D-glucofuranoside; the glucosides were then converted into the corresponding sulfones, and an α-D-glycofuranosyl phenyl sulfoxide was obtained, thought only in very small yield. (E. Zissis)

Amino Sugars

Several l-amino-l-deoxyheptitols were prepared and preliminary studies showed that some of them, at least, could be oxidized by Acetobacter suboxydans to produce 7-amino-7-deoxyheptuloses. (H. J. F. Angus)

Section on Medicinal Chemistry

Evaluation of Analgesics

Dose-range finding experiments were performed on 43 drugs most of which were from external sources. Of these, 42 were subjected to complete analgesic assay (Eddy modification of Woolfe-Macdonald hot-plate method) and acute toxicities were determined for 10. Nearly half of the 42 drugs assessed were sent to the University of Michigan for physical dependence studies in rhesus monkeys. Noteworthy results are the discovery of antagonism to morphine-like effects for two *levo*-isomers of the benzomorphan series which are strong analgesics in their own right, and of codeine-like physical dependence capacity and analgesic activity for the corresponding dextro-isomers usually inert in both respects. As each racemate had no physical dependence capacity (indicated in the 1964–1965 report), the *levo*-isomers must nullify the physical dependence property of their dextro counterparts as well as some of the pharmacologic effects of morphine. (E. L. May, N. B. Eddy, L. Atwell and W. Ness)

In Vitro Screening Methods for Anti-Inflammatory Properties

Based on the assumption that there is abnormal release of histamine through decarboxylation of histidine in the inflammatory process a commercial bacterial enzyme from *Cl. welchii* was found to decarboxylate histidine at 37°. However, aspirin, phenylbutazone and hydrocortisone had no inhibitory effect on this decarboxylation.

Albumin Coagulation. Mizushima's method, the suppression of heat-coagulation of certain globulin fractions by the standard anti-inflammatory agents, was found to be unreliable inasmuch as the solvent-buffer system which had to be used for phenylbutazone, indomethacin and hydrocortisone hemisuccinate gave almost as much suppression as the known anti-inflammatory agents.

Albumin-Trinitrobenzaldehyde Complex. The method of Whitehouse, the displacement of 2,4,6-trinitrobenzaldehyde from albumin (presumably from the terminal amino groups of lysine) by presently used anti-inflammatory agents results in a change in absorption at 425 and 526 mμ. Phenylbutazone and indomethacin were indeed positive but a definite drawback of the method is the quick deterioration of the trinitrobenzaldehyde in the buffer used. There was also too rapid change in transmission at the wavelengths used for quantitative estimations. This was considered to be the best of the three methods tried. (A. E. Jacobson)

Optical Resolutions of Benzomorphans

Because of the finding that α(−)-5,9-diethyl-2′-hydroxy-2-methyl-6,7-benzomorphan and β-(−)-2,9-dimethyl-2′-hydroxy-5-phenyl-6,7-benzomorphan are antagonists for their *dextro* counterparts and certain effects of morphine, α-2, 9-dimethyl-2′-hydroxy-5-propyl-and 5-ethyling resolved by various optically active acids.

2'hydroxy-2-methyl-6,7-benzomorphans are be-Mandelic acid appears to be superior to (+)-10-camphorsulfonic acid and (+)-3-bromocamphor-8-sulfonic acid. (E. L. May, A. E. Jacobson, and J. H. Ager)

Abnormal Stevens Rearrangement

Rearrangement of l-*p*-chlorobenzyl-trimethyl-1,2,5,6-tetrahydropyridinium chloride (PhLi) has given in addition to two expected products (2- and 4-*p*-chlorobenzyl-1,3,4-trimethyl-1,2,5,6-tetrahydropyridines), 2-*p*-chlorophenyl-1,3,3-trimethyl-4-methylenepiperidine. This third product is believed to be formed by abstraction of a benzylic proton followed by pyridine ring cleavage of the original quaternary to give an allylic carbanionpyridinium dipolar ion. Proton transfers and rotations about single bonds can lead to a different allylic carbanion which then ring closes. A comparable product is obtained when the *p*-position is unsubstituted. (A. E. Jacobson)

Rearrangement Products 9-Acetoxy- and -hydroxy-benzomorphans

Pyrolysis of 9-acetoxy- and 9-hydroxy-2,5,9-trimethyl-6,7-benzomorphan perchlorate at 185° gives a mixture of 1,2,3,4-tetrahydro-1,4,4-trimethyl-9H-indeno [2,1-b] pyridine (II), the yields and proportion depending upon pyrolysis time with short periods favoring II. As II (after isolation) could not be converted to I, a phenonium-ion intermediate from which II could be formed irreversibly and I reversibly, is implicated. Thionyl chloride treatment of 9-hydroxy-2,5-9-trimethyl-6,7-benzomorphan gave a 4% yield of II. (R. T. Parfitt, E. M. Fry and E. L. May)

A New Aromatization Method for β-Tetralones and a New Route to 2-Aminonaphthalenes

The pyrrolidine enamine of β-tetralone reacts with CNCl to give 1-cyano-β-tetralone. The same enamine with CNBr yields 1-(2-naphthyl-pyrrolidine). This has been extended to the preparation of other tertiary and secondary 2-aminonaphthalenes. The yields are uniformly good. (R. T. Parfitt)

Schiff-Bases and Oxazolidines

Condensation of N-methyl-4-piperidone (see 1964–1965 report) with 2-aminoethanols (primary and secondary) leads to reasonably stable spiro-oxazolidines with only a small percentage of tautomeric Schiff-base (in the case of pirmary amines) in equilibrium. With aromatic aldehydes only the Schiff bases exist. Both the Schiff bases and oxazolidines can be hydrogenated to substituted β-amino alcohols which are of interest in malaria and cancer chemotherapy. (J. H. Ager)

Potential Aldolase Inhibitors

Osmium tetroxide catalyzed hydroxylations of 1,4-dimethylenecyclohexanes were completed. Three diols resulting from this study are undergoing testing for antiviral activity. The preparation and characterization of 2-hydroxy-3-oxobutylphosphate have been achieved, and evidence for the isomeric 3-hydroxy-2-oxobutyl phosphate is at hand. This study is expected to point the way to increasing the accessibility of the biologically significant substances, dihydroxyacetone phosphate and glyceraldehyde phosphate hitherto accessible only as their dimethyl ketals. (J. G. Murphy)

Immunologic Studies in Clinical Hematology

Quinidine was catalytically reduced to dihydroquinidine and the latter 0-demethylated to dihydrocupreidine. This crude phenolic base was then converted to 6 aminodihydrocinchonine *via* the Bucherer reaction. Diazotization and compling with normal human serum gave a diazoprotein which has been tested for antigenic activity. (L. J. Sargent)

Dihydrocodeinone Isomers

The report of the formation of a "second" isomer during cyclization of 1,7-dibromodihydrocodeinonedihydromethine has been found to be in error. The "second" substance, recovered in less than 5% yield, is now known to be starting bromo ketone while the main product appears to be the 7-membered heterocycle re-

sulting from nitrogen closure to the 7-position rather than to the 5-position. (L. J. Sargent)

Polyethylenimine Studies

Cyanoethylation of polyethylenimine (PEI) in pyridine gives a quantitative yield of tractable material with the expected empirical formula. However, spectroscopic and other evidence indicate that this material is in part altered at the cyano groups which can be hydrolyzed to only 50% of the expected amide groups. Copper-binding studies give definite indication that PEI has a microgel-type structure. Its behavior is entirely different from that of the isomeric polyvinylamine which is probably linear. Moreover, added chloride ion gives spectral changes which, although slight, offer promise of use in determining the ratio of primary to secondary to tertiary anime groups in the polymer, in turn a measure of its branching. (T. D. Perrine)

Synthesis of 4-p-methoxy-1-vinyl-2-pyrrolidone

The material described in the 1964–1965 report as 4-*p*-methoxyphenyl-1-(2-dimethylaminoethyl)-2-pyrrolidone (I) was found to be a mixture of three products by thin-layer chromatography. Column chromatography effected separation of these products one of which was identified as I through the methiodide which was subjected to Hofmann elimination with silver oxide to give 4-*p*-methoxyphenyl-1-vinyl-2-pyrrolidone for polymerization. (W. R. Landis and T. D. Perrine)

Naphth- and Benz-3,4-dihydro-oxazines

Reaction of salicylic acid with dicylohexylcarbodiimide in a variety of nonpolar or slightly polar solvents gives a 20% yield of 3-cyclohexyl-2-cyclohexylimino-3,4-dihydro-4-0x0-2H-1,3-benzoxazine parallel with an earlier finding with 1-hydroxy-2-naphthoic acid which yielded the corresponding naphth [2,1-e]-1,3-oxazine. Lithium aluminum hydride reduction of the naphthoxazine gives 3-cyclohexyl-3, 4-dihydro-2H-naphth [2,1-e]-1,3-oxazine which is superior to aspirin, salicylamide and the 3-phenylsalicylamides as an analgesic (mouse-hot plate assay method) and whose structure was confirmed by synthesis. (E. L. May)

Section on Microanalytical Services and Instrumentation

Approximately 11,000 chemical and instrumental analyses were performed for about 150 research scientists of NIH and for several scientists in other government agencies, including Naval Medical Research Institute, Food and Drug Administration, and Department of Agriculture. These analyses, performed by 8 chemists and technicians, included many nonroutine determinations which required more than ordinary time and effort. Special assistance was given to many research workers in relation to work carried out in their own laboratories. The acquisition of a Perkin-Elmer Model 421 Infrared Spectrophotometer has improved the quality of service rendered in this area.

Section on Steroids

Study of Alkaloids from Solanum congestiflorum (natri)

The isolation of (25R)-22,26-imino-5α-cholest-22(N)-en-3β-ol (I) as the major steroidal alkaloid present in *S. congestiflorum* has led us to the study of other alkaloids present in the plant. The two others so far identified have proven to be the 16α-hydroxy-(II) and the 24-oxo (III) derivative of I. It has been found that I and II in the plant exists as glycosides with the sugars glucose, galactose and rhamnose. The isolation of the alkamine I is of considerable interest biogenetically since most of the Solanum and Veratrum alkaloids could conceivably arise from I by simple ring closure of hydroxylation and ring closure etc. Biogenetic experiments along these lines are contemplated. (Y. Sato, Y. Sato and R. Overton)

Synthesis and Reactions of Heteroisteroids

Novel heterocyclic steroids possessing biological activity are being actively sought. It was found that the lithium aluminum hydride reduction of 3′-methyl-isoxazolino [17,16d] -5-androsten-3β-ol ('65 report) affords the 16-

hydroxy-20-amino steroid in good yields. This constitutes an expedient procedure for the preparation of this intermediate. Fairly pure thiosemicarbazones of estrone, estrone methyl ether and their N-acetyl derivatives, which is said to offer some difficulty in preparation, was prepared for the Endocrinology Branch, NCI for biochemical studies. Related steroid thiosemicarbazones were prepared for antiviral activity. (Y. Sato and R. Overton)

Mass Spectrometry of Steroidal Glycosides

The trimethylsilyl ether derivative of solasodine monoglucoside prepared with SIL-PREP/$_{DMF}$ reagent gives rise to molecular ion peaks corresponding to the tetra and penta-(trimethylsilyl) ether derivatives in the mass spectrum. Studies are continuing to determine the feasibility of utilizing the method for determination of molecular weights of steroidal glycosides. (Y. Sato, Y. Sato and H. Fales)

Sterols from Parasites

Exploratory data indicate that the parasites *Trypanosoma cruzi* grown on blood free media contain cholesterol and/or ergosterol. No sterols were found in the media. (R. Overton, Y. Sato and T. von Brand)

Photoreduction of β-Estradiol

Irradiation of a solution of β-estradiol and sodium borohydride in ethanol with a high pressure mercury lamp (Vycor filter) gave 3α,17β-dihydroxy-5(10)-estrene as the major monomeric product. Two other monomers and polymeric material were also obtained. (J. A. Waters, M. Chaykovsky and B. Witkop)

Chemistry of Iresine celosia

Twelve compounds were isolated from the crude plant material. Preliminary chemical investigations were made and several fractions containing these compounds were submitted for biological evaluation (epilepsy and cancer chemotherapy). South American medical authorities have reported the value of the crude plant in treatment of certain types of these diseases. (J. A. Waters and Y. Sato)

Sterol Biosynthesis in Plants

Radioactive stigmasterol and β-sitosterol, isolated from soya bean plants following incubation with mevalonic acid-2-C^{14}, were reincubated with excised soya bean leaves. Extensive analysis by thin-layer chromatography and scanning for radioactivity indicated that stignasterol and β-sitosterol are not directly related biosynthetically via hydrogenase-dehydrogenase enzyme systems. (D. Johnson and J. A. Waters)

Metabolism of C^{14}-labeled Steroids by Adrenals from Rats with a Mammotropic Pituitary Tumor

Radioactive progesterone, corticosterone, and deoxycorticosterone respectively were incubated *in vitro* with adrenal slices from normal rats and rats subjected to long-term stress of tropic hormones of the anterior pituitary. Comparisons of the radioactive metabolite patterns, as measured by column chromatography and thin-layer techniques, has revealed significant abnormalities in steroid biogenesis by the stressed rat adrenal. The results obtained suggest that enzymatic deficiencies or stimulation in the adrenal may have resulted from the prolonged effects of ACTH, growth hormone, and prolactin. (D. Johnson and D. Francois)

Adrenal Metabolism of the Gerbil

Progesterone-4-C^{14} was incubated with gerbil adrenal tissue *in vitro* and the radioactive metabolites were analyzed by column chromatography and thin-layer techniques. Chromatographic identity with known compounds revealed the presence of 11-deoxycorticosterone, 18-hydroxy-11-deoxycorticosterone, and cortisone as progesterone metabolites. A difference in metabolite pattern was observed, depending on the use of ether or Nembutal as anesthetic. These studies indicate that the gerbil is well suited to adrenal steroid investigations and that its adrenal metabolism is different from that of the rat. (D. Francois and D. Johnson)

Studies on the Biosynthesis and Metabolism of Biogenic Amines

The neuro-transmittor, norepinephrine, arises from the amino acid, tyrosine, through the sequential action of three enzymes. The biosynthesis of norepinephrine plays an important role in the functioning of the sympathetic and central nervous system. The localization both intracellularly and grossly of this biosynthetic trio of enzymes is under investigation. (C. R. Creveling, B. Witkop and J. Daly)

Section on Metabolites

Batrachotoxin, the Strongest Venom Known

It has now been found that the major toxic principle of the Colombian arrow poison frog (Phyllobates latinasus, previously bicolor) is unstable, and even on storage in the cold room rearranges to a less active compound which according to mass spectrometry is isomeric and shows a different cracking pattern compatible with the assumption of the migration of a double bond close to the single nitrogen in the molecule. Efforts are being continued to prepare a crystalline salt or a heavy atom containing derivative of batrachotoxin or of the more stable rearrangement product. One of the major obstacles in obtaining suitable crystals was the paucity of venom. This has now been remedied by our last expedition into the Choco jungle of western Colombia, November 1965 to January 1966. Our collector, Mrs. Marte Latham, was able to amass more than 5,000 frogs (National Geographic Magazine, May 1966). Dr. John Daly has been able to improve the workup of the frog skins and has now active material equivalent to 180 mg. of crystalline batrachotoxin.

The Pharmacology and Toxicology of Batrachotoxin

The pharmacology and toxicology of batrachotoxin has received renewed attention because one individual was exposed to the venom and has suffered damage described as comparable to a mild case of poliomyelitis.

Venoms of Other Amphibians

Dr. John Daly has made a study of frogs in Panama, Venezuela, Colombia, and Puerto Rico and has noticed that frogs of the genus *Rana, Bradycephalus, Phyllomedusa, Hyla, Atelopus, Eleutherodactylus, Pleurodema, Phrynohyas, Prostherapis,* and *Dendrobates* contain toxic principles of unknown structure. The genera most closely related to *Phyllobates* are *Dendrobates* and *Prostherapis.* The examination of three species of *Prostherapis,* one additional species of *Phyllobates* and three *Dendrobates* has now established the absence of batrachotoxin, although less active steroidal alkaloids may be present.

The Synthesis and Biosynthesis of Dehydrobufotenine, the Major Indole Metabolite from the Parotid Gland of the South American Toad, Bufo Marinus

The structure of the novel tricyclic indole, dehydrobufotenine (*cf.* Marki, Robertson, and Witkop, J. Am. Chem. Soc. 1961) has now been confirmed by synthesis. The starting material, 5-benzyloxygramine, is nitrated exclusively at position 4, converted to the nitrile, and, after reduction of the nitro to the amino group, hydrolyzed by alkali to the acid and cyclized to the 6-membered lactam, which could only be reduced to the amine with diborane. The O-benzyl group was hydrogenolyzed and the resulting bis*nor*dehydrobufotenine quarternized to yield dehydrobufotenine. *Nor-* and bis*nor*dehydrobofotenine, which have also been prepared, are unstable and have not been detected in extracts of toad parotid glands. Dehydrobufotenine occurs in *Bufo marinus* and *Bufo bufo japonicus* together with the following related 5-hydroxyindoles: serotonin, N-methylserotonin, bufotenine, and bufotenidine. Tissue slices of the parotid gland of *Bufo marinus* converted tritium-labeled 5-hydroxy-DL-tryptophan, serotonin, N-methylserotonin and bufotenine to dehydrobufotenine. Optimal conversions were obtained with tritiated bufotenine. The biosynthetic pathway to dehydrobufotenine consists of decarboxylation of 5-hydroxytryptophan to serotonin, followed by consecutive N-methylations to bu-

fotenine. Finally intramolecular cycle-dehydrogenation or oxidative cyclization leads from bufotenine to dehydrobufotenine. (S. Senoh an J. W. Daly)

The Reactivity toward N-Bromosuccinimide of Tryptophan in Enzymes, Zymogen, and Inhibited Enzymes

The oxidation of tryptophan by N-bromosuccinimide (NBS) in α-chymotrypsin, acetyl-chmyotrypsin, diisoprophylphosphoryl (DIP)-chymotrypsin, N-*p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK)-inhibited-chymotrypsin, and chymotrypsinogen A was investigated over a pH range of 4.0–7.0. The native enzyme possessed 2–3 more moles of NBS-reactive tryptophan at pH 5.5-6.0 than the inhibited enzymes or the zymogen. Acetyl-chymotrypsin after deacylation behaved exactly like native chymotrypsin in its oxidizability by NBS. This suggests that the difference in reactivity of bound tryptophan is the result of a conformational change. Much smaller differences in reactivity were observed in the pH range 5.5-6.0 between trypsin, DIP-trypsin, and trypsinogen; however, a large difference was noticeable between trypsin and its complex with the inhibitor from beef pancreas, where at pH 5.0, two tryptophan equivalents in the complex were protected from oxidation by NBS. The pH effect in the reaction of NBS with proteins and the use of NBS in effecting selective modifications of proteins permits description of tertiary structure in chemical terms. (T. F. Spande and N. M. Green)

Novel Photocyclization of Tryptophan Derivatives

Brief irradiation of N-chloroacetyl-L-tryptophan with a high pressure mercury lamp with Vycor filter have the lactam of L-tryptophan-4-acetic acid, *viz.*, the tricyclic 4-caroxy-6-oxo,3,4,6,7-tetrahydro-1H,5H-azocine [4,5,6-c,d]-indole. The yield in neutral aqueous solution exceeds 40%. This reaction makes accessible 4-substituted tryptophans and tryptamines in a one-step procedure and opens up new avenues to analogs and homologs of dehydrobufotenine, as well as of lysergic acid. (O. Yonemitsu and P. Cerutti)

Photoreductions of Tryptophan

UV-irradiation of aqueous solutions of L-tryptophan in the presence of excess sodium borohydride leads to 4,7-dihydro-L-tryptophan (10%), 2,3-dihydro-L-tryptophan (4%), indole-3-propionic acid (2%), and a trace of tryptamine. The catalytic reduction of 4,7-dihydro-L-tryptophan gives the unstable 4,5,6,7-tetrahydro-L-tryptophan, whose racemate was obtained by alkaline decarboxylation of 2-carbethoxy-4, 5, 6, 7-tetrahydro-DL-tryptophan, ethyl ester. Birch reduction of L-tryptophan gave 4,7-dihydro-L-tryptophan, identical with the main product of the photoreduction, in 55% yield. (O. Yonemitsu and P. Cerutti)

Novel Photocyclization in the Tyrosine and Tyramine Series

N-Chloroacetyl-*meta*-tyrosine on irradiation undergoes photocyclization to the newbicyclic 7-membered lactam of 3-hydroxyphenylalanine-6-acetic acid. The same photocyclization carried out with N-chloroacetyl-tyrosine did not lead to identifiable products. However, N-chloroacetyl-*p*-O-methyl-tyrosine gave a novel yellow product, λ 350 mμ, whose structure is under investigation. (O. Yonemitsu)

Photoreduction of Histidine

By shorter irradiation time the photoreduction of histidine can be conducted in such a way that the formation of secondary transformation products is avoided. The acid-labile primary photoreduction product has been obtained in pure form by careful column chromatography and has been characterized by crystalline benzoyl and tosyl derivatives. The mass spectrum of these compounds is suggestive of dimeric structures. If this observation is substantiated by further determinations, the photoreductive coupling of two histidines in close proximity in a protein or enzyme becomes an interesting possibility. (Y. Fujita)

The Binding Characteristics of Partially Reduced Polyuridylic Acid

Selective photoreduction of uridine has now been extended to polyuridylic acid. The bind-

ing capability of poly U/H_2 U was examined by means of quantitative infrared spectroscopy of aqueous solutions. The interpretation of the spectra was based upon previous studies of the poly A-poly U system and upon the spectra of H_2 Up and other dihydrouracil nucleoside model compounds. A comparison of the binding characteristics of poly U/H_2 U with poly A as a function of the H_2 U content showed that the affinity of the two polymers decreases progressively with the extent of reduction of poly U. (P. Cerutti, H. T. Miles, and J. Frazier)

Template Activity of Uridyllic Acid—Dihydrouridylic Acid Copolymers

Dihydrouridylic acid (H_2 Up) has been described as a minor component of alanine-sRNA and serine-sRNA from yeast. Polymers containing various rations of uridylic acid and dihydrouridylic acid have been tested for template activity in both the amino acid incorporation system and the binding of aminoacyl-sRNA to ribosomes. The results show that dihydrouridine cannot substitute forA,G,C, or U in U-containing codons and apparently is not recognized in these assays for template activity. (F. Rottman and P. Cerutti)

Mechanism of the Two-step Reduction of Thymidine

Thymidine in its photoexcited state is reduced by sodium borohydride. The two-step reaction involves first photoreduction of the 5,6-double bond and, in a subsequent light-independent step, reductive cleavage between positions 3 and 4 of the dihydrothymine ring. The second reductive step is generally observed with dihydropyrimidine nucleosides. The product of dihydrothymine on reductive ring opening is 3-ureido-2-methylpropanol-1. The structure of this product rests on spectral and chemical evidence and on the synthesis by an independent route. The mechanism of the primary photoreduction was studied by the use of differently labeled reducing systems, ($NaBD_4/H_2O$; $NaBH_4/D_2O$; $NaBD_4/D_2O$) combined with nmr spectroscopy. The hydrogen donated by $NaBH_4$ enters to molecule at C–5, whereas the hydrogen added to C–6 originates from the solvent. (G. Balle and P. Cerutti)

Stereoisomerism and Stereoinduction in the Photoreduction of Thymidine

Dihydrothymidine has a new optically active center in the 5-position. The absolute stereochemistry of this new center can now be established by cleavage of the ribose residue by mild acid treatment and by measuring the ORD curves of the optically active dihydrothymines or the corresponding 3-ureido-2-methylpropanols as obtained by photoreduction or catalytic reduction, respectively. (Y. Kondo)

The Stereochemistry of 3-Methylproline

The known product of Michael condensation of crotonaldehyde with diethyl acetamidomalonate can be readily dehydrated to the enamide, or converted to the related N-acetyl-4,5-dehydro-3-methylproline ethyl ester, which was separated into *cis* and *trans* forms. These diastereoisomers were key intermediates in the correlation of *cis*- and *trans*-3-methylprolines with *allo*isoleucine and isoleucine. For example, the *cis* enamide N-acetyl-4,5-dehydro-cis-3-methyl-DL-proline ethyl ester was converted, by hydrogenation and hydrolysis, to *cis*-3-methylproline, while its reaction with ethyl mercaptan gave a mercaptal which was desulfurized to N-acetylalloisoleucine ethyl ester. These correlations confirmed the stereochemical assignments based upon preferential saponification, in which isomeric mixtures of N-protected 3-methyl proline esters gave a *cis* ester and a *trans* acid; the latter procedure was also useful for separation of the isomers. The NMR spectra of *cis*- and trans-3-methylprolines and their derivatives permitted complete assignments of individual proton peaks. By comparison with its published spectrum it was confirmed that the 3-methylproline in bottromycin A is *cis*. (A. B. Mauger and F. Irreverre)

Proton Magnetic Resonance Spectra of 3,4-Dehydroproline Derivatives

Slight changes in the chemical shifts of protons in 3,4-dehydroproline derivatives cause

the appearance of their proton magnetic resonance spectra to change markedly, and this can be effected by taking one compound in two different solvents or by observing closely related derivatives in the same solvent. The explanation involves a previously undescribed type of deceptively simple coupling. A first-order analysis of the ABMXX′ pattern for 3,4-dehydroprolinamide in deuterim oxide has been made. A full analysis was made of the ABXX′and ABXY patterns for this amide in deuterium oxide and deuterochloroform, respectively, after deuterium exchange of the labile H^2; a very large homoallylic coupling is required. Two conformations exist at 40° in solution for all N-benzyloxycarbonyl methyl esters of proline and its derivatives due to restricted rotation about the amide bond. Free rotation of the amide bond of corresponding N-acetyl and N-benzoyl derivatives still occured at −50°. The deceptively simple spectrum of N-benzyloxy-carbonyl-2,5-dihydroxy-Δ^3-pyrroline and that of its diacetate have been analyzed as an A_2X_2 system; a very small homoallylic coupling is required. (L. Johnson, A. Robertson, and W. Simpson)

Proline Analogs and Their Effects Upon the Biosynthesis ofActinomycins

Significant differences have been observed in the effect of *cis*- and *trans*-4-fluoro-L-prolines on cultures of growing *Streptomyces antibioticus*. Unlike proline hydroxylase of chick embryo the bacterial hydroxylase seems to be capable of hydroxylating both *cis*- and *trans*-fluoroproline. Considerable radioactivity is found in the 4-keto-proline, indicative of loss of HF from an unstable intermediary 4-hydroxy-4-fluoro-L-proline. (E. Katz and A. B. Mauger)

Analogs and Homologs of Proline and Hydroxyproline

An extensive review (277 references) on this field of growing importance has been compiled. It is presently serving as a guide for attempts to devise inhibitors of proline hydroxylase for which the minimal molecular weight required is under investigation. (S. Udenfriend and A. Mauger)

The Synthesis of Erythro-γHydroxyl-L-lysine and its Nonoccurrence in Collagen

threo-γ-Hydroxy-L-lysine, prepared *via* γ-chloro-L-lysine by photochlorination of L-lysine, was converted to the dicarbobenzyloxy lactone, opened to the amide, and oxidized to the γ-keto derivative. Catalytic hydrogenation and debenzylation yielded, after hydrolysis of the amide, 72% of the erythro acidlactone mixture and 28% of the *threo* pair, which were separated by ion-exchange chromatography.

Catalytic hydrogenation of ε-diazo-δ-oxo-L-norleucine (DON) gave a mixture of 25% erythro- and 75% threo-δ-hydroxy-L-lysine. By reaction with S-methylisothiourea, *erythro*-γ-hydroxy-L-homoarginine lactone, the diastereoisomer of the natural *threo* amino acid from *Lathyrus*, was prepared. Unlike *trans*-3-hydroxy-L-proline, the position isomer of natural 4-hydroxy-L-proline, neither *erythro*-γ-hydroxy-L-lysine, the position isomer of natural *erythro*-δ-hydroxy-L-lysine, nor its *threo* isomer are regular building stones of collagen. (N. Izumiya, Y. Fujita, and F. Irreverre)

Synthesis and Metabolism of 6-Hydroxycatecholamines

The following 6-hydroxycatecholamines (2,-4,5-trihydroxyphenethylamines or -phenethanolamines), potential metabolites of catecholamines, were synethesized: 3-*O*-methyl-6-hydroxydopamine, 6-hydroxynorepinephrine, 3-*O*-methyl-6-hydroxynorepinephrine, 6-hydroxyepinephrine, and 3-*O*-methyl-6-hydroxyepinephrine. Enzymatic *O*-methylation with catechol *O*-methyl-transferase with S-adenosylmethionine-^{14}C as donor of $^{14}CH_3$ gave radio-active ^{14}C-labeled 3-*O*-methyl-6-hydroxycatecholamines which were used for metabolic studies in the rat. The corresponding phenylacetic and mandelic acids, as well as the phenylglyclo, were identified as metabolites by comparison with synthetic compounds. Eleven new potential metabolites were compared and characterized. The relative substrate activity of some of these 3-*O*-methyl-6-hydroxycatecholamines with

monoamine oxidase was much lower than that of 3-*O*-methyldopamine or normetanephrine. (J. W. Daly, J. Benigni, R. Minnis, and Y. Kanaoka)

The Chemorelease of Norepinephrine from Mouse Hearts. Structure-Activity Relationships. Sympathomimetic and Related Amines

A rapid method for determining the chemorelease of cardiac norepinephrine in the mouse has been developed. Endogenous cardiac norepinephrine is prelabeled with 5 μcuries of norepinephrine-7-^{3}H. The cardiac norepinephrine-^{3}H remaining in the heart after 3 hours averages 340 mμcuries. Lower levels of radioactivity are found in heart when animals are injected 1 hour after the norepinephrine-^{3}H with a compound that causes chemorelease. The releasing potencies of a wide variety of sympathomimetic amines and related compounds have been determined. Structure-activity correlations have been established. (J. W. Daly and C. R. Creveling)

Drugs Affecting the Sympathetic and central Nervous Systems

Endogenous cardiac norepinephrine in mice has been prelabeled with a 5-μcurie injection of norepinephrine-^{3}H, and the effect of various classes of compounds on the normal physiological depletion of norepinephrine-^{3}H has been studied. The effect of a variety of tranquilizers, antidepressants, ganglionic blocking agents, hypotensive agents, sympatholytics, and compounds that inhibit key enzymes in the biogenesis and metabolism of norepinephrine have been ascertained. The releasing and release-inhibiting activities of close to 100 representatives of these drugs have been measured. (J. W. Daly and C. R. Creveling)

The Depletion of Norepinephrine-^{3}H from Heart by α-Methyl-m-tyrosine. A Novel and Convenient Method for Assaying the Inhibition of Aromatic Amino Acid Decarboxylase in vivo

Cardiac norepinephrine of mice was prelabeled with 5 μcuries of norepinephrine-^{3}H. After 1 hour 10 mg/kg of α-methyl-*m*-tyrosine was administered subcutaneously. After 3 hours the activity of cardiac norepinephrine-^{3}H was 50% of that of control animals. This release of norepinephrine which requires enzymatic decarboxylation of α-methyl-*m*-tyrosine to the active releasing agent α-methyl-*m*-tyramine is blocked by inhibitors of aromatic amino acid decarboxylase. The resulting decrease in α-methyl-*m*-tyrosine-releasing activity is a direct measure of inhibition of the enzyme *in vivo* and provides a convenient method for determining the effectiveness of decarboxylase inhibitors in intact animals. (C. R. Creveling and J. W. Daly)

Preparation of Gramicidin A, B, and C

By countercurrent distribution and redistribution the commercially available peptide antibiotic, gramicidin, was resolved on a preparative scale. The group of lipophilic peptides contained gramicidins A, B, and C, each of which consisted of a pair of congeners, *i.e.*, valine-gramicidin (80–95%) and an isoleucine-gramicidin (5–20%). The sum of aromatic amino acids was always four, namely, four tryptophan in gramicidin A, three tryptophan plus one phenylalanine in gramicidin B, and three tryptophan plus one tyrosine in gramicidn C. A more hydrophilic, strongly antibiotic group of peptides, designated gramicidin D, contained five to six additional amino acids. Complete amino analyses, including time-dependence studies of hydrolyses, have been carried out over the entire range of 999 transfers. (E. Gross)

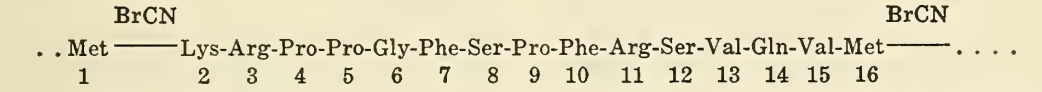
Release of Kinin Activity from Human Kininogens and Fresh Plasma by Cyanogen Bromide

The two human kininogens I and II have been treated with cyanogen bromide, a reagent which under acidic conditions selectively cleaves methionyl bonds in peptides and proteins. Kinin activities were estimated by the muscle contraction of estric rat uterus. As a control the stability and recovery of kallidin were tested under the same experimental con-

ditions as were used for cleavage of the Kininogens. In a series of experiments 90–98% of the kallidin activity was recovered. Solutions of 100 λ of Kg I and Kg II, respectively, in 1 ml of 0.25 *N* hydrochloric acid containing 1 mg of cyanogen bromide were incubated at 35° for 20 hours. The reaction products were then lyophilized and assayed for kinin activity. Unfractionated native human plasma with cyanogen bromide releases kinin activity to the extent of 10% of the activity observed after treatment with urinary human kallikrein.

Kininogen	Kinin Activity (expressed as γ kallidin/mg protein)	
	after cleavage with CNBr	after cleavage with human urinary kallikrein
Kg I	2.5	8
Kg II	0.8	6

The methionyllysylbradykinin (1–11) is pictured as a unit within the polypeptide chain of the precursor protein:



(R. Axen, J. V. Pierce, and M. E. Webster).

Selective Cleavage ofCysteine Peptide Bonds

Peptides which contain N-alkylated residues of cysteine were synthesized. Cleavage of the aminoacyl bond of cysteine in model peptides is as high as 90–100%. The cleavage of S-alkylcysteine-containing peptides proceeds *via* two different mechanisms: (*a*) at low temperatures an oxazolinium bromide is formed. Hydrolysis leads to the formation of the-O-acyl derivatives which have to be hydrolyzed under conditions which prevent an ON-acyl shift from taking place. (*b*) At high temperatures β-elimination is predominant. Water is added to the acrylic acid derivative. Hydrolysis results in the formation of pyruvic acid and the amide of the amino acid that preceded cysteine.

Cleavage of Cysteine Peptide Bonds in Insulin and Ribonuclease

The disulfide bonds of insulin were reduced by treatment of the hormone with dithioerythritol. Various alkyl halides were employed to alkylate the liberated mercapto groups. The methylated chains of insulin are poorly soluble in aqueous solution. Presently the most promising alkylating reagent appears to be aminoethylbromide. Yields of conversion of S-alkylcysteine amount to 75%.

The S-protein of bovine pancreatic ribonuclease was cleaved with cyanogen bromide. Liberated homoserine lactone was removed by gel filtration. The disulfide bonds of the resulting product were reduced with dithioerythritol. The mercapto groups were methylated by treatment of the reaction mixture with methyl iodide and the fragments separated by gel filtration. The NH_2-terminal fragment (one residue of S-methylcysteine) was cleaved with cyanogen bromide. The amino acid analysis was low in serine indicating that one residue of serine has been removed in part with the S-peptide.

Antibiotic Nisin

The peptide antibiotic nisin is rich in sulfur-containing amino acids: methionine, lanthionine, and β-methyllanthionine. Little is known about the structure of the antibiotic. The high thioether content makes the peptide a candidate for fragmentation *via* the cyanogen bromide reaction. A commercial product called Nisaplin contains approximately 2% of nisen. From this material partially purified nisin has been obtained and analyzed. A study of the elution pattern of the lanthionines on alayzer columns has led to the separation of three optical isomers of synthetic β-methyllanthionine.

Active Carboxy Group of Pepsin

Pepsin contains only three amino acids, the peptide bonds of which can be cleaved specifically with an enzyme. Selective nonenzy-

matic cleavage appears to be required to establish the primary structure of the enzyme. Pepsin was reduced with dithioerythritol and carboxymethylated. Treatment of CM-pepsin with cyanogen bromide converted all residues of methionine to homoserine. Reduced pepsin was also methylated and then treated with cyanogen bromide. Seventy-five percent of the residues of S-methylserine were converted to serine. *p*-Bromophenacyl bromide has been reported to inactivate pepsin reversibly. The proposed involvement of disulfide bond was ruled out. By carrying pepsin through a series of chemical events it was shown that the enzyme contains *one* residue of active aspartic acid: *p*-bromophenacyl bromide esterifies the β-carboxyl group of the active aspartyl residue. (E. Gross and J. L. Morell)

Improvements in the Methodology of Amino Acid Analysis

The first automatic integrator had been designed and was built prior to the advent of accelerated systems for amino acid analysis. The threshold-type detection to start and stop integration is not applicable to accelerated systems. The integrator has been modified by adding a second type detection. The slopes of a peak that represents a solute which is eluted from a column are now monitored. This type of ON–OFF control of integration is fully satisfactory for the acelerated analytical systems. The automatic regeneration of analytical columns has been improved and extended to additional systems. A dual valve system has been designed to add automatically samples to the top of columns. The sample has to reach the resin without being diluted. This is accomplished by a special, plunger-type connector which is in direct contact with the resin. The "Robot Chemist" ninhydrin analyzer has been modified to handle twice the number of ninhydrin analyses per day. The computer program for the calculation of the amino acid composition of peptides and proteins has been revised. Details, such as minimum molecular weights per residue, numbers of individual residues, and molecular weight calculations are now readily available. (E. Gross and J. L. Morell)

LABORATORY OF EXPERIMENTAL PATHOLOGY

Introduction

In July, 1965, Dr. Benjamin Highman, Chief, Section on Pathologic Anatomy, was assigned as PHS Liaison Officer to the Armed Forces Institute of Pathology (AFIP) and was appointed Chief of the Institute's Radiopathology Division "I." He also remained a member of the staff of the Laboratory of Experimental Pathology, NIAMD, continuing with research projects active at the time of his assignment to the AFIP.

The duties of the Liaison Officer are varied. He serves on the Intra-Mural Interagency Advisory Group of the AFIP, which meets weekly. He keeps the pathologists of the PHS informed about the various courses, educational aids, and other activities of the AFIP that may be of interest to them. In general, he is concerned with matters of mutual interest to the PHS and the AFIP.

As Chief of the Radiopathology Division "I," he takes part in and has administrative responsibilities for the work of the Radiologic Pathology Branch and the Radiation Pathology Branch. He participates in the educational program for radiology residents given by the Radiologic Pathology Branch; he usually devotes several hours monthly in giving lectures correlating the radiologic and pathologic pictures. He has also undertaken several research projects related to radiation pathology.

Following Dr. Highman's assignment, the activities of the Section were handled by the Chief of the Laboratory. Assistance to the Division of Indian Health, PHS, the Hospital of the Coast Guard Academy, New London, and to several Federal Prisons in the form of pathologic consultations and examination of tissues submitted for diagnosis has continued unaltered. The Laboratory staff trained in pathology was greatly helped through voluntary and generous participation in this activity by Dr. Ruth Kirschstein (Chief of Pathology, DBS) and Dr. Richard Asofsky (LGAR–NIAID)

In its research activities, the Laboratory staff has made increasing use of the available electron microscopes, a trend expected to con-

tinue. It necessitated a larger staff of technical assistants in the preparation of material. The additional use of bacterial and tissue culture systems in investigations by other members has made it possible to utilize positions previously used in the general tissue laboratory for these special purposes. For the same reason, a reduction in the preparation of microscopic slides for investigators outside the Laboratory and the Institute became necessary. A satisfactory solution to this problem was found when the outside laboratories agreed to provide personnel and our Laboratory training and technical equipment to handle such material.

The Laboratory is host to two foreign guest workers, Dr. Iwao Hirono and Dr. Nestor Piva. Dr. Hirono, Associate Professor of Pathology at Gifu University, is the holder of an Eleanor Roosevelt International Cancer Fellowship; Dr. Piva, Head of the Department of Pathology, in the Institute for Experimental Medicine, Aracaju-Sergipe-Brazil, is sponsored by the Brazilian Government. Dr. Hirono's work is concerned with studies of carcinogenetic effects of cycasin and Dr. Piva's area of interest concerns special problems related to the histochemistry of schistosomes.

Pathology of Rheumatic Diseases

The elasticity of aging human cartilage, obtained at necropsy, was studied by an indentation technique. Costal cartilage was stiffer than articular tissue at all ages. The Young's modulus of rib cartilage was greater in middle aged than young adults; by contract the values for patellar cartilage were not significantly different in the two age groups. Nor was the recovery from deformation diminished. In osteoarthritic fibrillation, there was a marked softening as well as a measurable unrecovered strain. These observations contradict a widely held, undocumented view that senescent impairment of the elasticity of articular cartilage is a predisposing factor in the development of degenerative joint disease. (Dr. Sokoloff)

Motion of the embryonic limb was found necessary for the proper development of animal joints. Movement was prevented in chicks, beginning of the seventh day of incubation, by three techniques: administration of botulinum toxin, decamethonium chloride, or extirpation of the lumbosacral spinal cord. Although primordial development of the joint anlage proceeded to a certain point, the joint cavity and certain accessory articular structures, such as the cruciate ligaments and some sesamoid bones, failed to develop. Variable cartilaginous ankylosis ultimated supervened. (Dr. Drachman [Pratt Institute, Boston, Mass.] and Dr. Sokoloff)

It was shown that, over short time intervals, the friction of cartilage sliding against glass is nearly unaffected by changes in load, as would be expected for a weeping bearing.

A theoretical investigation has shown that lubrication of bearings by osmotic type forces arising from adsorbed layers of long chain polymer molecules (like synovial mucin) is thermodynamically possible. (Dr. McCutchen)

Animal Joint Lubrication

The coefficient of friction during sliding can best be analyzed by breaking it down into its components, namely: viscosity of the lubricant, incremental deformation or plowing, and adhesion. In a dogs ankle, fluid viscosity had little or no affect on friction loss; plowing was appreciable at low speeds and decreased with increasing speed; adhesion was much lower with synovial fluid than other lubricants due to a lubricity producing factor in it. The coefficient of friction decreased with increasing loads at constant speed. The coefficient of friction was low in the joint since the load was mainly carried by self-pressurized fluid in the water rich articular cartilage. The friction losses during dynamic or cyclic loading, similar to that produced when walking, were comparable to those obtained with constant loads. The addition of hyaluronidase to synovial fluid did not change the lubricating quality of synovial fluid. Purified hyaluronic acid in an isotonic saline solution gave a friction loss similar to the saline solution and 2.5 times that obtained with synovial fluid. (Mr. Linn)

Cytogenetics

Experiments on Reutilization of Chromosomal DNA by Cells Grown In Vitro

This project is an attempt to determine whether cells in culture will reutilize foreign DNA in the form of isolated single chromosomes. Chinese hamster fibroblast cultures labeled with H^3. The metaphase cells were separated, fragmented and the chromosomes isolated. These were then inoculated into hamster cell cultures and human blood cultures. Autoradiographic preparations obtained from these cultures showed weak labeling on several chromosomes in many mitosis. In one experiment, which requires confirmation, no labeling was detected when the cell culture was given a large excess of unlabeled base (thymidine). Thus, it seems that fibroblasts and lymphocytes growing *in vitro* reutilize foreign chromosomal DNA. Tentative findings indicate that this DNA is first digested and the constituents enter the cells' metabolic pool of purines, pyrmidine, etc. This appears to be relevant to recent claims that animals can incorporate foreign nucleic acids into their systems. (Dr. Tjio)

Meiotic Chromosome Studies in the Human Male

In the meiotic prophase the chromosome is differentiated into morphologically distinct regions (chromomeres) which are constant in size and position. These regions have been described and correlated with certain genotypic expressions in many organisms, especially in corn (*Zea mays*) of which elaborate gene maps have been obtained.

This study is aimed at obtaining similar gene maps of the human male meiotic prophase, in particular the pachynema. Experiments are being carried out to obtain well preserved pachytene chromosome preparations suitable for mapping the structural morphology. Human testicular biopsy material has been provided by the Surgical Department of the George Washington Hospital. (Dr. Tjio)

Characterization and Quantitation of Enzymes in Histomchemical Systems

A continuing study of the characteristics of hydrolytic enzymes demonstrated using histochemical substrates in both histochemical and biochemical systems has resulted in the idenification of a wide variety of hitherto undescribed aminopeptidases residing predominantly in the particulate fraction of the cell. Aminopeptidase B, an exopeptidase selective for N-terminal arginine and lysine residues of peptides and polypeptides, has been highly purified (over 1000 fold) from rat liver and shown to be the first described peptidase activated by an anion (Cl^-). This enzyme is capable of catalyzing the conversion of Kalledin X to Bradykinin without further degradation of the nonopeptide. The possible significance of cellular anionic changes in potentiating the formation of Bradykinin are under investigation.

In addition to the previous finding of a Co^{++}-activated aminopolypeptidase from rat and hog kidney, a new class of enzymes acting on dipeptide naphthylamides (e.g. Ala-Ala-β-naphthylamide) with a high degree of substrate specificity have been found. These enzymes do not attack the N-terminal amino acid, but have a selectivity for the N-terminal dipeptide moiety. None of the members of this group have been found to be anion or cation activated. Efforts to characterize enzymes acting on the histochemical substrates acetyl- and α-halogenated acetylnaphthylamides have resulted in the identification of kynurenine formamidase as one of the chief effectors of the hydrolytic reaction in rat tissue homogenates (liver and kidney). Other as yet poorly-defined enzymes also appear to be implicated in the hydrolysis of this class of substrates. (Dr. Glenner and Drs. Vanha-Perttula and Hopsu [Turku University, Turku, Finland])

Histochemical Localization of ATPase Activity

Studies on the lead-salt method of Wachstein and Meisel for localization of tissue ATPase activity have confirmed previous reports by other investigators that both fixation and the presence of lead ions markedly inhibit tissue ATPase activity as measured by biochemical techniques. Further studies have demonstrated that lead alone may catalyze the nonenzymatic hydrolysis of ATP under conditions employed

for the Wachstein-Meisel procedure. In addition, localization of lead deposits to plasma membranes occurred only with those lead and ATP concentrations which gave significant nonenzymatic breakdown of ATP by lead. These studies indicate that localization of lead deposits to specific tissue sites does not constitute sufficient evidence for the presence of ATPase activity in those sites. (Dr. Moses and Drs. Rosenthal and Beaver [Vanderbilt University, Nashville, Tenn.])

Studies in Oxidative Phosphorylation

A derangement of oxidative phosphorylation may be a factor contributing to the elevated serum enzyme levels observed after animals are subjected to stress. Studies of the mechanisms by which chemical reagents depress this function in mitochondria had shown that these compounds are bound to the protein of the organelle. It was demonstrated that mitochondria containing the bound uncoupling reagents were more susceptible to the attack of proteolytic and lipolytic enzymes and this may be a factor in the cellular pathology of stress. It was shown that serum albumin not only could restore oxidative phosphorylation to chemically damaged mitochondria, but could also rapidly restore the more sensitive parameter, respiratory control. Electron microscopy revealed that serum albumin also prevented the morphological deterioration induced by the uncoupling reagents. Cellular and serum proteins may play a similar role in the recovery of stressed animals. (Dr. Garbus and Dr. Weinbach [LPD–NIAID])

Mechanism of Protection by 5-HT in Tryptophan Deficiency

It had previously been noted in a study of experimentally induced cardiac lesions of the carcinoid type in guinea pigs that 5–hydroxytryptamine (HT) reduced the incidence of myocardial lesions as well as the mortality of the tryptophan deficient animals. The "protective effect" of 5–HT was limited to the myocardium and had no influence on the endocardial alterations in these animals. In an attempt to evaluate the pathogenesis of the myocardial lesions experiments were performed to study changes in the myocardium prior to microscopically visible injury.

It was found that a tryptophan deficient diet in guinea pigs produced a decrease in total phosphorylase and that this depression was prevented completely by exogenous 5–HT and in part by supplementing the deficient diet with DL–tryptophan. The protective effect of 5–HT appeared to be time dependent since it was no longer seen after tryptophan deficiency was established.

The mechanism by which 5–HT protects the mycardium of guinea pigs on a tryptophan deficient diet is still uncertain although the results of the experiments indicated that 5–HT increased phosphorylase *a* activity by converting phosphorylase *b* to *a* since total phosphorylase was not altered. The essentially similar effect of exogenous 5–HT on reserpinized and nonreserpinized guinea pigs in increasing phosphorylase *a* activity showed that this effect was still obtained after depletion of endogenous 5–HT and norepinephrine following reserpine injection. When comparing the increases in phosphorylase after 5–HT administration between the two groups, the hearts of nonreserpinized animals showed a greater increase in the percentage of conversion of phosphorylase *b* to *a*. Whether the slightly lower response of reserpinized animals is due to the formation of abnormal binding centers for 5–HT as suggested by E. Kuntzman, *et al.* (*Fed. Proc. 15: 450, 1956*) is unknown. (Dr. Spatz)

Fluorescein Studies

Localization of Antigens in Tissues

During the past year, studies involving the localization of endogenous antigenic substances by means of specific fluorescent antibody have been extended to include the enzyme α-glycerophosphate dehydrogenase in the heart muscle of the rat where it is distributed in mitochondria surrounding the fiber. This distribution has been shown in relation to the A band of the muscle fiber which is identified by staining with fluorescent anti-myosin globulin. The distribution of the enzyme is similar to that

of glyceraldehyde phosphate dehydrogenase with which it is closely linked in the glycolytic cycle of the msucle metabolism. (Dr. Emmart)

Localization of prolactin in rat pituitary and in a transplantable mammotropic pituitary tumor using fluorescent antibody was completed demonstrating the presence and distribution of prolactin in acidophilic cells of tumor tissue, especially in those cells containing erythrosiniphilic granules. Bioassay of the isolated rat tumor prolactin indicates that it contains 0.15 International units per mg. Precipitin reactions in the test tubes show that it precipitates readily with antibody to ovine prolactin. A comparison of the electrophoretic pattern of ovine prolactin and rat tumor prolactin shows that they have different rates of migration and that the tumor prolactin contains some impurities. (Dr. Emmart and Mr. Turner and Dr. Bates [LNE–NIAMD])

Localization of Prolactin in Fundulus Heteroclitus, L.

It has been established that antiovine prolactin will precipitate with fish prolactin. This antibody has therefore been used to localize prolactin in erythrosinophilic cells of the rostral pars distalis of the teleost, *F. heteroclitus, L.* Further studies in immunochemistry of fish prolactins are in progress. (Dr. Emmart and Dr. Pickford [Yale University, New Haven, Conn.] and Dr. Wilhelmi [Emory University, Atlanta, Ga.])

Studies on Nucleic Acids and Protein Synthesis

Autoradiographic and cytochemical studies regarding structure and function of the nucleolus in relation to nucleic acid and nucleoprotein synthesis have been continued. In order to develop suitable methods for electron microscopic investigation of nuclear and nucleolar ultrastructure, a study of the effect of fixation on the autoradiographic localization of nucleoprotein synthesis in nucleoli was undertaken. The effect of temperature and pH of formaldehyde, gultaraldehyde and of acid alcohol fixation on the localization of H^3-lysine, H^3-arginine and H^3-uridine was studied in PPLO-free HeLa cells under conditions of selective inhibition of nucleolar RNA synthesis Actinomycin D and of ribosomal protein synthesis with Puromycin. It was found that the temperature of fixation with PO_4 buffered formaldehyde and glutaraldehyde alters the intranuclear distribution of H^3-lysine and H^3-arginine after brief labeling, without apparent loss of nuclear uptake. Fixation at 37° C, as compared with 0° or 23° C, results in a shift to chromatin of about 25% of the ratio of nucleolar to nuclear grain count/unit area ($\alpha < 0.01$). A similar redistribution was found when cells were formalin-fixed at acid pH, with acetic:ethanol (Carnoy), or were eluted after fixation with hypertonic alkaline Tris buffer. This shift of nucleolar label appears to involve ribosomal protein, since a proportional inhibition, with loss of nuclear uptake, occurs after treatment with Puromycin (200 mgm/ml) or with Actinomycin D (0.05 mgm/ml). In the former case inhibition occurs under conditions not inhibitory to nucleolar RNA synthesis, but in the case of Actinomycin there is a parallel selective inhibition of nucleolar RNA, shown by R. P. Perry (NCI Monograph No. 14, 73–91, 1964) and others to be a ribosomal precursor. By removing the inhibitor, the effect of Puromycin can be reversed only where cells are fixed at 0° or 23° C. These findings, together with previously reported data, are interpreted as evidence for the rapid incorporation in the nucleolus of amino acid label into a basic ribonucleoprotein participating in the synthesis or stabilization of the ribosome complex. This ribonucleic protein unstable under some conditions of fixation previously employed and solubilized in aldehyde fixative at slightly elevated temperature probably as a result of thermal denaturation of associated RNA. (Dr. Suskind)

An autoradiographic and virologic study, in collaboration with Dr. G. Rabotti (LVO–NCI) designed to investigate the sites of viral nucleic acid synthesis and viral protein synthesis of Rous sarcoma virus in transformed (neoplastic) and nontransformed chicken fibroblast cultures is in progress. Preliminary results point to a nuclear site of viral nucleic acid synthesis. (D. Suskind)

Studies on Soluble Ribonucleic Acid (sRNA)

A new method for the chromatographic fractionation of different sRNA species has been developed. A gradient of increasing salt and pH is employed to elute sRNA from DEAE-Sephadex. There is evidence that resolution is dependent upon a change in the sRNA from a partially denatured state at the beginning of elution to the native state at the end. The method has a large input capacity and provides good resolution of certain sRNA species. It is convenient to use and can be adapted either for preparative or analytical work. (Dr. Smith)

Electron Microscope Studies

Studies in Amyloidosis

Electron microscopic evidence has indicated the morphologic identity of amyloid fibers obtained from both the primary and secondary disease. Recent electron microscopic studies on negatively stained extracts from human amyloidotic tissue revealed the presence of two distinct types of particles. One was a 100 Å wide rod, while the other was a small pentagonal structure (unit structure) 90 Å in diameter both morphologic units were present in all preparations obtained from various tissues of patients with either primary or secondary amyloidosis. Narrow electron dense bands divided the rods at intervals into smaller segments with the average distance from the center of one segment to the center of the next being approximately 40 Å. Frequently, however, the width of the electron dense bands between segments varied irregularly, suggesting that the segments were easily disunited. When preparations containing predominantly long rods were sonicated, a large increase in the number of unit structures was evident. The unit structures seemed to be single or double segments of rods lying on their flat side, thus affording an end view of the amyloid rod. They appeared to be composed of five globular sections surrounding a hollow core. Purification of this periodic component of amyloid to afford a single protein band on disc electrophoresis and reconstitution of the rod has been achieved. (Dr. Glenner and Dr. Bladen [LHP–NIDR])

Fine Structure and Cytochemistry of Rabbit Marrow Cells

A descriptive analysis of granulocyte develment and of the origin, nature, and fate of their characteristic granules (especially those of the heterophil); localization of acid and alkaline phosphatases in this tissue.

The mode of eosinophil granules formation has been detailed within the past year and incorporated into this completed study. Small vacuoles of moderately dense, flocculent contents arise from Golgi lamellae. Apparently the contents of these vacuoles condense centrally, resulting in the characteristically dense and homogeneous substance of eosinophil granules. Fusion of additional vacuoles and further condensation result in large spherical granules which mature individually, acquiring the angular profile and included crystalloids which are typical of eosinophil granules in this species. (Drs. Wetzel and Spicer and Dr. Horn [Vanderbilt University, Nashville, Tenn.])

Myeloid Elements

Investigation into the fine structural aspects of granulocyte development and cytochemistry have been extended to include investigation of human granulocytes in health and disease. Phosphatase cytochemical methods, ultrastructural demonstration of mucosubstances and electron microscope autoradiography with $S^{35}O_4^{=}$ and with tritiated arginine have been utilized to characterize leukocyte granules of humans and experimental animals. (Drs. Wetzel and Spicer and Dr. Horn [Vanderbilt University, Nashville, Tenn.])

Studies of Human Lymphocytes after Exposure to Phytomitogens

In vitro transformation of human peripheral small lymphocytes with phytomitogens has been studied. The physiochemical, biologic and morphologic features of cells stimulated with pokeweed mitogen (PWM), an extract of *Phytolacca americana* reveals that this material has properties which differ from phyto-

hemagglutinin (PHA). Pokeweed mitogen stimulation is maximal after 72 hours in culture. With optimal concentrations of PWM 50–60% of the initial cell inoculum transforms. At 72 hours the cell population is comprised of small- and medium-sized lymphocytes, large PHA-like blast cells, and a unique intermediate-sized cell type. The fine structural features of these intermediate cells include an eccentric nucleus, with clumped heterochromatin, a well developed Golgi apparatus, ribosomal aggregates, and a well developed rough-surfaced endoplasmic reticulum. These fine structural features differ importantly from those consistently seen in PHA transformed cells, and are comparable to those of cells which have been described as early antibody-producing cells. (Dr. Douglas, Drs. Chessin and Borjeson [LI–NIAID], and Mr. Hoffman)

Fine Structural Characterization of Neuromelanin

Neuromelanin

Electron microscopic and cytochemical and rhesus monkey substantia nigra and locus coeruleus have been concluded. Melanin and other pigments from various anatomical sites were also examined for the purpose of comparison with the neuromelanin pigment. A substructure different from that of other melanin pigments was found in neuromelanin which more closely resembled neuronal lipofuscin pigment. Neuromelanin granules, however, contained a reducing substance in the form of a very electron dense component—a structure not found in neuronal lipofuscin pigment granules. (Dr. Moses and Drs. Ganote and Beaver [Vanderbilt University, Nashville, Tenn.])

Experimental Porphyria

Allylisopropylacetamide (AIA) induced porphyria, in rats is characterized by hepatomegaly and increased production in liver of the porphyrin precursors, -aminolevulinic acid and porphobilinogen. The latter has been shown to be secondary to induction of liver δ-aminolevulinic acid synthetase, the rate-limiting enzyme of heme biosynthesis. Light and electron microscopic studies on this experimental model, which has been extensively investigated biochemically, revealed marked hypertrophy of hepatic parenchymal cells and little evidence of cellular proliferation. Electron microscopically the enlarged hepatic cells exhibited increased numbers of secretory vesicles, extensive development of the smooth endoplasmic reticulum, and striking nucleolar enlargement—changes which can probably be explained on the basis of increased secretory activity, drug detoxification and enzyme induction with increased protein synthesis. Both the morphologic and metabolic effects of AIR were blocked by the simultaneous administration of either Actinomycin D or Puromycin. A combined biochemical electron microscopic and autoradiographic study of amino acid and nucleoloid uptake under these conditions is in progress. (Drs. Moses and Suskind and Dr. Tschudy [ODir-NCI])

Cytochemistry of Bacterial Phosphatases

The localization of several phosphatases in *E. coli* has been studied in an attempt to confirm and extend biochemical findings in this system. Substrate specificity, genetic differences among various strains, and pH optima have been used experimentally to distinguish among the enzymes. Selective localization of cyclic diesterase and glucose-6-phosphatase to the space between the cell wall and the plasma membrane, often forming prominent polar caps, is of particular interest in view of (1) the lack of significance attached to this region, and (2) recent data on the selective permeability of the cell wall. This region may thus serve to compartmentalize metabolic events in a manner analogous to intracytoplasmic membrane-limited cisternae in metazoan cells. An alkaline phosphatase appears to be localized primarily to the surface of these organisms, but a pH-dependent penetration factor has not been excluded. (Drs. Wetzel and Spicer, and Dr. Heppel [LBM–NIAMD])

Temperature Effect on Lipids of Leishmania

Although primarily a biocehmical analysis of shifts in fatty acid composition at elevated culture temperature, the initial identification

of cytoplasmic droplets as lipid and supportive morphological evidence was provided by this Laboratory. (Dr. Greenblatt [LPB–NIAMD] and Dr. Wetzel)

Mouse Thyroid Gland Cytology

This is a descriptive morphological study of this organ; unique features include ciliated follicular epithelia, several unusual types of follicular cell, and the elaboration of acid mucosubstance. (Dr. Wetzel and Dr. Wollman [LPHY–NCI])

Thyroid Parafollicular (Light) Cells

Preliminary histological findings in long-term (16 weeks postoperative) parathyroidectomized animals indicate an increase in the thyroid light cell population relative to normal animals. These data, when correlated with reports of thyrocalcitonin accumulation under the same experimental conditions, support the concept that thyroid light cells are the seat of thyrocalcitonin production and storage. Cytologically most of the light cells in the experimental animals are essentially filled with typical secretory vesicles. (Dr. Wetzel and Drs. Gittes and Wells [Surg-NCI])

Mucosaccharide Histochemistry

Investigation of the histochemical characteristics of epithelial mucosubstances has been extended, applying a variety of histochemical methods specific for carbohydrate to the study of secretions in salivary glands of several species including ungulates, primates, dogs and cats. A number of sulfated mucosubstances unfamiliar and unexplored by biochemical techniques have been demonstrated and partially characterized by these methods. In addition, various types of sialomucins have been identified in separate loci. (Drs. Leppi and Spicer)

The epithelial secretions in the dog's stomach have been investigated in regard to the identification of the types of mucosaccharides formed in the different epithelial cells. (Dr. Spicer and Dr. Sun [VA Hospital, Phoenix, Ariz.] and Dr. Hollander [Mount Sinai Hospital, New York, N.Y.

A technique employing dialyzed iron staining of cryostat sections has been developed for localization of acid mucosubstances at the ultrastructural level and this procedure has been applied to investigation of mucosaccharides in several sites. (Mrs. Wetzel, Drs. Wetzel and Spicer)

Staining of Paneth cell granules in the mouse small intestine has been investigated by correlated light microscope and electron microscope techniques. Acid mucosubstances including sialo- and sulfomucin have been observed in the rim of the granules and basic protein and an acid mucosubstance in the core of the granules. (Dr. Spicer, Mrs. Staley, Mrs. Wetzel and Dr. Wetzel)

Cytochemical studies on the sweat glands have been undertaken correlating light microscope and electron microscope techniques. These investigations concern particularly the localization of acid mucosubstances and enzymes in the sweat gland epithelium. (Drs. Grand and Spicer)

Correlated light and electron microscope studies on the mucosubstance in a coccolithophorid has revealed sulfate-containing mucosaccharides associated with foci of calcium deposition in the cytoplasm of this organism and in its intercellular matrix. (Dr. Douglas, Dr. Isenberg [Long Island Jewish Hospital, N.Y.], and Dr. Spicer)

Immunocytochemical methods employing the ferritin-labeled antibody technique at the fine structural level reveal selective localization of ferritin-labeled protein in the medial A-bands of rat skeletal muscle apparently on a nonimmunospecific basis. (Dr. Douglas, Dr. Gottlieb [LMP–NIAMD], Dr. Strauss [LI–NIAID], and Dr. Spicer)

Electron microscopy of the thymus of the red-eared turtle, *Pseudemys scripta elegans*, has revealed the presence of cells with the sarcomeric and myofilamentous structure of striated muscle. These myoid cells have been shown to have reactivity with serum gamma globulins of patients with myasthenia gravis. (Dr. Douglas and Dr. Strauss [LI–NIAID]). Fine structural features of thymic changes following acute irradiation of the rat, with particular emphasis on the role of medullary cells

in recovery, are in progress. (Dr. Douglas and Mr. Hoffman)

Microspectrophotometric Studies of Basic Protein

Objective confirmation of a visually observed metachromatic shift in certain basic proteins stained with the acid dye, Biebrich scarlet, has been confirmed by microspectrophotometric measurements. The absorption curves obtained show a maximum at about 510 mμ for strongly basic proteins in elastica, heterophil granules, thyroid colloid and other sites which stain orthochromatically. Absorption curves show a maximum at 485mμ in mucigenic epithelial cells, nuclei and mast cells which stain metachromatically with this procedure. (Drs. Douglas and Spicer, and Dr. Bartels [E. Leitz, Inc., New York, N.Y.])

Iron Staining of Acid Mucosubstances

Selective ultrastructural staining of acid mucosubstances in sites containing histochemically identifiable sulfo- and sialomucins has been obtained with colloidal iron solution in the rectosigmoid region of mouse colon. Specific localization of acid mucosubstances has been noted in intracellular sites, including: globules within colonic goblet cells and deep crypt mucous cells, small vesicles of the superficial nongoblet epithelial cells, and Golgi lamellae within each of these cell types. Extracellular material, presumed to be acid mucosubstance, was found on the surface of the epithelial microvilli and on the lumenal surface of capillary endothelium. Similar material formed a reticular network surrounding stromal cells, collagen bundles, and various colonic connective tissue elements. Specific staining depended upon the pH of the iron-containing solutions, and the optimal value was found to be approximately 2.0. (Mrs. Wetzel, Drs. Wetzel and Spicer)

Fine structural alterations of a degranulating nature have been observed in mast cells of the uterine cervical wall in the mouse during pregnancy and after treatment with exogenous hormones of pregnancy in ovariectomized mice. The altered granules coincide with: (1) the increased histochemical stainability of acid mucopolysaccharides in the ground substance of the cervix; and (2) with the increased dilatability of the cervical canal and softening of the cervical wall, both of which are necessary to facilitate the passage of fetuses through the cervical canal at birth. (Dr. Leppi)

Studies on Endocarditis

It was shown previously that subcutaneous injection of epinephrine in oil may induce in rats a severe myocarditis and sterile valvular lesions which can be largely prevented by prior treatment with the adrenergic blocking agent, Dibenamine hydrochloride. Accordingly, several series of rats were given either one or two intravenous injections of a broth culture of *S. mitis* or *Staph. aureus*. Each series was divided into a control group given bacteria alone, a group given 2 daily doses of 5 mg/kg epinephrine in oil subcutaneously beginning the day before the first bacterial injection, and a third group receiving, in addition, 2 doses of 50 mg/kg Dibenamine hydrochloride 1 and 2 days before the first dose of epinephrine in oil. The animals receiving bacteria and epinephrine in oil developed a significantly higher incidence of bacterial endocarditis than either controls or rats given Dibenamine prior to epinephrine in oil. The incidence of bacterial lesions involving the renal papilla was also higher in rats given epinephrine in oil than in controls. All groups had a high incidence of focal interstitial nephritis.

It is suggested that a relative and localized hypoxia induced by epinephrine in oil may be a major factor contributing to the increased susceptibility of epinephrine-treated rats to bacterial endocarditis and renal bacterial papillitis. (Dr. Highman and Dr. Altland [LPB–NIAMD])

Studies on Dimethyl Sulfoxide

Because of the unusual ability of dimethyl sulfoxide (DMSO) to act as a carrier of drugs and other agents in facilitating their passage through membranes and even intact skin, studies were initiated to determine the effect of

DMSO on alterations in serum enzyme levels induced by exercise and other stresses.

Administration of 4.5 g/kg dimethyl sulfoxide (DMSO) i.p., without exercise increased slightly serum transaminase levels within 24 hours. DMSO combined with exercise (5 hours) increased serum enzymes as much as nine-fold and intensified all 5 isoenzyme bands of SLDH. In addition the isoenzyme band of malic dehyrogenase associated with the mitochrondria was found in the serum. Nineteen hours after terminating exercise, fatty changes in the liver and thigh muscles occurred in all 6 rats given DMSO and in only 3 of 6 untreated rats. These results suggest that DMSO in exercised rats enhances the permeability of cellular and mitochondrial membranes, facilitates the release of tissue enzymes into the circulation, and may increase the incidence of fatty changes in the liver and in thigh muscles.

Studies have been initiated on the effects of DMSO in rats exposed to a cold environment and to high altitude hypoxia. (Dr. Highman, Drs. Altland and Nelson [LPB–NIAMD], and Dr. Garbus)

In a study with Drs. Hansell and White at the Armed Forces Institute of Pathology, it was shown that DMSO has a radioprotective effect in rats. (Dr. Highman)

Studies on High Altitude Hypoxia

In collaboration with Dr. Altland (LPB–NIAMD), studies have been completed on gas and acid base characteristics of unanesthetized rats exposed to gas mixtures low in oxygen in order to provide basic respiratory data at levels of hypoxia shown previously to produce pathological changes in heart, kidney, and sex organs.

In another study with Dr. Altland, it was shown that ground level exercise training prior to the performance of 4-hour daily periods of exercise at high altitude significantly reduced enzyme and tissue changes and increase reproductive performance and survival.

Studies are nearing completion on the serum enzyme and tissue changes in rats exposed nearly continuously to high altitude hypoxia. (Dr. Highman, Drs. Nelson and Altland [LPB–NIAMD])

Studies on Hamycin

In a study of hamycin toxicity in dogs with Drs. Emmons, Utz, Williams and Witorsch (NIAID), this polyene antifungal antibiotic was administered over a period of two months in daily oral doses of 10, 20 and 50 mg per kg. Azotemia with subsequent death occurred in four of six dogs receiving the high dose and in only one of eleven animals receiving the lower doses. Transient diarrhea developed during the first week of drug administration in all dogs receiving 20 and 50 mg per kg per day. Histopathologic findings were limited to the kidney, lesions occurring in all six high dose animals and two of eleven lower dose animals. The toxicity of orally administered hamycin, clinically and perhaps histopathologically, appears similar to that of intravenously administered amphotericin B. (Dr. Highman)

Cycasin Research

Experimental studies with the glycoside cycasin (β-D-glucosyloxyazoxymethanol) and its aglycone (methylazoxymethane) have continued throughout the year. The availability of substantial amounts of the crystalline glycoside has made it possible to prepare the aglycone as needed, a considerable advantage because of its relative instability on storage. Without the collaborative effort of the germ-free unit of LNE of NIAMD, several significant studies would not have been possible.

Studies in Germ-Free Rats

Cycasin when fed to germ-free (g.f.) rats is nontoxic and noncarcinogenic. The glycoside is excreted nearly quantitatively in urine and feces indicating that the g.f. rat cannot deglucosylate the glycoside. The aglycone is toxic and carcinogenic in g.f. rats. It was postulated, therefore, that the enzymic hudrolysis of the glycoside was dependent on a bacterial flora capable of providing a β-glycosidase necessary for hydrolysis. Studies are now in progress in which g.f. rats are monocontaminated with pure bacterial cultures previously tested for their ability or inability to deglycosylate cycasin. The effect or various bacterial strains on the hydrolysis of cycasin is estimated from

survival rates, determination of urinary and fecal excretion of the nonhydrolyzed glycoside and histopathologic examination of the viscera. (Drs. Spatz and Smith, Mr. McDaniel [LNE–NIAMD], and Dr. Laqueur)

Mutagenic Effect

Methylazoxymethanol (MAM) which is known to methylate nucleic acids, is a good bacterial mutagen. Several histidine requiring mutants were caused to revert to histidine in dependence, at many times the spontaneous reversion rate, when they were grown in the presence of MAM. As a mutagen MAM appears to have a specificity similar to that of other alkylating agents such as nitrosoguanidine and dimethylsulfate. (Dr. Smith)

Transplacental Induction of Tumors With Cycad Material

This study is a logical follow-up to previous observations in which the successful production of nephroblastic tumors had been found to be dependent on the young age of the animals. Since comparable neoplasms in man occur almost exclusively in small children, the possibility of a chemical induction of such tumors has been entertained for a long time.

Exposing female rats at various times of pregnancy and at various concentrations to the crude cycad material for 2–3 days has resulted in tumors of uterus, brain, kidney, colon, small intestine, breast and lung. Of particular interest was one litter in which 4 of 5 siblings had tumors of the small intestine at similar sites. The overall incidence of transplacentally induced tumors has been 27% thus far.

A high mortality among the offspring was noted in several litters and further investigation indicated that the mammary glands of mother rats were less developed and less functioning when compared with normal rats at comparable days of lactation. A study is now under way to examine the contribution of mother and offspring to the decrease in lactation by using foster nursing. (Drs. Spatz and Laqueur)

Prevention of Acute Toxicity of Cycasin

One of the biologic effects of cycasin has been the production of large numbers of chromosomal aberrations in onion roots exposed to low concentration of the glycoside (Teas, *Science, 149:* 541, 1965). At the concentrations used, the effect was comparable to a dose of 200 r gamma rays. Among several agents known to have radioprotective actions, cysteamine was found to be most effective producing survival rates of 90% from a dose of cycasin lethal in unprotected rats within 48 hours. The high survival rate was dependent on a relatively short period of time during which systeamine was effective prior to cycasin administration (10 minutes). If extended to 30 minutes, the survival rate decreased to 20%; if cysteamine was administered after gastric installation of cycasin, it was nonprotective. The survivors are being observed now for possible development of tumors. (Dr. Hirono)

Transplantation of Cycasin Induced Neoplasms

Several cycasin induced renal neoplasms and one MAM induced renal tumor in a germ-free rat have been successfully transplanted during the past year. One of these, now in the 13th transplant generation has required successively smaller inoculums for takes. Attempts to convert these tumor lines into ascites tumors are continuing. The majority of the primary tumors thus far were obtained from Fischer rats which had received a single subcutaneous injection of 2.5 mg of cycasin on the first day of life. (Dr. Hirono)

LABORATORY OF CHEMICAL BIOLOGY

The research of the Laboratory of Chemical Biology centers around the biological aspects of proteins. Various groups in the Laboratory are investigating covalent structure, genetic control of biosynthesis, factors concerned with the formation of specific tertiary structure, relationships between structure and function, and the nature of the molecular lesions in chemistry and in synthetic control mechanisms in health and disease.

Structure-function relationships in proteins

It has been shown in previous years, both by members of this laboratory and by other

groups, that the primary sequence of protein molecules determines the final three-dimensional conformations of the biologically active macromolecules without the participation of other detectable genetic information. For example, fully reduced protein molecules in which the cross-linked parent substances have been converted to random polypeptide chains can undergo essentially complete reoxidation with reformation of specific disulfide bonds and with regeneration of full enzymatic activity. Furthermore, a variety of functional groups on the side chains of the amino acid residues can be modified by, for example, acylation, peptidylation, and esterification without interfering with this refolding process. The results are consistent with current hypotheses that suggest that the major forces that direct and stabilize the internal structure of a protein molecule are given by hydrophobic residues that reside in the essentially nonaqueous interior of the macromolecule. Among such stabilizing residues are certain of the tyrosine residues that occupy sterically hindered positions in the structure. It has now been shown (Goldberger and Scheraga) that a variety of iodinate ribonuclease derivatives that retain high levels of enzymatic activity are no longer able to refold after reduction. These experiments thus differentiate the essential role of certain residues in activity as opposed to their residues in activity as opposed to their importance as steric factors in three-dimensional stabilization. A detailed analysis of a large number of examples from our own work and from the literature indicates a strong correlation between "permissibility" of mutations that lead to the replacement of hydrophilic residues by other hydrophilic residues or hydrophobic residues with other hydrophic residues in the three-dimensional structure of the protein in question. In short, mutations that change "inside-type" residues to "outside-type" residues are inconsistent with the formation of the biological activity is basically a problem in geometry rather than a problem in linear arrangement; the latter need only lead to approximately the same geometric solution (Epstein, Anfinsen, Goldberger). In some instances, prosthetic groups may have an additional modifying effect on the conformation assumed by the polypeptide chain of the protein in the absence of the prosthetic groups. Schechter and Epstein have studied such a system by measuring structural parameters of myglobin durings its renaturation in the presence and absence of heme. The heme group clearly causes a slight, but undoubtedly extremely important, further change in the conformation of this protein, over and beyond the conformation of the apoprotein itself.

At a higher level of structural organization, studies have been made of the specific aggregation of subunits in the protein β-galactosidase, which contains in its native form four monomer units of 135,000 molecular weight. Mutants in *E. coli* have been selected that produce either monomers or dimers and that independently are devoid of enzymatic activity. These two forms can, however, undergo *in vitro* complementation during which the two kinds of faulty subunits can, in a sense, repair one another with the production of tetramers that now show activity. By labelling one of these forms with fluorescent dye, the kinetics and stoichiometry of the complementation reaction can be studied. This method will shortly be applied to a broader study of complementation between a variety of other inactive mutant forms. (Steers, Shifrin, Craven, Anfinsen)

Another protein system composed of a number of monomer units aggregated into a larger biologically whole that has gone under investigation is the protein phytochrome. This macromolecule is the essential photochemically active substance in plants that is concerned with the control of morphogenesis and flowering. The highly purified phytochrome (Steers, and Correll of the Smithsonian Institute) has been examined by chemical and physical methods and it has been shown that the protein exists in several aggregated forms, each reflecting a different state of plant activity. The form characteristic of exposure to red light has a molecular weight of about 200,000 while that present in far red light is about 1,200,000. Both forms dissociate into subunits of approximately 40,000 in the presence of denaturing agents.

Since occasional mutations lead to inactive proteins that still demonstrate immunologic cross reactivity with the native protein, it is of interest to study the immunologic cross reactivity as a function of the location of the genetic lesion within the genetic material that determines the structure of the protein molecule. Such studies have been carried out with a homogeneous isomerase which is one of the enzymes involved in the biosynthesis of histidine in *Salmonella*. Eighty-five mutants of this organism were grown under conditions in which the defective protein was synthesized, and the presence or absence of immunologic cross reaction was estimated by double diffusion experiments. The mutants were also studied after exposure to a variety of mutagens, or following growth in the presence of a suppressor strain that would counteract the effects of certain so-called amber or ochre mutations. An analysis of the over-all data indicated that those inactive mutants that retain immunologic cross reactivity represent either "missense" or possible "in-frame" deletions. Cross reactivity was not retained by strains involving amber and some ochre mutations, and by mutations that appear to involve genetic deletions or frame shifts. (Goldberger and Margolies)

In connection with the studies mentioned above on the reformation of specific disulfide bonds in reduced protein chains, the disulfide-sulfhydryl interchange enzyme previously described has been purified to a state of homogeneity from beef liver microsomes. The protein, having a molecular weight of 42,000, contains three half-cystine residues, one of which must be in the SH-form for activity. The portion of the amino acid sequence of the protein that contains this essential SH group has been isolated from trypsin digests, and its sequence is now essentially completely known. The reduced enzyme is capable of attacking and catalyzing the rearrangement of incorrect disulfide bonds that have been deliberately introduced into several protein substrates, including ribonuclease, soy bean trypsin inhibitor, lysozyme and trypsinogen. The enzyme is capable of catalyzing renaturation in the absence of added mercaptoethanol, and the efficiency of the renaturation process appears to be a function of the number of disulfide bonds in the protein under consideration. It is planned to investigate the distribution of this enzyme and its comparative sequential chemistry in the neighborhood of the active center SH group in a variety of marine organisms in collaboration with Dr. Francesco DeLorenzo. (DeLorenzo, Fuchs, Anfinsen, Venetianer)

Structure, function and synthesis of a nuclease from Staphlococcus aureus

The following advances have been made since last year's report:

The total amino acid sequence of the nuclease has now been elucidated. The protein contains 149 amino acid residues and is devoid of cysteine and disulfide bridges. Reconstruction of the sequence was carried out by isolation and sequence determination of the peptide fragments produced by cyanogen bromide cleavage and by digestion with trypsin and chymotrypsin. The calculated molecular weight from the sequence information agrees with values obtained by amino acid analysis of the total protein and by physical methods.

The single tryptophan residue and the three histidine residues of the nuclease have been attacked with specific chemical reagents to investigate their possible role in the active center of the protein. Treatment with N-bromo succinimide destroys the tryptophan moiety specifically and leads to parallel loss in activity. Photooxidation causes destruction of the histidine residues and once again causes inactivation in parallel. The carboxyl terminal glutamine residue may be removed by digestion with carboxypeptidase and full activity is retained.

Since a major aim of the work in the Laboratory is to establish unequivocally that the primary amino acid sequence yields the tertiary structure of proteins, experiments have been initiated aimed at the total organic synthesis of the protein. Peptide fragments of the sequence are being synthesized by application of a solid phase method based on that developed by Merrifield and his colleagues. Amino acids are added in a stepwise fashion, employ-

ing the activated succinimide esters. Efforts are under way to determine the optical specificity of the synthetic methods of examination of the synthesized peptide fragments with leucine aminopeptidase and carboxypeptidase.

X-ray crystallographic study of crysals is being carried out collaboratively with Drs. Hazen and Cotton at MIT. Native crystals have given diffraction patterns up to 1.5 A, and photographs permit the determination of the space group and the number of protein nomomers per unit cell. Efforts to prepare heavy metal isomorphous replacements are in progress. (Anfinsen, Taniuchi, Hazen, Cotton)

The conformational papameters of the nuclease were elucidated by determination of the molecular weight (by high-speed and low-speed ultra-centrifugal equilibrium techniques), intrinsic viscosity, diffusion coefficient, optical rotatory dispersion, sedimentation velocity, isoionic and isoelectric points, frictional ratio, axial ratio, viscosity increment, and β-papameter. The molecule is a compact globular protein without disulfide bridges, with an axial ratio of 3.7 to 1. The effective hydrodynamic unhydrated particle is a prolate ellipsoid with a frictional ratio of 1.16 and a β value of 2.19. The molecular weight is 17,000. (Suriano, Heins, Anfinsen)

Mechanisms of control of biosynthesis of histidine in Salmonella typhimurium

The synthesis of histidine in *Salmonella* occurs under the catalysis of 10 enzymes in a sequential pathway. Each enzyme is under the control of a specific gene and the 10 genes are linked in a so-called "operon" system in the genetic material of the host cell. When histidine is limited in the growth medium, the entire process undergoes what is known as derepression, and the required anzymes for synthesis are then produced in quantity. Two of the proteins in the series have been subjected to careful purification procedures. The first, isomerase, was referred to above in connection with immunologic studies. This protein has been carefully studied with respect to its physical and chemical as well as its antigenic activity. A second protein, phosphatase-dehydrase, is now nearly in a homogeneous state. This protein is of particular interest since genetic control of both the phosphatase and dehydrase actions appears to be by a single gene. It seems likely that these two catalytic activities are part of one physically integrated unit composed of several polypeptide chains. Purification of this and other enzymes in the histidine operon group to a high degree of purity will shortly permit a detailed study of the kinetics and mode of synthesis by employing pulse labelling techniques. (Goldberger, Margolies, Henderson)

In another study on this system, an attempt has been made to test directly the hypothesis that so-called deletion mutants cause the physical deletion of part of the chromosomal DNA. A mutant was studied involving a genetic deleletion between two of the genes (D and A) in the operon. The time interval between the appearance of D and A enzyme activity, normally about 7 minutes, had disappeared. These results are consistent with deletion of DNA material, with annealing of the two loose ends thus produced. (Goldberger, Berberich)

In the synthesis of histidine by *Salmonella* the synthesis of the 10 enzymes involved proceeds in a sequential manner. Thus, the enzyme responsible for the first step appears shortly after derepression by limitation of histidine in the growth medium, then the second and so on. Investigations for a number of mutants involving faulty synthesis of one or another of the 10 enzymes now shows that this derepression in a temporal sequence occurs only in those mutants that are capable of producing the intermediate 4-amino-5-imidazole carboxamide ribonucleoside, whereas simultaneous derepression without a sequential series of time lags in observed in those mutants incapable of producing this intermediate. The addition of 4-amino-5-imidazole carboxamide ribonucleoside to cultures of mutants ordinarily characterized by the simultaneous mode of derepression served to shift the mode from simultaneous to sequential but did not influence the derepressed rate of enzyme synthesis. In organisms in which the sequential mode is ordinarily observed, adenine effected a shift to

the simultaneous mode, causing all the enzymes to derepress at the same time, but did not influence the derepressed rate of enzyme synthesis. Chloramphenicol, at low concentrations, reduced the growth rate of derepressed cells by a factor of 2 to 3 and decreased the rate of derepression of the histidine enzymes in all organisms tested. In the presence of chloramphenicol, adenine shifted the mode of derepression of *hisE11* and LT2 from sequential to simultaneous, just as it did in the absence of chloramphenicol. However, adenine did not eliminate the effect of chloramphenicol on the rate of derepression. The data presented here suggest that the histidine operon is transcribed into a polycistronic message which, under conditions where sequential derepression is observed, is translated from one end only. Under conditions in which simultaneous derepression is observed, the translation of this message is initiated at the beginning of each cistron.

Biochemical studies preliminary to the investigation of genetic defects in higher organisms, including man

With the aim of establishing a chemical basis for the ultimate study of protein synthesis and its genetic regulation in the tissue of higher organisms, metabolic and cytologic work has been carried out on livers of mice and rats. Intact liver cells have been prepared with the use of tetraphenylboron, a compound that breaks intracellular potassium-dependent bonds. These intact separate cells have been shown to exhibit the capacity to incorporate labelled amino acids, but this incorporation appears to be resistant to inhibition by puromycin and depends upon added energy sources for optimal incorporation. The population of individual liver cells has been examined for the distribution into discrete ploidy classes. Cells that differ two-fold in ploidy differ two-fold in volume. There appears to be little change in cell volume during aging. These studies will furnish important guide lines for investigations on mice, shortly to be initiated, involving the biochemical lesions in certain dominant and recessive traits that exhibit their effect during early stages of differentiation. (Epstein, Friedman) A group of patients with Werner's syndrome, a recessively inherited disease that simulates premature senility, were investigated clinically in collaboration with Drs. Motulsky and Martin of the University of Washington. An extensive review of the clinical, pathological and biochemical features of the disease was carried out, and their relationship to the features of "natural aging" defined. It was found that in some respects the lesions in Werner's syndrome were similar to those of aging, although they might be more severe. In other regards, including the severe skin changes, cataracts, peripheral wasting, and predisposition to diabetes, the findings were unique. An approach to further biochemical investigations, using skin fibroblast cultures, was initiated. (Epstein)

LABORATORY OF BIOCHEMICAL PHARMACOLOGY

Studies with EDTA-treated *E. coli*

Previous work by Dr. Leive had shown that treatment of *E. coli* with EDTA results in a non-specific increase in permeability, which permits entry of several unrelated molecules, including the drug actinomycin.

Inhibitory Effect of Actinomycin

The ability of EDTA-treated *E. coli* to be inhibited by actinomycin has permitted a number of different studies related to RNA that were not previously possible in the organism:

The half-life of messenger RNA was shown to be about 1.5 minutes. Messenger RNA was shown to account for 1–3% of the total bacterial RNA.

A ribosomal precursor particle, including 23 S RNA, was found. Newly synthesized 23 S RNA gradually was incorporated into a 40–45 S particle, and only later was incorporated into a 50 S ribosome.

Studies on the induction of β-galactosidase supported the hypothesis that inducer acts by stimulating the formation, and not the utilization, of specific messenger RNA.

Surface Changes in EDTA-*Treated* E. Coli

In addition to the permeability change EDTA treatment causes rapid release of about half of the lipopolysaccharide (endotoxin) layer of the cells. In chemical analysis this lipopolysaccharide is identical to material extracted from the cells by more conventional means.

Bacteriophage studies

Studies in EDTA-Treated E. Coli

Previous work by Drs. Korn, Leive, and Protass showed that extremely low concentrations of actinomycin prevent the formation of bacteriophage T4 in EDTA-treated *E. coli.* This was surprising since these concentrations do not affect other RNA, DNA or protein biosynthesis. Further work was carried out on these effects; as little as 5 molecules of actinomycin per T4–DNA molecule are effective. (D. Korn, J. Protass)

T-phage adsorption is not inhibited in EDTA-treated cells; on the other hand, temperate phage adsorption is inhibited. This suggests that there are fundamental differences between receptors for T-phage and for temperate phage adsorption. (Korn and Protass)

In T-phage infection messenger RNA for a late enzyme is not produced until the time that the enzyme activity appears in the infected cells, indicating that the mechanism controlling the late expression is at the level of phage DNA *transcription* (i.e., not that of mRNA *translation.* (Korn andProtass

Lysogenic Induction

During studies of the synthesis of the two known λ-directed enzymes, DNA-exonuclease ("early") and lysozyme ("late") were studied. It was found that (*a*) exonuclease synthesis is independent of phage DNA replication, (b) lysozyme synthesis does not occur if DNA synthesis is inhibited. These and other studies indicate that these two enzymes are encoded in different operons of the λ-genome, and that the λ-repressor acts directly upon the early operon only.

Studies were also carried out with the r_{11} mutants of phage T4, that cannot propagate in K12(λ). This was shown to be due to the presence in the cell of at least that part of the λ genome that controlled repressor synthesis (i.e., rather than loss of bacterial function). (D. Korn and J. Protass)

Ribosome structure and function

E. coli ribosomes can be purified by DEAE-chromatography, yielding preparations with less protein. These DEAE-ribosomes can bind phenylalanyl-sRNA, and participate in polypeptide synthesis *in vitro.* (A. Furano)

Dye-stacking experiments indicate that the RNA in the intact ribosome is essentially single-stranded, and thus is in a quite different conformation from the isolated ribosomal RNA which has significant amounts of double-helical structure. (A. Furano and D. Bradley)

Even though ribosomes have polyamines, these exchange with added polyamines (contrary to data in the literature). These findings demonstrate that data on the polyamine content of ribosomes reflect the conditions of isolation, and not necessarily the polyamine concentration *in vivo.* (C.W. Tabor and P. Kellogg)

Amine studies

The spermidine "conjugate" in *E. coli* has been purified. It contains gluthathione in peptide linkage with spermidine. (C. W. Tabor and H. Tabor)

An enzyme has been purified 800-fold from *E. coli* that converts spermidine to a "conjugate" in the presence of glutathione. (C. W. Tabor and H. Tabor)

Further purification has been obtained for the Serratia enzyme that degrades spermidine to Δ^1 pyrroline and 1,3-diaminopropane. (C.W. Tabor and P. Kellogg)

The primary product formed by the action of beef plasma amine oxidase on spermine or spermidine is secondarily converted to acrolein and 1,4-diamino butane by heating. (C.W. Tabor and P. Kellogg)

A monoamine oxidase has been purified $>$ 450 fold from rabbit serum, and character-

ized. Of particular interest is the finding that protonated species of the amines interact with the enzyme (in contrast, the nuprotonated form reacts with the human monoamine oxidase). (C. M. McEwen)

Serum levels of monamine oxidase in 119 cases of liver disease indicated a positive correlation of elevated enzyme levels with hepatic fibrosis, portal hypertension, and portal-system collateral circulation. This may possibly offer promise as a simple screening test for cirrhosis. (C. M. McEwen with Dr. Donald O. Castell, Dept. of Medicine, U.S. Naval Hospital, Bethesda Md.)

Polysaccharides and glycoproteins

A new rhamnose nucleotide, UDP-L-rhamnose, has been isolated and characterized from a bacterial source. (V. Ginsburg)

Tracer experiments have shown that the ribose in lipopolysaccharide (*Salmonella artis*) is formed by the same pathway as RNA ribose. (R. Kaufman)

Control of enzyme levels in mammalian tissues

Animal tissues contain four distinct hexokinases, including a liver specific high K glucokinase and three different low $K_m$hexokinases. The latter have been purified and characterized. Each purified enzyme type, regardless of tissue source, has certain unique properties that distinguish it from other hexokinase types. (R. T. Schimke, L. Grossbard and E. W. Sweeney)

The inactivation of α-glucosidase of *Saccharomyces cerevisiae* had been studied as a model of "deadaptation". This inactivation requires energy, and is prevented by a cycloheximide, an inhibitor of protein synthesis. (R. T. Schimke and E. W. Sweeney)

Burn—shock studies

Burns

Studies were continued on the role of bacterial infection in the mortality observed after burns.

E. coli monocontaminated mice or *α-hemolytic streptococcus* and pasteurella decontaminated mice show no difference in mortality after burns from germ-free mice. (K. Markley and E. Smallman)

Burned mice were 300–1000 times more sensitive to *E. coli* and *S. typhosa* endotoxin than non-burned mice. Mice tolerant to a lethal dose of endotoxin showed a lower shock mortality after burns. (K. Markley and E. Smallman)

Simple methods were devised for standardizing fatal pseudomonas infections in burned mice. Six drugs were found to be effective in treatment. In view of the importance of bacterial infection in clinical burns, this method may be very important in the evaluation of new therapeutic agents. (S. M. Rosenthal)

Tourniquet Trauma

Somewhat surprisingly germ-free mice showed a significantly decreased shock mortality compared to conventional mice. Both groups were very sensitive to *E. coli* endotoxin. Mice made tolerant to endotoxin were protected partially against trauma, and mice surviving tourniquet trauma were protected against endotoxin. These studies implicate endotoxin as an important factor in shock mortality, in addition to the electrolyte and fluid factors previously reported from this laboratory. (K. Markley and E. Smallman)

Sulfur-containing compounds

It has been previously reported that the enzyme glutathione reductase has two reducible bonds per unit of its flavin content, only one of which is involved in its catalytic function. From recent experiments a major new concept has developed according to which the second reducible bond, which appears to be a disulfide, does function in a regulatory mechanism although it does not participate in the catalytic mechanism. When this bond is reduced the enzyme ceases to function. Its reduction is hindered by TPN which therefore may be classified as an activator. (S. Black, S. Hopper and Blondel Hazel)

Leprosy studies

Growth of acid-fast organisms have been observed in macrophage cultures after inocu-

lation with biopsy material from human leprosy cases. A few serial passages were successful. (Y. Chang and R. Anderson in Dr. Knight's laboratory)

Improvements have been made in the growth conditions of *Mycobacterium lepraemurium* in macrophages, permitting a generation time of 7 days, which is comparable to the growth rate of this organism in mice. (Y. Chang and R. Anderson)

LABORATORY OF PHYSICAL BIOLOGY

The Laboratory has experienced a productive year, with no major changes in general orientation. Research highlights for fiscal 1966 are summarized briefly below, grouped in one of several possible series of informal categories.

Molecular Structure

A major interest of LPB continues to be physicochemical studies of molecular structure and interaction at levels of organization ranging from interatomic forces in simple inorganic molecules to tertiary and quaternary structure of proteins and polymers. Last year's Report went into some detail about the principles of operation of NMR (nuclear magnetic resonance spectroscopy), EPR or ESR (electron spin resonance spectroscopy), IR (infrared spectroscopy), ORD (optical rotatory dispersion), CD (circular dichroism) and other physical methods commonly used by LPB scientists: that material will not be repeated here. Mention should be made, however, of our rapidly growing use of electronic computer technology for collecting and analyzing data.

In a continuing effort to specify unique intramolecular force fields for hydrides and fluorides of Sn, B and related Group IV–VI elements it was found that Coriolis coupling constants could be used to supplement rotational distortion and vibrational amplitude data. (Levin, Abramowitz, Ziffer, Berney, Comeford)

Isotopically labeled oxyhalide complexes of Cr, Mo and W have been investigated by ESR in an attempt to obtain electron density measurements and in-plane bond parameters. (Kon, Sharpless) Free radicals of pyruvic acid derivatives were examined by ESR to identify isomers and ESR spectra of irradiated ribonuclease, gelatin, myoglobin and lysozyme were studied to determine optimum exposure to tritiated H_2S for γ-ray radiolysis. (Kon, Cahnmann, Matsuura, Riesz, White)

NMR spectra of the iodo, methoxy, nitro and other analogs of DDT have been analyzed in a continuing study of the resonance of the aromatic rings. (Sharpless)

By studying the UV spectra and optical activity of various acylchymotrypsin derivatives and related model compounds it has been possible to determine that the normal s-trans configuration of the C=O bond, with respect to the C=C in acrylic substrates, is isomerized to the s-cris configuration. This explains why the more energetic cis configuration is favored when the substrate complexes at the active site of the native enzyme. (Charney, Bernhard)

UV spectra of substituted ethylenes at various temperatures have been recorded to provide a basis for a quantum mechanical explanation of the so-called mystery spectral band of olefins. (McDiarmid, Charney)

Instrumentation and theory have now been completed for electric field induction of dichroism and birefringence in DNA and polypeptides, from which information on tertiary conformation may be expected. (Yamaoka, Charney)

Similar plans for gaseous HCl are also about to bear fruit. (Needham, Charney)

NMR studies of base pairing of nucleosides have shown specific hydrogen bonding similar to that between polynucleotides and have provided thermodynamic and kinetic data for elucidating internally hindered rotation of amino and methylamino groups in substituted cytosines. A new technique for IR spectroscopy at 20° K is affording much improved band resolution. (Becker, Miles, Shoup, Gramstad, Bradley)

The three photodimers of the cyclohexenone piperitone have been studied by NMR and identified as head to head dimers. The stereoisomerism at the junctions of the 6- and 4-membered rings has been determined as both cis in two of the dimers and at least one trans

in the third (Ziffer). A similar stereochemical analysis is now being applied to steroidal compounds. (Ziffer, Williams)

ORD measurements of non-planer conjugated transoid dienes confirm the validity of a rule, derived in this laboratory, that connects the sense of skewness of the chromophore with the sign of the Cotton effects. A heteroannular cisoid diene, however, has given a Cotton effect of sign opposite to that predicted, and will require further investigation. (Weiss, Ziffer, Sharpless)

An analysis of ORD curves of the combination of the dye acridine orange with DNA and polyglutamic acid showed that four Cotton effects are required for unique specification of the band structure in both cases. (Yamaoka and Resnik). In the course of this work an important source of artifact in certain ORD instruments was discovered. (Resnik, Yamaoka)

Surface Phenomena

Besides work involving excitation and permeability changes, much LPB research impinges on, implicates or implies controlled passage of material across cellular and subcellular membranes. The Laboratory also supports investigations aimed at providing defined and precisely controlled physicochemical models for certain properties of biological membranes.

Work has continued on defined synthetic liquid ion-exchange membranes which exhibit high selectivity and transmissivity for most inorganic and organic ions. The behavior of such membranes has also been treated theoretically in relation to carrier transport and in comparison with biological membranes. (Sollner, Shean)

Exploration has begun of long-range repulsive forces, amounting to as much as 25 dynes/cm^2 over distances as great as three microns, which arise between charged glass surfaces in liquid media. Such forces are important in colloidal dispersions and organized structures. (Sollner, Gottlieb)

The forces which maintain structural integrity and reactivity of manomolecular layers have been studied with phosphate films spread on thin oil membranes. The role of hydrogen bonding in phosphate group aggregation with H^+ decrease has been defined, and a 10 cycle/sec fluctuation in membrane resistance has been induced by low D.C. voltages. (Gershfeld)

Physiology and Biochemistry

Dimethyl sulfoxide, an industrial solvent with a remarkable spectrum of biomedical effects, was found to intensify the effects of exercise upon rats, as reflected in liberation of various tissue enzymes into the serum, and pathological changes. (Altland, Highman, Nelson, Brubach, Barbus, and Thompson)

Rat lysosomes were found to be ruptured by hypoxia before serum enzyme concentration increases suggesting that lysosomal enzymes are important in initiating hypozia pathology. (Nelson, Parker)

In studying protein metabolism during insect metamorphosis it was found that nearly twenty distinct small peptides occur in larval blood, that blood protein concentration increases some 300-fold during larval growth and that larval blood proteins are probably transferred intact into adult tissue. (Levenbook, Bauer, Chen)

Excitation and Coupling

Processes by which one type of energy is transduced into another or one cell structure triggers activity in another site have increased several LPB investigators.

Previous work on the linkage between absorption of light in the squid retina and the generation of electrical current in visual elements is being supplemented by chemical studies of the visual pigments. The results suggest that retinaldehyde links to the protein opsin via the ε-amino group of lysine (F. Hagins, Gross, Bauer, W. Hagins). Changes in Cotton effects of rhodopsin with exposure to light suggest a three point association, with no change in secondary structure of the peptide part of the photopigment. (Hagins, Ziffer). A number of phospholipids associated with rhodopsin have been identified. (Adams)

In striated muscle the electrical excitation that leads to contraction is thought to be con-

ducted into the muscle via infoldings of the surface membrane and to eventuate in release of Ca^{++}. It has now been found that the calcium release depends on depolarization of the "sarcoplasmic reticulum" (Podolsky, Costantin). By comparing the response of skinned slow and fast frog muscle fibers to Ca^{++}, the 10X difference in contraction rates has been shown to be intrinsic to the fiber, not the activation mechanism. (Costantin, Podolsky)

A number of sophisticated optical methods localization of and measurement of molecular orientation of chlorophyll and protein in subcellular structures of green cells have been developed. Preliminary work indicates that these techniques will provide new data for the elucidation of pathways of light-activated energy transfer. (Olson, Jennings)

Biological Interactions

The study of the dynamic influences of cell on cell in an organism (as opposed to a culture) or of organism on organism in a community (as opposed to a population) represents the opposite extreme to the biochemical or ultrastructural dissection of the cellular unit. Several examples have been investigated recently in LPB.

The simple multicellular animal *Hydra* includes species which are colorless and species in which many of the cells contain symbiotic green algae. By freeing green hydras of their algae the process of reinfection and the relative infectivities of algae separated from host tissue, of several species of free-living green algae, and of algae migrating from grafted green tissue, have been studied. (Park, Ortmeyer, Greenblatt)

The colonial marine soft coral *Renilla* is favorable for study of nervous conduction at its simplest level because the individual zooids are connected by a non-polarized slowly-conducting nerve net. By local electrical stimulation and recording of induced bioluminescence the kinetics of the responses of the two classes of individuals in the colony have been determined and some details of the coordination of excitation established. (Hanson, Buck)

A unique example of insect communal activity, in which thousands of fireflies flash in rhythmic synchronism for long periods, was studied in Borneo and Thailand. Coordination was found to be precise within 30 msec in a 500 msec cycle, to depend on visual feedback, and to involve central nervous delay and adjustment of endogenous excitation with respect to the preceding mass flash. (J. Buck and E. Buck)

Biological Ultrastructure

Our electron microscope facility has made possible a variety of findings, ranging from evidence supplementing or confirming results from other methods to full-dress cytological investigations.

In studies of frog striated muscle an improved test for intracellular localization of sodium was developed (Tice). Glucocoricosteroids were found to destroy the mitochondria of both red and white muscle of the rat, but affect fibrillar structure only in red muscle. (Tice, Engel)

A comparative study of the photogenic organs of certain American and Far Eastern fireflies has revealed numerous systematic differences, the most striking being the intracellular penetration of air tubules and direct nerve-photocyte connections in the oriental forms. (Peterson)

Electron micrographs of human sickle cell anemia erythrocytes and of extracted S-hemoglobin suggest that the pigment molecules can stack in chains, the chains aggregating in parallel to form six-stranded hollow molecular cables. (Murayama)

Ox liver catalase has been shown to consist of approximately spherical units which give a tetramolecular orthorhombic unit cell in dry crystals and a hexagonal bimolecular association in wet crystals. Thyroglobulin molecules do not form true crystals but long chains of linearly associated spherical units in random side-by-side array. (Labaw)

Electron micrographs were supplied as illustrations for several investigations described elsewhere. (Hanna)

Cellular Biology

Cells as functional units have received an unusual amount of attention from LPB investigators this year.

In a continuing attempt at exhaustive biochemical characterization of a particular cell type, a comprehensive analysis of carbon metabolism in the green flagellate *Euglena* has been completed. As part of a survey of extraordinary metabolic capabilities in microorganisms, *Thiobacillus thiooxidans* has been found able to grow in endogenous H_2SO_4 and survive at an acidity of pH 0.0, and *Halobacter salinarium*, growing in a required concentration of 4 M NaCl, has been shown not to excrete large amounts of nucleic acid as had been reported in the literature. Kempner, Miller)

In the luminous bacterium *Achromobacter fischeri* a "noise spectrum" analysis has shown that the cell does not emit photons in packets (Hanson, Hagins), and continuous recording of light emission during culture growth demonstrates a wide but systematic discrepancy between cell number and luminous output. (Hanson, Kempner)

The rat hemoparasite *Trypanosoma lewisi* cannot ordinarily establish itself in mouse blood, but by blocking the mouse's immunological defenses by administering rat globulins the parasite is enabled to grow in the foreign host. (Greenblatt, Clipper)

LABORATORY OF BIOPHYSICAL CHEMISTRY

The main interest of this laboratory is the study of the correlation between structure and function of proteins with special interest centered on those involved in blood clotting and muscular contraction.

The main aspects of the clotting of fibrinogen have now been clarified. Fibrinogen can carry out its role in hemostasis and wound healing only if two enzymes act on it. Thrombin, the clot forming enzyme of plasma, splits off two peptides from fibrinogen, and as a result, the fibrin molecules form a network. The clot stabilizing enzyme (Laki-Lorand Factor) introduces bonds between amino groups of one fibrin molecule and the asparagin and glutamine groups of another. Apparently, only those asparagin groups are involved that carry a sugar moiety. (Laki, Gladner, Chandrasekhar)

From these basic foundations, we can now branch out to inspect the broader biological significance of this clotting process.

It is now well established that the peptides released from fibrinogen have physiological activity. This could be conveniently followed by their action in protentiating bradykinin. It turned out that in a number of peptides, a region containing -Asp. Tyr- residues, in others, the region containing -Asp. Ser- residues, could be implicated in this activity. Using sympathectomized animals, a direct vasoconstrictor action of these peptides could also be demonstrated. (Gladner, Osbahr, Colman, Laki)

Since our pioneering work, which established the amino acid sequence of the peptides released during clotting of bovine fibrinogen, the sequence of many other of these peptides originating from various animals is known. The similarities in any two of these peptides may be correlated to the time elapsed since these separated from a common ancestor. It was found that if the logarithm of the amount of correspondence (similarity) is used for plotting, a straight line resulted. In contrast to a similar plot of other proteins (cytochrome C, for example), the plot of these peptides cannot be extrapolated beyond the time of the appearance of the vertebrates. From this, we concluded that before the appearance of the vertebrates, a different clotting system may have operated. It is now an established fact that in Limulus (horseshoe crab), the clotting proteins are not freely distributed in the plasma, but are confined to cells circulating in the blood. These cells release the clotting material when they come into contact with endotoxin. Since these cells in the Limulus are the prototype of the platelets of vertebrate plasma, it is now understandable that a serious condition arises if the platelets become sensitized to endotoxin and release their clotting proteins. (Laki)

It appears that clot formation is involved, not only in hemostasis and wound healing, but also in inflammation reactions. Under the in-

fluence of cortisol, an enzyme can be isolated from the skin of a number of animals, which clots bovine fibrinogen by splitting off an elongated version of peptide B. (Gladner, Houck, Murtaugh)

The molecular weight of the liver enzyme, transglutaminase, isolated from guinea pig liver by Folk's group, has been established. (Carroll, Mitchell) This enzyme acts on fibrinogen as the clot stabilizing enzyme.

It is now becoming increasingly evident that without a detailed knowledge of the structure of the contractile proteins, the mechanism of muscular contraction cannot be understood. Therefore, our attention was centered on the molecular aspects of these proteins.

Actin and tropomyosin were modified by enzymatically introducing the amine, putrescine, into the glutamine residues of these proteins. The liver enzyme, transglutaminase, used in these experiments, could be demonstrated to occur in many other tissues. In addition to introducing amines into these proteins, this enzyme could bind actin and tropomyosin together by using actin as the amine donor and tropomyosin as the acceptor. This enzyme may thus function as a last step in protein synthesis by connecting the subunits of a larger protein together. (Laki, Derrick)

Methylated, SH-reduced and S-carboxymethylated tropomyosins were prepared to clarify the conformation of this highly charged and helical constituent of muscle. (Bodwell)

Two years ago, it was shown in this laboratory that methylation of glycerol-treated muscle completely inhibited its ATPase activity, but reduced the shortening response by only 40%. Pyrophosphate, adenosine diphosphate and other large cations became contracting agents for these methylated fibers. This finding prompted us to ascertain which end-groups or residues are methylated. We are investigating this problem by the use of C^{14}-containing dimethyl sulfate (methylating agent). (Bowen)

We have demonstrated that actin and tropomyosin contained an acetylated N-terminal group. These findings gave us further chemical means with which to establish minimum molecular weights for these proteins. (Laki, Alving)

I would like to list now experiments that are not directly related to the topics discussed above, but knowledge gained from these stimulated our way of looking on some of the problems.

The molecular weight of a small, bacterial nuclease was determined by sedimentation and diffusion measurements and found to be consistent with the value calculated from the chemical composition. Using viscosity measurements, this protein, which requires calcium ion for its enzymatic activity, was found to undergo a thermal transition to a more unfolded form at about 50° C., but this transition is prevented or reversed by the presence of small amounts of Ca^{++}. (Carroll, Anfinsen, LCB)

A relatively crude model of lysozyme, based on the assumption that charged groups occur in clusters, gave a chain configuration practically identical with that determined from X-ray crystal analysis. (Saroff)

The excretion of abnormal amounts of cystathionine in the urine occurs in patients which may have various congenital defects and mental retardation. The demonstration of an abnormally high concentration of cysthathionine in tissue extracts of a case of cystathioninuria prompted the hypothesis that this syndrome resulted from a deficiency of the enzyme cystathionase. We tested this hypothesis and found that cystathionase activity was markedly reduced in the liver of a patient suffering from cystathioninuria. (Mudd, NIMH; Finkelstein, VA; Laster, CI; Irreverre)

The discovery of the new amino acid, *cis*- and *trans*-3-hydroxyproline both found occuring in an antibiotic polypeptide, Telomycin, and the *trans*-form, in vertebrate and invertebrate collagen, led to the investigation of the amino acid composition of the proteins and cell wall of the Telomycin producing streptomyces. Whereas the organism synthesizes the two diastereoisomers of 3-hydroxyproline in Telomycin, neither of these two amino acids is incorporated into the proteins or cell wall of the organism.

An unusual amino acid was also isolated from the hydrolysates of the proteins and cell walls of the streptomyces by column and paper chromatography and identified as muramic acid.

A very sensitive, modified Sakaguchi spray for the detection of arginine and monosubstituted guanidine compounds was developed. Due to its sensitivity and stability of color, this spray has been found useful for locating arginine and arginine-containing peptides specially in fingerprinting or sequence studies in proteins.

It was found that polystyrene sulfonic acid, a synthetic model for charged proteins, bound cupric ions quite strongly without causing precipitation. The indication of binding comes from the change of the absorption spectrum of the copper upon the addition of polymer. Barium ion is able to desplace the bound copper and return the absorption characteristics of the free cupric ion. (Carroll, Eisenberg)

LABORATORY OF MOLECULAR BIOLOGY

Structure and Function of Glutamate Dehydrogenase from Bovine Liver

Succinylation of about 4 lysines per chain of the bovine liver enzyme yields an electrophoretically more rapidly moving but still enzymatically active protein. Mixture of the succinylated protein with the native protein (each containing 6 subunits) should permit the detection of exchange of subunits between the two forms of the enzyme. However, electrophoretic analysis of such a mixture showed no exchange even under conditions where allosteric modification of the protein is going on. We conclude that the allosteric change in glutamate dehydrogenase mediated by GTP and DPNH does not involve dissociation of the individual subunits (MW 50,000) from the native enzymatically active molecule (MW 300 to 400,000).

Reaction of the enzyme with fluorodinitrobenzene suggests that only a single lysine is reactive under the conditions tested. The peptide containing this lysine will be isolated and its composition and sequence investigated to provide further evidence for the conclusion presented last year that the subunits of this allosteric enzyme are chemically indentical. (G. Tomkins, J. Kallos, E. Appella)

Enzyme Induction in Bacteria

Continued studies on the lactrose (lac) operon have suggested that the enzymes are induced in sequence rather than simultaneously as a result of the sequential transcription of the *lac* genes. Delay in transcription of the A gene (for thiogalactoside transacetylase) results in a slower production of this enzyme with respect to that of β-glactosidase. A delay in transcription can be induced either by inhibitors of protein synthesis or by nonsense mutations (amber or ochre) in the gene. The delay induced by the latter means can be corrected by suppression of the nonsense mutations. These findings can be rationalized by a model relating messenger RNA transcription in simultaneous enzyme synthesis. (G. Tomkins, P. von Knippenberg, D. Alpers)

Cysteine Biosynthesis

Two enzymes have been identified in extracts of *E. coli* and *S. typhimurium* which catalyze the conversion of L-serine and inorganic sulfide to L-cysteine. The first enzyme catalyzes the reaction: L serine + acetyl CoA to form O-acetyl L-serine. The second enzyme catalyzes the reaction O-acetyl L-serine + SH to give L-cysteine. The gene locus controlling the first enzyme is the Cys E region of the salmonella chromosome. The gene controlling the second enzyme is not yet known. The first reaction is inhibited by the end product L-cysteine. This is the first demonstration of the mechanism of cysteine biosynthesis in microorganisms. (N. Kredich, G. Tomkins)

Enzyme Induction by Steroid Hormones

A newly established line of hepatoma cells growing in tissue culture has been developed. The cells grow exponentially with a doubling time of 24 to 30 hours. Dexamethasone 10^{-5}m or other glucocorticoids induce the synthesis of tyrosine-α-ketoglutarate transaminase from 5 to 20-fold within 6 to 8 hours after their

addition. Induction occurs in dividing or resting cells and is inhibited by inhibitors of either protein or of RNA synthesis. Inhibition of RNA synthesis after maximal levels of the enzyme are reached causes a further stimulation of enzyme synthesis. It is thought that the inducer acts at two levels, the nuclear level and the cytoplasmic level to regulate the synthesis of the protein. The enzyme has been crystallized from rat liver and its physical and enzymological properties studied. An antibody prepared to it shows immunological identity with uninduced rat liver enzyme as well as induced and basal tyrosine transaminase in the hepatoma cells. Experiments involving radioactive amino acid incorporation into immunological precipitates suggests that the increase in enzyme level is due to an increase in the rate of synthesis of the protein. Experiments using C^{14} and tritium labelled amino acids suggests that in addition to tyrosine transaminase several other proteins are also induced by the hormone. Studies on RNA synthesis show minimal if any increase in the rate of messenger RNA production. Other classes of RNA do not appear to be affected by the hormone. (G. Tomkins, E. B. Thompson, S. Hayashi, T. Gelehrter, B. Peterkofsky, D. Granner)

Active Transport of Amino Acids in Salmonella Typhimurium

Further work has been done on the transport systems for amino acids in *Salmonella*. Radioactive lipids have been obtained after exposure for one minute of whole cells to radioactive amino acid. At least four fractions could be distinguished by column chromatography. Two of these liberated the intact amino acid upon mild alkaline hydrolysis. The identification of these radioactive intermediates is under way. Involvement of this fraction into the process of transport is being investigated.

The histidine permease mutant has been mapped (50% linkage with Pur F). The aromatic permease mutant has been partially mapped (between pro A and gal). (Giovanna Ferro-Luzzi Ames)

Amino Acid Incorporation into Protein in Vitro: Mechanisms and Possible Metabolic Controls

Experiments in model systems showing that the rate of protein synthesis directed by synthetic polyribonucleotides can be controlled by reversible alterations in ordered structures of the polymers have been extended. Investigations of the mechanisms of the final step in protein synthesis have been initiated. (E. S. Maxwell, S. Raeburn, F. B. Howard, H. T. Miles, and L. Barnett)

Chemical and Structural Investigations of Nucleic Acids and Related Substances

A new polynucleotide, poly 2-aminoadenylic acid, has been prepared by chemical synthesis of the 5′-pyrophosphate and enzymatic polymerization. The complex of this polymer with poly U provides the first example in addition to the natural G–C pair of a two-stranded helix stabilized by three interbase hydrogen bonds. Physical studies show that the additional hydrogen bond increases the stability but that other specific attractive factors must be important in G–C affinity.

GTP and ATP form helices with complementary polynucleotides if and only if divalent cations are present. This Mg-dependent substrate template pairing is probably important in nucleic acid synthesis.

Dihydrouridine residues in copolymers of U and H_2U do not bind to poly A and reduce the ability of the normal uridine residues to do so.

Specific base pairing of monomeric nucleosides has been demonstrated in organic solvents by nuclear magnetic resonance. The G–C interaction is far stronger than any other, including other pairs which also form three hydrogen bonds. (H. Todd Miles, F. B. Howard, R. V. Ravindranathan, E. D. Becker (LPB), Dr. R. R. Shoup (LPB), Dr. P. Cerutti (LC), Dr. M. Chamberlin (Univ. of Calif.), Mr. J. Frazier)

X-ray Diffraction Studies on Proteins

X-ray diffraction studies on λ-chymotrypsin have been continued. A number of new heavy atom derivatives have been discovered. One of

these, phenyl mercury acetate, has been located in two-dimensional projections and three-dimensional data is being collected to confirm this interpretation.

Several heavy atom derivatives have been discovered for crystals of DIP trypsin and these are being examined in detail. (David R. Davies, Gerson Cohen, Enid Silverton, Hazel Braxton)

A Search for DNA Recombination in Vitro

Ultracentrifugation and standard enzymological techniques are being used to find and characterize an enzyme which produces covalent links between DNA molecules. Assay systems have been developed which involve the separation of two DNA's which can be differentially eluted from hydroxylapatite columns. Attempts to detect linkage using crude *E. coli* extracts have so far been equivocal. (Martin Gellert)

Structure of Polyadenylic Acid at pH 7

We have carried out a study of some of the solution properties of poly A. Samples of the polymer have been fractionated according to molecular weight by a combined variation of salt concentration and temperature. An investigation has been made of the molecular shape as a function of salt and temperature. Under ideal solvent conditions, poly A which is 80% in the ordered form behaves in a manner characteristic of a classical random coil. The finding is entirely consistent with the predictions of the earlier spectral analyses of poly A thermal denaturation. (Henryk Eisenberg, Gary Felsenfeld, Marc Leng)

Optical Properties of Nucleic Acids

The analytical spectral techniques developed in earlier years have been brought to a high degree of precision. As one byproduct of the investigation it has become possible to analyze any of the spectra of nucleic acid samples (native, denatured, or hyperchromic spectra) to determine the concentration and base composition of the DNA.

In the case of the hyperchromic spectrum and the spectrum of the high temperature form, it is only necessary to make observations at four wavelengths to determine concentration and composition with an accuracy comparable to that of any of the other techniques for determining base composition. The method has the advantage of being much more rapid than any other.

We have also used the spectral methods to analyze in detail the denaturation of λ phage DNA. We find that it is possible to detect the internal heterogeneity of base sequence which was first revealed by other investigators using ultracentrifugal methods. It appears that the spectral analysis will permit rapid determination of such internal heterogeneity, and of homologies of sequence between different but related DNA's. (S. Hirschman, G. Felsenfeld)

Interaction of DNA with Polycations

The interaction between DNA and synthetic basic polypeptides has been studied. Preliminary results reveal that polylysine can be made to interact preferentially with A–T rich DNA, while polyarginine does not. The interaction of DNA with spermine has also been investigated. Contrary to reports of other investigators, spermine exhibits no selectivity with regard to the base composition of binding sites. (Marc Leng, Gary Felsenfeld, Shalom Z. Hirschman)

Internal Proteins of Bacteriophage T2

The internal proteins of T2 bacteriophage have been further purified. Current work indicates that the acid soluble fraction contains two components and the acid insoluble fraction, two or more components.

The acid-soluble components are well resolved on DEAE. Based on P–60 and P–100 acrylamide columns, both are approximately 15,000 molecular weight. Pulse-labelling infected cells and then isolation of the proteins from the resulting phage indicates both components are synthesized early in the infection, one having roughly the kinetics of an "early enzyme" and the other being made even sooner in the infection. The kinetics have been essentially substantiated when the proteins were

isolated by direct T.G.A. extraction of infected cells.

The acid-insoluble components are partially resolved by acrylamide columns. A minor peak appears in the size range corresponding to ca. 30-50,000 molecular weight, a major peak at about 15,000 mol. wt. and indication of a third smaller material. All the acid-insoluble materials are made before or during the period of early enzyme synthesis. (Steven B. Zimmerman, G. Felsenfeld, G. Sandeen)

Effect of Glucosylation on the Kinetics of Endonuclease Degradation of DNA

It has been demonstrated viscosimetrically that the initial rates of degradation of DNA by pancreatic DNAse and endonuclease I are in the order T*2=T*4>T2>T4≈T6 with relative rates of 4:2:1. Deoxyribonuclease II was found to degrate T*4 and T4 DNA at the same rate.

These data show that the kinetics of enzymatic degradation are sensitive to rather subtitle differences between the DNA's of the various bacteriophages.

The most likely explanation of these results is that the different rates are due to the extent of glucosylation although on account of the complex glucosylation patterns of the T-even bacteriophage DNA's it is not possible to determine whether the mode (stereochemistry) of glucosylation also influences the kinetics. (Philip D. Ross, William B. Uphold)

Viscosity Studies of the DNA of Bacteriophage Mutants

T5—Differences in the intrinsic viscosity of intact DNA isolated from a series of T5 bacteriophage mutants have been found. These differences would correspond to a difference in molecular weight of 9% between T5+ and the deletion mutant T5 st-0. This result is in excellent agreement with a 7% MW difference calculated from sedimentation constant by Rubinstein. The fact that s and $[\eta]$ vary in the same direction enables one to conclude from hydrodynamics alone that these differences are a true size effect. A difference of 7% has been demonstrated by direct measurements of length in the electron microscope (McHattie & Thomas). This work establishes that the viscosity technique is capable of detecting small differences in the size of bacteriophage DNA molecules.

T4—Small, reproducible differences have been found in the intrinsic viscosity of intact DNA isolated from various rII mutants of bacteriophage T4. When the phage are grown on *E. coli B* a correlation between the intrinsic viscosity of the DNA and the genetic deletion size is found, however the size differences calculated from the viscosity are much larger than estimates of the size by genetic techniques. When the phage are grown on other bacterial strains different sorts of results are obtained indicating that the viscosity is influenced in part by the complex physiology of growth conditions which reciprocally may reflect a property of the genetic mutation. (Philip D. Ross, Robert L. Scruggs)

Chemistry and Genetics of Hemoglobin and Other Proteins

1,2-cyclohexanedione was found to react with the guanido group of arginine to form a stable derivative. Modification of the arginyl residues of proteins with this compound blocked the action of trypsin at the arginine positions but not the lysine positions.

Factors affecting denaturation and dissociation of hemoglobin were studied. It was found that alkali denaturation is inhibited by sulfhydryl blocking agents and by mercaptoethanol. Although human adult hemoglobin, fetal hemoglobin, and bovine adult hemoglobin differ greatly in their resistance to denaturation by alkali, they were found to be very similar with regard to dissociation into half-molecules at high pH.

The amino acid substitutions of two abnormal hemoglobins were determined. Hemoglobin Hopkins-1 has an aspartyl residue in place of a lysyl residue in position 95 of the beta chain, and hemoglobin J has an aspartyl residue in place of glycyl residue in position 16 of the beta chain. (H. A. Itano, A. J. Gottlieb, S. Yamada, and E. R. Tudor)

Biochemical Control Mechanisms in Histidine Biosynthesis

The pathway of histidine biosynthesis in *Salmonella* typhimurium has been shown to be unbranched and to involve 10 enzymes. Assays for the ten enzymes have been developed. The genes for these enzymes are in a cluster on the *Salmonella* chromosome.

Transfer-RNA and the control of the histidine operon. (J. R. Roth, D. Silbert, G. Fink, P. E. Hartman, and B. N. Ames)

The control mechanism that regulates the rate of synthesis of the group of histidine biosynthetic enzymes made by this cluster of genes is being examined to try and understand the mechanisms of "repression." Four classes of regulatory mutants have been obtained which are derepressed for the enzymes of the histidine operon. Their properties suggest that histidine-tRNA is important in repression and not free histidine. One of these regulator genes is the gene for the histidine activating enzyme. The mutants in this gene have an enzyme with a decreased affinity for histidine. Another of the genes involves the histidine-tRNA.

The translation of the histidine operon. (R. G. Martin, D. B. Berkowitz, and H. J. Whitfield, Jr.)

We have continued the study of polarity (i.e. mutants in one gene which affect the other genes in an operon distal to the operator region) in an attempt to understand the biochemical basis for it. A random group of 65 mutants in the C gene (the aminotransferase) have been examined in this regard. These mutants have been divided into chain terminating types containing the UAG (amber) or UAA (ochre) triplets, missense and frameshift classes. A method has been developed for classifying mutants into these groups. All the amber and ochre and frameshift mutants are polar: none of the missense mutants are polar. The frameshift mutants behave as if they are polar not of themselves but rather because they produce nonsense condons beyond the point of the mutation. The effect of the frameshift mutation does not extend beyond the limits of a given cistron *i.e.* the enzymes subsequent to the block are present, albeit in reduced amounts.

A model for translation based on the hypothesis that the degree of polarity is a function of the type of proximal chain initiator has been developed.

Frameshift mutagenesis in *Salmonella.* (B. N. Ames, H. J. Whitfield, Jr.)

A frameshift mutation is one where bases are added or deleted from the DNA so that the translation of the genetic message which occurs in groups of 3 bases is out of frame. The agents that cause these frameshifts (acridines such as proflavin) do not work in bacteria. Numerous polar mutants in the histidine operon are not ambers or ochres and appear to be frameshift mutants. A number of them are revertable by a class of aza-acridines, benzacridines, acridines, and quinolines with a polyamine side chain. Our evidence so far indicates that these mutagens specifically cause frameshifts and not transitions or transversions. We have been learning about the specificity of mutagenic agents using these mutants.

Purification and protein chemistry of two of the enzymes of histidine biosynthesis. (M. J. Voll and R. G. Martin)

The first enzyme and the aminotransferase have been purified and their protein chemistry has been investigated.

Mechanism of Lambda Bacteriophage Induction

Lysogenic induction and curing constitute two processes in which a specific recombinational event occurs as a consequence of the expression of certain genes of a temperate bacteriophage. Studies are being undertaken to identify the genes involved and determine their roles in this event.

Methods have been developed for readily determining (*a*) whether a reagent can cause lysogenic induction, (*b*) genetic requirements for the curing of one bacteriophage by another. A thermosensitive phage mutant (designated 434hy C_t) is used in the latter method. Considerable attention has been devoted to the unusual characteristics of this phage. Some of the properties of 434 hy C_t support the following conclusions:

The phage repressor is unstable even at low temperature, more unstable at high temperature.

Derepression leads to a reversible phase of phage development which is then followed by an irreversible phase. A system responsible for curin appears in the reversible phase. In addition, certain bizarre properties of 434hy C_t are being investigated. (M. Yarmolinsky)

MATHEMATICAL RESEARCH BRANCH

As of February 24, 1966, the Office of Mathematical Research has been designated the Mathematical Research Branch.

Research and consultation have continued along the general lines set forth in the summary of last year. Primarily, the work has consisted of generalization and extension in depth of previously initiated studies in mathematical biology and related mathematics and application of methods, mathematical models and theories previously developed to selected biological problems. New approaches and methods have been brought into play and the demands of application have entailed modification of formulations and some more or less tangential studies. However there have been few significant departures from the general areas of research and the broad objectives outlined in previous summaries.

The main areas of research are: Mathematical formulation and analysis of models for dendritic neurons; mathematical and computational studies of visual systems; general theory of transport processes with special reference to renal concentrating systems; mathematical description of the transport-diffusion-chemical kinetic problem for substances in the blood-capillary complex; mathematical problems arising from the rate behavior of metabolic systems; mathematical and computational methodology for compartmental and related systems.

Several theoretical preductions concerning synaptic activity of dendrites have received support from recent anatomical and physiological studies. A theoretical analysis of electric potentials in rabbit olfactory bulb led (Rall and Shepherd in 1964) to the hypothesis that the secondary dendrites of mitral cells deliver synaptic excitation to the dendrites of granule cells and that the depolarized granule cell dendrites then deliver synaptic inhibition to the mitral dendrites. This hypothesis met the requirements of the then existing physiological data for the mammalian olfactory bulb. However, dendro-dendritic synaptic interactions of this kind had not been previously observed or postulated for any nervous system, and the hypothesis remained tentative until the results of electron-micrographic studies of this region (external plexiform layer of olfactory bulb) should become available. Thus, it was exciting to learn from an independent electron-micrographic study (Reese and Brightman, NINDB) that there are synaptic contacts between granule and mitral dendrites, and moreover, that these contacts are unusual in that neighboring contacts (between the same pair of dendrites) have opposite synaptic polarity (presynaptic-to-postsynaptic) as judged by the locations of synaptic vesicles. This anatomical finding fit the requirements of the physiological hypothesis so well, that collaborative discussions were undertaken to explore and writeup the implications. What has emerged is the concept and evidence for a new kind of synaptic pathway for inhibition; it provides a negative feedback to the mitral cells; it can provide for the lateral inhibition that is needed for sensory discrimination; it can provide for adaptive inhibition which can adjust a sensory system to a wide range of input intensity. Also the granule cells of olfactory bulb, like the amacrine cells of vertebrate retina, possess no axons; this new hypothesis provdes an interpretation of how such cells could perform an important task without generating a nerve impulse. Thus, the implications extend beyond the olfactory bulb, and are being explored. An entirely different set of computations has been carired out to provide theoretical predictions being tested by new experimental data on cat motoneurons obtained by colleagues in the spinal cord section (NINDB). Several cases of good agreement between theory and experiment have re-

sulted and are being written for publication. (Dr. W. Rall)

The Fuortes-Hodgkin model for visual response of the *Limulus* eye, extended by the addition of a second feedback loop was studied further. Suitable adjustment of the parameters of the second slow loop gives good agreement with responses to trains of pulses. A paper on the one loop model appeared in the Journal of Physiology. A simple set of instructions for use of the SAAM program for models of the Fuortes-Hodgkin type was written.

Some of the relations between graph theory and matrix theory which are particularly relevant to compartmental systems were explored, and some new theorems on reciprocity derived. A paper has been prepared.

As an aid to embryological studies of the lenses of chicks, an equation was fitted to the lens profiles. This is useful in determining volumes, surface areas, and also as a compact way of describing the course of development. (Mrs. R. B. Marimont, NINDB, Associate Member MRB)

Work has continued on various aspects of the renal concentrating system. One question has been whether a counter current multiplier with active sodium transport limited to the medulla can account for the sodium concentration profile in the medulla. It has been shown (Stephenson, Nature 206, 1215, 1965) that in a two-loop system (which included the loop of Henle and the medullary vessels) this is not possible. This work was extended to systems with any number of loops in which trans-tubular water as well as salt movement is allowed. This work is scheduled to appear in the Biophysical Journal. A study of the effect of diffusion and trans-tubular water movement on counter current multiplier efficiency has been begun. A thermodynamic analysis of certain features of counter current multipliers has also been initiated. Finally, preliminary analytical work on random walk models of counter current multipliers has been done with the objective of programming some of these models for machine computation. (Dr. J. Stephenson, NHI, Associate Member MRB)

A numerical method and the corresponding computer program have been developed to solve for the oxygen concentration along a capillary and in the surrounding tissue. The consumption of oxygen in the tissue is assumed to obey non-linear (formal "Michaelis-Menten") chemical kinetics. By changing the kinetic parameters it is possible to explore the oxygen concentration profiles for the cases of zero, first and second order kinetics, all for a given oxygen consumption, arterial oxygen saturation and blood flow. Several other features of the system have been explored. In particular the influence of the chemical kinetic parameters on the mean intercapillary distance, subject to a prescribed oxygen consumption which avoids oxygen debt, has been explored and, for the first time, it is now possible to study the influence of the kinetic parameter on the mean capillary length. The interplay of kinetic parameters, mean intercapillary distance, mean capillary length and cardiac output for a given oxygen consumption which avoids oxygen debt suggests an interesting and complex optimization problem. The numerical method consists, briefly, of writing the differential equations as one non-linear system which is solved by a generalized n-dimensional Newton-Raphson technique, each linear step of which is solved by an iterative over-relaxation method. Rigorous convergence proofs and error bounds for the method have been formulated.

An effort was made in the above formulation to incorporate the best estimates of the minetic parameters the O_2-hemoglobin kinetics in whole blood. A mathematical model for the Roughton-Gibson experimental set-up was developed which allows more information to be extracted from their data.

A description of gas exchange at the lung alveolar-capillary level is being developed which will presumably eliminate some apparently incompatible results which have appeared in this field. (Dr. Jose Gonzalez-Fernandez)

The Moore-Penrose pseudoinverse for a matrix has, from some points of view, been well studied. Recently (the first in 1960, the last in 1964) other classes of generalized inverses

have been defined. As yet there has been little work on their properties and, with the exception of the thesis of C. A. Rohde (May 1964) and a paper by Rohde scheduled to appear this year, nothing approaching a systematic comparative study of these various classes of inverses. Such a study was begun last year. Our terminology is: The matrix B is a j-inverse of A if it obeys the first j of the equations (1) $ABA=A$, (2) $BAB=B$, (3) $AB=E_1=E_1^*$, (4) $BA=E_2=E_2^*$. The 4-inverse, the Moore-Penrose inverse, is the only unique one but the others are far simpler to construct. It can be shown that for many purposes (in particular linear estimation, certain analytical properties, etc.) the character of the results is independent of the choice of generalized inverse. In other contexts one can get computational and algebraic simplicity at a small price (or for free) in terms of the breadth of conclusions if it can be shown that a j-inverse shares certain properties with the 4-inverse or the proper inverse of a non-singular matrix. There are properties of a proper inverse which though well nigh trivial (the inverse of a hermitian matrix is hermetian; the inverse of the inverse is the matrix, transposition and inversion are commutative operations, etc.) do not necessarily hold for a generalized inverse and we must give up certain conclusions and disallow certain operations when these properties fail. In practice then it is extremely important to know what properties of A are inherited by B, when A and B commute, when they have common invariant subspaces, when the (non-zero) roots are reciprocally related, and so on. Many such questions are settled by the following result: Let $T_1(AC) = T_1(A)T(C)$, $T_2(AC) = T_2(C)T_2(A)$ and let M have the properties of T_1 and/or T_2 and additionally $M(A^*) = M(A)^*$. It has been shown for what value of j it is true that $T_1(B)$ or $M(B)$ is a j-inverse of $T_1(A)$ or $M(A)$ when B is a j-inverse of A. These transformations include conjugate-transpose, adjoint, compound matrices, compound adjugate, and induced matrices. Similar results have been obtained for equivalence unless it includes similarity, congruence and multiplication by a non-singular matrix. The existence of each higher inverse has been shown to follow from that of a 1-inverse and the importance of this lies as much in computational application (for the proof is constructive and quite simple) as in theory and moreover is the basis of the theorem: The existence of a 1-inverse, B, with any of the properties (i)–(iv) implies the existence of a j-inverse, for every j, with that property where (i) B hermitian when A is hermitian (ii) B commutes with A, (iii) E_1 or E_2 normal, (iv) B symmetrizable when A is symmetrizable. Results too numerous to list here have been obtained on conditions under which A and B are commutative or quasi-commutative, have common eigenvectors have common principal vectors associated with non-zero roots and have reciprocally related non-zero roots. Finnally, an equality has been derived based on a j-inverse in which includes the famous Kantorovitch inequality as a special case and which provides a novel and exceedingly simple discussion of the conditions for strict equality. (Dr. John Z. Hearon)

A new addition to the SAAM program has been programmed. This is a PREREAD section containing about 50 subroutines and has new and more extensive data input capability. It also has compiler capability and permits the entering of mathematical equations together with the data. This PREREAD is essential for the new extended capabilities being incorporated into SAAM for ON-LINE computer operations.

As a result of new iodine kinetics studies, further structure in the thyroid system has been recognized and a new iodine kinetics model has been developed. The new model contains a delay phase that did not seem to be necessary in previous studies. The new model was also tested and found compatible with past studies.

Collaborative studies on a metabolism and absorption of Ca have also continued. (Dr. Mones Berman)

CLINICAL INVESTIGATIONS

During this fiscal year, a number of organizational rearrangements have been completed within the branches of NIAMD Clinical Inves-

tigations. At the outset of the year, Dr. Robert Gordon replaced Dr. G. Donald Whedon as Chief of the Metabolic Diseases Branch. Dr. Whedon continues his interest in clinical investigation within the Branch, but is no longer required to divert time from his responsibilities as Institute Director to carry out the duties of Branch Chief. Within the branch, three sections were separately delineated. The Section on Mineral Metabolism is now headed by Dr. Gerald Aurbach, formerly an investigator in the Laboratory of Nutrition and Endocrinology. In addition, the branch includes the Section on Gastroenterology, headed by Dr. Leonard Laster, and the Section on Physiology and Clinical Nutrition under Dr. Gordon. New sections have also been organized within the Clinical Endocrinology Branch. The Section on Endocrine Biochemistry is headed by Dr. Jacob Robbins, while the Section on Diabetes and Intermediary Metabolism is under the leadership of Dr. Stanton Segal. Finally, effective March 1, 1966, separate sections within the Arthritis and Rheumatism Branch were recognized. Dr. Jarvis Seegmiller has become Chief of the Section on Human Biochemical Genetics, while Dr. John Decker heads both the Branch and the Section on Connective Tissue Disease.

Dr. John Decker joined the staff of the Institute on September 19, 1965 to fill the vacancy created by the death of Dr. Joseph Bunim. Before joining the Institute, Dr. Decker had been Associate Professor of Medicine at the University of Washington School of Medicine in Seattle, and head of the Division of Arthritis. Before his appointment to the medical faculty of the University of Washington in 1958, Dr. Decker had been a Research Fellow of the Arthritis and Rheumatism Foundation at the Massachusetts General Hospital under Dr. Wlater Bauer. Dr. Decker had served the NIAMD previously as a member of the Arthritis Training Grant Committee, and is an active member of the American Rheumatism Association, for which he has edited the "Primer on the Rheumatic Diseases." At the Clinical Center, Dr. Decker will continue his interests in clinical and laboratory investigations of rheumatoid arthritis, lupus erythematosus, and related rheumatic diseases.

On July 1, 1965 a new group of 11 Clinical Associates joined the Institute, replacing 8 who had completed their two-year period of duty. These men have, as always, been the mainstay of patient care activities, and have made major contributions to clinical and laboratory investigations. The high caliber of the junior investigators who have carried out the function of Clinical Associate within the NIAMD has been of the greatest importance in assuring the excellence of the Institute's clinical research program. Other temporary members of the clinical investigations staff during this year include 12 visiting scientists and guest workers from overseas.

The NIAMD has made two beds within its nursing units available to the National Institute of Dental Research, in addition to accommodating its own patients. During the period March 1, 1965 through February 29, 1966, 480 patients were admitted for a total of 17,066 hospital days. The average patient stay was 46 days, and the average census was 67% of capacity. During the same period, 1446 outpatients were seen for study and treatment.

ARTHRITIS AND RHEUMATISM BRANCH

The Branch has been divided into two sections in the interest of more clearly delineating functions. Dr. Seegmiller now heads the Section on Human Biochemical Genetics; his interest in gout continues but, in addition, the sec- deals with a variety of other heritable metablic disorders. Dr. Decker directs the Section on Connective Tissue Disease which is concerned with rheumatoid arthritis, systemic lupus erythematosus, and related disorders, often grouped as the "collagen diseases."

Clinical Studies of Natural History of Disease

Lymphocyte Function in Sjogren's Syndrome

Sjogren's syndrome continues to be a focus of activity and is heavily represented among patients admitted for study. A reduced lymphoblastic response of patient lymphocytes cultured with phytohemagglutinin has been observed; the defect is most pronounced in pa-

tients with accompanying pseudolymphoma or rheumatoid arthritis. The finding has been substantiated and extended by the further observation that skin sensitization to dinitrochlorobenzene is not as readily or as often induced as in normals. (Drs. Talal, Barth, Levinthal, NCI, and Waldorf, NCI)

Immunological Reactivity in the Elderly

In the above studies it was noted that skin sensitization was more likely to occur in patients without circulating "autoimmune" antibodies and vice versa. Elderly normal individuals often show such antibodies and studies of circulating antibodies and skin sensitization are now in progress in a group of 150 such people. (Drs. Decker, Talal, Waldorf, NCI, and Willkens, University of Washington in Seattle)

Variations in Rheumatoid Factor Titer

Using data and sera assembled at the Clinical Center since 1957, an effort is being made to relate the clinical status of the patient to changes in the titer of rheumatoid factor over a mean time span of 5½ years. The results will be pertinent to prognostic problems and to identification of possible antigens. (Dr. Decker)

Clinical and Metabolic Evaluation of Cystinosis

Detailed clinical and metabolic studies of children with cystinosis continues. The identification of a peripheral retinal lesion, present before the classical biochemical lesions are detectable, holds considerable promise in terms of preventing damage by early treatment. Two modes of therapy—with penicillamine and with a synthetic low cystine diet—are under evaluation. (Drs. Seegmiller, Schneider, Crawhall, and Wong, NINDB)

Therapeutic Studies

Prophylactic Synovectomy in Rheumatoid Arthritis

Surgical synovectomy is being carried out on one randomly selected joint of a pair of symmetrically diseased joints neither of which show erosive disease by X-ray. The results of this long-term study should provide information on the efficacy of the procedure in preventing damage as well as some data on appropriate selection of cases of surgery. (Dr. Decker and Dr. Peterson, George Washington University)

Melphalan in Systemic Amyloidosis

This disease, commonly accompanied by a bone marrow picture suggestive of multiple myeloma, is invariably fatal. Therapy with melphalan, perhaps the "best" agent for myeloma, is under trial. The tendency of ^{131}iodide to localize in parencymal organs involved by amyloid is being studied. (Drs. Barth and Decker)

Lymph Drainage in Systemic Lupus Erythematosus

It is possible to cannulate the thoracic duct and exteriorize its entire flow. This makes possible the removal of lymphocytes, immunoglobulins, or both. The procedure has been done in a single patient with severe systemic lupus erythematosus with nephritis. The subsequent favorable changes in renal function were sufficiently encouraging to conduct more extensive trials. (Drs. Decker, Cohen, and Irvin, NCI)

Mechanisms of Disease

Purine Biosynthetic Pathways

The influence of a variety of naturally occurring metabolites on glycine incorporation into uric acid and on total uric acid synthesis is under study in an effort to explain the regulatory defect which is considered to be the cause of overproduction of uric acid in certain individuals with gout. Adenine markedly inhibits glycine incorporation into uric acid and the effect of orotic acid is to be tested. (Drs. Seegmiller, Rosenbloom, and Kelley)

Induced Renal Retention of Uric Acid

Acute renal function studies have shown marked renal retention of uric acid upon infusion of β-hydroxybutyrate or sodium acetoacetate while the third major "ketone body," ace-

tone, had no effect. The finding probably explains the hyperuricemia which has been observed in patients who are totally fasted, on high fat diets, or in diabetic ketoacidosis. (Dr. Seegmiller)

Role of Infection in Rheumatic Disease

Efforts to isolate mycoplasma or bedsonia from the synovial fluids and tissues of patients with rheumatoid arthritis or Reiter's syndrome, respectively, have not been fruitful but are continuing. Serological studies, on the other hand, indicate more reactivity to a specific mycoplasma, M. salivarium in rheumatoid sera, and more complement fixing reactivity to a bedsonia antigen in Reiter's sera than in appropriately matched control sera. (Drs. Decker, Chanock (NIAID), Barile and Hopps (DBS))

Experimental Amyloidosis

Casein-induced mouse amyloidosis has been successfully produced and the effect of various modifications of the immune apparatus, such as theymectomy, irradiation, and cytotoxic drugs, is under study. In addition, efforts are being made to study 131 iodide-amyloid interactions in the experimental disease. (Drs. Barth and Decker)

Ribosomes in Hormonal Control of Protein Synthesis

Studies of amino acid incorporation by ribosomes from hypophysectomized or thyroidectomized rat livers demonstrated that the ribosomal reading of a synthetic messenger RNA was defective. The results suggest that the hormonal control of protein synthesis occurs, in part, at least, at the ribosomal level. (Drs. Garren and Richardson)

Mechanism of Action of Adrenocorticotrophic Hormone

The action of ACTH on the adrenal is blocked by inhibitors of protein synthesis. When protein synthesis was blocked it was found that the side chain cleavage of cholesterol, the first step in its conversion to corticosterone, was inhibited. It was thus proposed that ACTH facilitates the synthesis of a protein which is responsible for initiating the transformation of cholesterol to corticosterone. (Drs. Garren, Davis, NHI, and Ney, Vanderbilt Medical School)

Studies on Immunoglobulins

Antibody Synthesis in the Spleen

Some noteworthy properties of membrane-bound ribosomes as compared to free ribosomes of rat spleen have been found. In a cell-free system, protein synthesis by the bound ribosome is sharply inhibited with chloramphenicol and is not readily induced by synthetic messenger. The reported effect of chloramphenicol on antibody production *in vivo* suggests that the observed effect in the cell-free system is, in fact, an antibody protein synthesis but this point continues under study. (Drs. Talal and Plotz)

Macroglobulin Subchains

The previously discovered probability that γM was composed of 5 rather than 6 subunits has been amply confirmed by (a) disulfide bond reduction and consequent chain separation with dithiothreitol and by (b) the results of partial tryptic digestion of the molecule. The latter procedure showed the molecule to be divalent and completely analogous to the structure of γG. Using depolarization of fluorescence, the γM molecule was found to be composed of loosely bound, non-interacting subunits, possibly explaining the uniquely high agglutinating activity of γM antibodies. (Drs. Metzger, Miller, Perlamn, Edelhoch, Stone and Bladen and Mage, NIDR)

Macroglobulin Catabolism

Radioiodine-labelled γM and subunits of γM produced with dithiothreitol, were given to animals and to patients and their catabolism was followed. The subunits showed a much shorter half-life than did the whole molecule. In sharp contrast to the situation with γG and its subunits, there was no evidence of competition between γM and subunits of γM for catabolism. (Dr. Cohen)

Studies on Canine Immunoglobulins

A thorough study of canine immunoglobulins is being carried forward and has provided information of six different moieties, one of them in the macroglobulin class. Canine colostrum contains the same six but in different proportions. The isohemagglutinins of canine A_2 blood group are also being classified. The metabolism of these molecules is of importance because of the occurrence of several model diseases in dogs, particularly hemolytic anemia and systemic lupus erythematosus. (Dr. Johnson, and Drs. Swisher and Trobold, University of Rochester)

Miscellaneous

Carbon-Fluorine Bonds in Biology

Enrichment methods have produced a bacterium which can break the carbon-fluorine bond, and can use fluorinated organic compounds as the sole source of carbon. Fluoride accumulates in the growth medium. Attempts to isolate the microbial enzyme responsible for cleavage are continuing. These studies are of interest in relation to the known effects of fluorine substituents in delaying the catabolism of a variety of organic compounds, such as the synthetic corticosteroids. (D. Goldman)

METABOLIC DISEASES BRANCH

Research on Human Physiology and Nutrition

Clinical Studies of Total Energy Balance

The NIAMD Metabolic Chamber, which has been used in the past for studies on total energy metabolism by indirect calorimetry, is being re-instrumented to take advantage of newer electronic devices for data collection and processing. New plans also include provisions for measurement of carbon isotopes in expired air, so that a wide range of studies of oxidation of labeled precursors may be undertaken. (Drs. Thompson and Gordon)

Effects of Heat Stress on Potassium Balance

Investigation of the effect of a high sodium, low potassium rice diet (similar to diets common in Southeast Asia) on the potassium balance of normal volunteers has continued. On this diet, healthy men experience a 10 to 15% decrease in potassium content over 1 to 2 months. Loss of potassium is increased by heat exposure and sweating. However, none of the diseases of hot climates, some of which were considered possibly to be due to potassium depletion, were reproduced by the experiment. (Drs. Gordon, Thompson, Waller (NIAMD Extramural), and Cage (NCI))

Mechanisms of Water and Electrolyte Secretion by the Human Sweat Gland

In the course of the foregoing study, it was noted that as serum potassium levels fell, sweat potassium followed in proportion. This observation suggests that potassium enters sweat by passive diffusion from extracellular fluid. This information, together with a review of literature on the structure and function of sweat glands, has led to a hypothesis which attributes to lactate ion the primary role in establishing the electrochemical gradients that move water and ions from body fluids to the sweat duct. This hypothesis has been peepared for publication, and is being tested in current experimental work. (Drs. Gordon, Thompson, Waller (NIAMD Extramural), and Cage (NCI))

Clinical Investigation of Calcium Metabolism

A kinetic model was devised to describe calcium absorption in man; in collaboration with Dr. Mones Berman a computer program was established to analyze this compartmental model. This kinetic analysis has been applied to study several clinical disorders of calcium metabolism. Both calcium and parathyroid hormone appear to influence calcium absorption in man. Further preliminary observations suggest that the decreased urinary losses of calcium induced by high phosphate intake is mediated through an effort to decrease bone resorption.

Patients with idiopathic osteoporosis were tested with regard to lactose tolerance and jejunal biopsy specimens were obtained; flat lactose tolerance tests were associated with intestinal lactase deficiency as measured by direct

enzymatic analysis with the biopsy specimens. Eight percent of osteoporotic subjects over the age of 50 were deficient of the enzyme whereas subjects without evidence of bone demineralization in the same age group showed normal enzyme activity in the intestine. (Drs. Birge, Keutmann, Aurbach and Whedon)

Bone Cell Metabolism

Fetal bone cells have been isolated and cultured *in vitro*. Collagen synthesis was measured by determining C^{14} hydroxyproline in protein. An interesting observation has been made that ascorbic acid stimulates collagen synthesis in this preparation; the action of ascorbic acid is probably related to its proposed function in catalyzing hydroxylation of proline. (Dr. Birge)

Chemistry and Physiology of Parathyroid Hormone

Parathyroid hormone has been isolated in pure form and digested with trypsin. Analysis of tryptic peptides of the hormone as well as other peptide fragments produced by different chemical enzymatic means yielded information to assign each tryptic peptide to a position in the molecule; this assemblage of peptide subunits thus placed in linear order has become a working model for the structure of the hormonal polypeptide. A minimal area of the molecule requisite for biological and immunological activity has been isolated from weak acid digests of the pure hormonal molecule. This fragment accounted for a sequence 20 amino acids long at the carboxyl-end of the polypeptide chain. Further studies showed that the methionine, tryptophan and tyrosine within the acid fragment region are each critically important for biological activity. Studies on the secretion of parathyroid hormone have been carried out by measuring parathyroid hormone in the blood of ruminants with a highly sensitive radioimmunoassay. From these experiments it can be concluded that blood calcium is the major if not only physiological regulator of parathyroid secretion. Some purification of human parathyroid hormone has been accomplished after extracting human parathyriod adenomas with 8 molar urea. It has been shown that human parathyroid hormone behaves similarly throughout fractionation, gel filtration on Sephadex and chromatography on cellulose ion exchanges. A further finding has been that the sedimentation rates of bovine and human parathyroid hormone are similar in the ultracentrifuge. (Drs. Aurbach, Keutmann, O'Riordan, Potts (NHI) and Sherwood (NHI)

Studies on the Action of Thyrocalcitonin

The action of thryocalcitonin, a hypocalcemic factor extracted from the thyroid gland, was studied by kinetic analysis of the distribution of calcium–45 injected intravenously into rats. The effect was examined with a kinetic model set up through the computer program and developed by Dr. Mones Berman. The experimental findings could be reproduced in the model system when the return of unlabeled calcium from a reservoir switched off. The most likely location of this reservoir is bone and it is apparent that thyrocalcitonin acts by suppressing bone resorption. (Drs. O'Riordan and Aurbach)

Studies of the Small Intestine

Whippple's Intestinal Lipodystrophy

The long-term study of the pathophysiology of untreated and treated Whipple's disease continues. The female patient described in last year's report whose disease relapsed 3 years after she had been treated with antibiotics and steroids for 4 months responded favorably, but slowly, to reinstitution of the same treatment. Her relapse was unusual in that she developed hypoalbuminemia (due in part to exudative enteropathy), hypochloesterolemia and hypocarotenemia but no steatorrhea.

A new patient was added to the series bringing the total number studied to 8. He was treated only with antibiotics and his response was satisfactory. He is the second patient in the series to be treated without steroids.

Of the remaining 6 patients, one has died of gastric cancer, and the others continue to remain in remission. Dspite their general good health, they all continue to show PAS-positive macrophages in the lamina propria of the

small intestine. (Drs. Laster and Heizer, NIAMD; Dr. Waldmann, NCI)

Other Diseases

It is generally accepted that extreme flattening of the mucosa of the small intestine is characteristic and diagnostic of a sensitivity to dietary gluten. During investigations of gluten-sensitive enteropathy in this laboratory several patients were studied who had a flat mucosa but proof of their sensitivity to gluten was lacking or indefinite. One patient with flat mucosa and hypogammaglobulinemia improved her intestinal function when she was fed a gluten-free diet. However, after restoration of a normal diet her intestinal function has remained relatively normal for more than one year. Another patient with malabsorption and a flat intestinal mucosa responded only slowly and partially to a gluten-free diet, but began to improve dramatically after treatment with antibiotics, a measure which should not improve a patient with a typical gluten-sensitive enteropathy. Another patient with malabsorption and a flat intestinal mucosa responded well to a gluten-free diet but has also developed wide-spread cancer which may be a reticulum cell sarcoma. These deviations of clinical behavior in patients with a flat small intestine mucosa are under study.

Because of the finding of unsuspected malabsorption in 2 patients with Hodgkins disease, a study of the small intestine in Hodgkins disease has been started. To date, 3 of 6 additional patients studied have had functional and/or structural abnormalities of the mucosa of the small intestine. Attempts are in progress to determine whether a patient of this type, with a flat intestinal mucosa, is gluten-sensitive. (Drs. Laster and Heizer, NIAMD; Drs. Carbone and Vietzke, NCI)

Intermediary Metabolism of the Intestinal Mucosa

Studies of the synthesis of 27-carbon sterols by scrapings of mucosa from the guinea pig small intestine were extended to show that purified preparations of sodium taurocholate and sodium glycocholate, the predominant bile salts of guinea pig bile, are capable of inhibiting sterol biosynthesis in the mucosal scrapings *in vitro*. At 20 mM each bile salt all but obliterated sterol synthesis by mucosa of guinea pig ileum. At 4mM inhibition was still observed, but to a lesser extent. These findings suggest that bile salts, which are most actively absorbed by the ileum, may contribute to the regulation of intestinal sterol synthesis. (Drs. Ockner and Laster, and Mrs. Woodson, NIAMD)

Ultrastructure of the Human Intestinal Mucosa

A collaborative study of the ultrastructure and chemical composition at the subcellular level of the human small intestine mucosa has been initiated with Dr. A. J. Tousimis (Contract No. PH 43–66–10). The ultimate purpose of the collaboration is to apply electron probe microanalysis, electron microscopy, electron microscope histochemistry and electron microscope radioautography to studies of the human intestinal mucosa in health and disease, and in the presence of various forms of chemical and physical injury.

Since the methods for electron microscopy used in this study represent partial modification of conventional procedures, an exploration of the normal human small intestine mucosa was undertaken with the new techniques. Epithelial cells of the intestinal mucosa adhere to each other at the apical region of their lateral surfaces. This region is termed the junctional complex and it comprises the zonula occludens, zonula adherens and the desmosome. Biopsies from more than 50 subjects have been studied to determine variations in the conformation of the unit membrane in the zonula occludens of normal human intestinal mucosa. Effects of chemical injury on the zonula occludens are also under study.

In preparation for the application of electron probe microanalysis to biological tissues and to the human intestinal mucosa in particular, methods have been developed to render observations quantitative. Preliminary studies of the presence and distribution of sulfur and iron in the normal human intestinal columnar cell have been completed. (Drs. Tousimis, Bio-

dynamics Research Corp.; and Laster, NIAMD)

Inborn Errors of Metabolism

Homocystinuria

As new cases of homocystinuria continue to be discovered, it becomes evident that this inborn error is a relatively frequent cause of mental retardation, second only perhaps to phenylketonuria. The initial demonstration by this group that the underlying defect in homocystinuria is a deficiency of activity of cystathionine synthase was based on studies of 2 patients with the disease. The findings have now been extended to show that 3 additional patients with homocystinuria are markedly deficient in cystathionine synthase activity. In each of 2 families the parents of a patient with cystathionine synthase deficiency were shown to have a partial reduction in activity of this enzyme to about 60% of the mean control value. These heterozygous parents have neither clinical manifestations of the disease nor abnormal amounts of homocystine in the urine. Additional studies indicate that the brain of a patient with homocystinuria is deficient in cystatnionine synthase activity. The demonstration by this group that a patient with marked deficiency of cystathoinine synthase activity has an impaired capacity to convert the sulfur atom of L-methionine to urinary inorganic sulfate, has led to the development of a clinical test for marked deficiency of cystathionine synthase activity. (Dr. Laster, NIAMD; Dr. Mudd, NIMH; Drs. Finkelstein and Irreverre, NIAMD)

Cystathioninuria

Cystathioninuris was discovered in 1958. It appears to be a familial disorder, the clinical manifestations of which have not yet been fully defined because of a paucity of cases. Mental retardation is a feature of the disease. One such patient was studied by this group and it was shown that activity of the enzyme cystathionase was markedly reduced in an extract of liver from the patient. This enzyme catalyzes the hydrolytic cleavage of cystathionine to cysteine, α-ketobutyrate, and ammonia. The claim of Frimpter, that addition of pyridoxal phosphate *in vitro* stimulates cystathionase activity in extracts of liver tissue from patients with cystathioninuria, could not be confirmed in this case.

Crystalline rat liver cystathioninase has been reported to catalyze deamination of homoserine to α-ketobutyrate and ammonia. The extract of the liver from the cystathioninuric patient was tested and a deficiency of this enzymatic activity was found. Thus, the evidence suggests that in man a single protein catalyzes both cystathionase and homoserine dehydratase activities, and that there are at least 2 enzyme activity deficiencies in cystathioninuria. (Drs. Finkelstein, NIAMD; Mudd, NIMH; Laster and Irreverre, NIAMD)

A New Disorder

During the course of screening procedures for the detection of patients with homocystinuria and cystathioninuria a mentally retarded patient was found to excrete a compound in the urine which, to the knowledge of this group, has not been reported to occur in human urine. The identification of the compound and of the enzymatic defect underlying the disorder are in progress. The patient's family history suggests the abnormality is a familial one. (Drs. Irreverre and Laster, NIAMD; Dr. Mudd, NIMH; and Dr. Heizer, NIAMD)

Intermediary Metabolism

In considering the possibility that metabolism of the small intestine mucosa is abnormal in patients with gluten-sensitive enteropathy because of an immune mechanism, the study of a model system was considered. The plan was to test whether the reaction between human platelets and human antibodies to platelets alters platelet metabolism. Two parameters of platelet metabolism were tested and were found to be unaffected by the addition of antibodies. In the course of these studies it was observed, however, that the addition of thrombin to platelets *in vitro* results in marked and prolonged stimulation of glucose oxidation via the glycolic pathway. Additionl studies suggest that thrombin acts on one or more steps earlier in the pathway than the one in

which pyruvate appears. Thrombin may be acting on the hexokinase reaction or on the transport of substrate into platelets. As an associated or independent effect, thrombin also inhibits galactose oxidation. In testing whether thrombin stimulates oxidation of glucose by platelets by inducing the synthesis of new enzymes, evidence was obtained to show that platelets are capable of protein synthesis. To the knowledge of this group, this has not been shown before. Thrombin did not appear to act on protein synthesis in platelets. (Drs. Warshaw, Laster, and Shulman, NIAMD)

CLINICAL ENDOCRINOLOGY BRANCH

The Branch underwent an administrative reorganization during the past year, largely as part of an effort to expand its activities in the field of diabetes research. Two sections were created: the Section on Diabetes and Intermediary Metabolism, and the Section on Endocrine Biochemistry. None of the permanent staff was on a prlonged leave-of-absence during 1965–1966, but one served as Visiting Professor at the University of Naples in the spring and early summer. A number of visitors from abroad joined the Branch: one from Belgium, two from England, three from Italy and two from Japan.

Thyroid Biochemistry

Iodide Transport

In order to understand more fully the matter of iodide transport, attention was turned to tissues which do not "trap" iodide. The Ehrlich ascites tumor of mice was chosen as a source of isolated cells of this type. These cells were found to exclude iodide and related anions (Br^-, ReO_4^-, $WO_4^=$). The efflux of iodide from the ascites cell was dependent on the metabolic integrity of the cell and was inhibited by cardiac glycosides, quinidine and certain nucleotides. The relationship of iodide transport across the ascites cell membrane to that in the thyroid cells is not yet known. (Drs. Wolff and Salvatore)

The mechanism of the antigoitrogenic effect of antithyroid anions (ReO_4^- and ClO_4^-), when given with prophylthiouracil. Although prophylthiouracil appeared to augment thyroid enlargement produced by TSH injection, this effect was not diminished by simultaneous feeding of the anions. The mechanism of their antigoitrogenic effect remains obscure. (Drs. Wolff and Alexander)

The mechanism of iodide transport in the thyroid was investigated from the standpoint of the roll of the cell membrane. Phospholipases A and C inhibited iodide transport. Although certain phospholipids prevented this effect, they were not capable of restoring transport after the membrane had been damaged. Further studies of this subject are in progress. (Drs. Wolff and Larsen)

Iodination Reactions and Thyroxine Synthesis

In an attempt to clarify the process of iodination and thyroxine synthesis in biological iodoproteins, studies have been undertaken with proteins of known structure which do not normally contain iodine.

The rate of iodination of lysozyme with I_3^- at pH 8.5 was more rapid in 8M urea than in water. Only two of the three tyrosines were iodinated in water but all three in urea. Monoiodotyrosine was produced at low iodine levels (<2 moles of I_2/mole of lysozyme), but diiodotyrosine was the major product at higher levels. Thyroxine was not found. Iodohistidine (mainly monoiodohistidine) was formed after on tyrosyl residue had been fully iodinated. The quantitative importance of iodohistidine formation had not been appreciated previously. (Drs. Wolff and Covelli)

Iodination of bovine pancreatic ribonuclease in water produced three iodotyrosyl residues and one iodohistidyl, whereas in urea two additional tyrosyl residues and one additional histodyl residue were iodinated. The histodyl residue iodinated in water was proven to be His–119 by studies in which His–119 was blocked by carboxy-methylation and also by hydrolysis with subtilisin. The initial loss of enzyme activity during iodination is probably due primarily to tyrosine iodination. (Drs. Wolff and Covelli)

Iodination of human serum albumin was studied by difference spectrophotometry in

water and urea. The conversion of "buried" tyrosyl to "buried" iodotyrosyl groups was demonstrated. This indicated that groups normally in the interior of the protein molecule can react, presumably at the surface, under certain conditions and then return to the interior. This finding may have relevance to the biological iodination of thyroglobulin, since interior iodotyrosyl residues occur in the native molecule. (Drs. Edelhoch and Perlman)

Further clarification was obtained from the model reaction in which 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) and 3,5-iodotyrosine (DIT) couple to form thyroxine. Preliminary studies on DIHPPA by UV- and NMR-spectoscopy defined the relation between its keto and enol forms in various buffers and solvents. O_2 is requried for the formation of thyroxine from DIHPPA and DIT. It was shown that the O_2 is utilized to form a stable oxidized intermediate of DIHPPA, which is then able to react with DIT in the absence of O_2. This intermediate is not a free phenoxy radical such as that formed by the oxidation of DIHPPA with permanganate. The intermediate, however, gives rise to a different free radical at a slightly alkaline pH or at neutral pH in certain organic solvents. Investigations on the nature of this free radical and of the oxidized intermediate are now under way. (Drs. Cahnmann, Nishinaga and Kon)

Iodoproteins

In a further search for abnormal thyrolobulins, such as that found in South African cattle with congenital goiter, five lines of the Wollman transplantable rat thyroid tumor have been investigated. One of these (line 1–8) contains considerable amounts of abnormal thyroglobulin. This iodoprotein has a sedimentation coefficient of about 8S, is excluded from Sephadex G–200 and forms a soluble complex with antithyroglobulin antibody. A partial purification has been achieved by filtration through granulated 7% agar. Purification of a quantity suitable for molecular studies is under way. (Dr. Robbins)

In the course of this work, a rapid and simple method for gel filtration analysis of thyroid extracts was devised. It employs a thin layer of G–100 or G–200 Sephadex on a glass plate, and provides a satisfactory separation of thyroglobulin, iodinated serum protein and particulate iodoprotein. (Drs. De Nayer and Robbins)

Protein Synthesis in the Thyroid Gland

The thyroid gland is a useful tissue in which to study protein synthesis since it produces in large quantities a unique protein, thyroglobulin, which is the site of thyroxine synthesis. Work is now under way to define the simplest subcellular system for thyroglobulin synthesis. Thyroid ribosomal fractions have been prepared which are capable of incorporating amino acids into protein in the presence of "pH 5 enzyme", an energy source, and either endogenous or synthetic messenger RNA. Immunochemical identification of the product is under study. It is hoped that this simplified system will be useful in studying the polymerization of thyroglobulin subunits, and in the investigation of control mechanisms for thyroglobulin synthesis. (Drs. Kondo, Robbins, and Rall)

Proteolytic Enzymes in the Thyroid Gland

Further study on the neutral protease in rat thyroid gland revealed that it resembled chymotrypsin in its specificity. On this basis it was considered possible that the activity originated in mast cells, which are known to contain a chymotrypsin-like protease. This was, indeed, found to be the case. The role of mast cells in the rat thyroid is not understood, but it is not considered possible that this enzyme can be involved in the hydrolysis of thyroglobulin *in vivo*. The enzyme has been partially purified, and has an approximate molecular weight of 23,000. (Drs. Pastan and Almqvist)

Measurement of Iodocompounds in Biological Fluids

Further progress has been made in the resolution of iodoamino acids by resin column chromatography. The failure to separate thyroxine and triiodothyronine in serum has been shown to be due to serum albumin, which adsorbs to the resin, precipitates in the presence

of the organic solvent, and cause accretion of the resin granules. The problem may be avoided by hydrolysis of the serum prior to chromatography or the sequential use of two columns. (Dr. Lewallen)

Pituitary Hormones

Growth Hormone

Studies on the molecular properties of bovine growth hormone have been continued. The molecule dissociates into halves in alkali, with a pK of 11.05. A major increase of fluorescence also occurs, with a higher pK, and indicates a molecular alteration in addition to dissociation. (Drs. Edelhoch and Condliffe)

During the study of a series of patients with acromegaly, who are being evaluated with respect to response to radiation therapy, it was found that they tended to have depressed levels of PBI in serum. This has been traced to a depression of thyroxine-binding capacity of the thyroxine-binding globulin. A concomitant increase in thyroxine-binding prealbumin occurs. The mechanism of these alterations is not yet known. (Drs. Roth and Hollander)

By the use of radioimmunoassay of growth hormone, two growth retarded children were shown to have isolated deficiency of growth hormone. Prior to the availability of immunoassay, such isolated deficiencies of pituitary hormone were difficult to identify with precision. (Dr. Roth)

Gonadotropins

A fourth case of carcinoma of the lung with gynecomastia has been studied. Gonadotropic activity was recovered from the blood and the tumor. Since this tumor was clearly not a trophoblastic tumor, this case adds significantly to the syndrome of gonadotropin-secreting lung carcinoma. (Dr. Rosen)

A radioimmunoassay for human follicle-stimulating hormone (FSH) has been developed, using double antibody precipitation to separate free and bound hormone. The assay has a sensitivity which permits measurements on plasma, and physiological studies employing the assay are now under way. (Drs. Rosen and Schlaff)

Thyrotropin

In order to determine the initial site of interaction of thyrotropin (TSH) with its target tissue, TSH was incubated briefly with thyroid slices. The hormonal response persisted despite extensive washing of the tissue, but could be abolished by anti-TSH antibodies. Therefore, the relatively intact hormone appears to be bound extracellularly, at least in the early stages of its interaction. Analogous findings have been obtained with insulin, which becomes similarly bound to striated muscle. This type of binding may be a general property of peptide hormones. (Drs. Pastan, Roth and Macchia)

Vasopresssin

Progress has been made on the development of a radioimmunoassay for vasopressin. Specific antibodies against pure arginine vasopres sin (AVP), lysine vasopressin and oxytocin have been produced by injecting these small peptides without prior coupling to larger molecules. ^{131}I-labeled pure peptide could be purified to high specific activity since it is differentially absorbed to dextran gels compared to unlabeled peptide. The assay is highly specific, and is able to detect blood levels of 50 $\mu\mu$g/m of AVP. It has been possible to measure the hormone in blood after hemorrhage, severe dehydration, and other conditions causing elevated blood levels. Further improvement in precision and sensitivity is under way, and the physiologic and diagnostic application of the assay is being explored. (Drs. Roth and Klein)

Insulin, Glucagon and Carbohydrate Metabolism

Regulation of protein metabolism by insulin and glucagon

Studies have continued with the isolated, cyclically perfused rat liver. Perfusion with insulin at 2.4 μg/hr. inhibits the release of L-valine-^{14}C from prelabeled livers by 40%. Since valine is not metabolized or synthesized by the

liver, its release represents protein degradation. Glucagon at 10 μg/hr. increases valine release slightly, but in the presence of insulin causes a 60% increase in degradation. Valine entry into the liver is not affected. Protein breakdown is not affected by glucose concentration from 1 to 4 mg/ml, and is reduced by omission of red blood cells from the perfusate. Similar insulin effects have been ob tained with the perfused rat hind limb, indicating that the effects are not confined to the liver. The alterations are large enough to suggest that these hormones significantly affect protein metabolism *in vivo*. (Drs. Mortimore and Glinsman)

Regulation of hepatic carbohydrate metabolism

Hepatic glycogen breaks down rapidly *in vitro*. In the perfused liver, glycogen breakdown was nearly complete after 60 minutes of perfusion with oxygenated Krebs-Ringer bicarbonate buffer. Addition of red blood cells to the perfusate markedly decreased glycogen breakdown, roughly in proportion to the oxygen uptake, and this effect was dependent on glucose concentration. The red cell effect is probably localized at an early step in glucose utilization. Since liver apparently lacks competitive glucose transport, as indicated by the lack of effect of 3-0-methy glucose, the red cell effect may be at glucose phosphorylation. Insulin, on the other hand, inhibits glucose release by a mechanism which is not dependent on glucose concentration in the perfusate, and hence is probably localized at a site or sites within the glycogen cycle. (Drs. Mortimore and Glinsman)

Glucose Transport

A study of the characteristics of glucose transport in intestinal mucosa has been initiated in normal and diabetic humans. With the use of α-methyl glycoside as a model sugar, Na^+ dependent active transport has been shown in normal intestine.

Lactrose transport in patients with lactase deficiency has also been examined. Lactose does not appear to be transported into the intestinal mucosa in this disorder. (Drs. London and Segal)

Galactose Metabolism and Galactosemia

The enzyme galactose-1-phosphate uridyl transferase in rat liver has been investigated with a new assay developed for this study. The enzyme is strongly inhibited by both substrates-galactose-1-phosphate and UDP-glucose-1-phosphate. Enzyme activity is present in the fetal liver at 18 days, increases progressively to 10 days after birth, and then falls to the adult levels by 45 days. This activity parallels that of galactokinase. (Miss Bertoli and Dr. Segal)

A new pathway for galactose metabolism has been found in mammalian liver. Galactose is oxidized to galactono-lactone which is con verted to galactonate. The latter is then oxidized to the 3-keto derivative, which is decarboxylated to form D-Xylulose, a sugar capable of entering the pentose phosphate pathway of glucose metabolism. The enzyme galactose dehydrogenase, which catalyzes the first step, has been purified 100 fold. Both oxidation steps require DPN.

In rats, large doses of ^{14}C-galactonate are extensively oxidized to $^{14}CO_2$. Although small doses are excreted in the urine unchanged, loading the animal with galactose causes even tracer doses of galactonate to be metabolized. This new pathway, therefore, appears to operate *in vivo*. (Drs. Cuatrecasas and Segal)

Amino Acid Transport

In a continuation of studies on the defect in cystinuria, a third variant of the disease has been found, based on amino acid trans port in intestinal mucosa. The transport of diabasic amino acids may be completely or partially defective, as may that of cystine. Three of the four possible combinations have been found.

Furthermore, cysteine transport is normal in mucosa which is completely usable to transport cystine. This finding was made possible by the discovery that dithiothreitol could maintain cysteine in a reduced state under

aerobic conditions, and thus enable direct study of its transport. Studies with kidney slices indicate that cysteine and cystine may have separate transport mechanisms. Once transported into the tissue, however, cystine is converted to cysteine. This has been demonstrated by forming the mixed disulfide with N-ethylmaleimide. (Drs. Crawhall and Segal)

Investigation of the relationship between transport of the dibasic amino acids and cyst(e)ine in kidney cortex slices has shown that they have independent influx mechanisms. The dibasic amino acids and cysteine, however, share a common efflux mechanism. (Drs. Schwartzman and Segal)

The possible participation of a phosphatidyl peptide as the carrier in amino acid transport was investigated. The finding that the rate of labeling of the amino acid pool was greater than that of the lipid fraction is inconsistent with the stated hypothesis. (Drs. Schwartzman, Crawhall and Segal)

Protein Structure

The 19S immune globulin and its reduced and digested subunits has been studied by polorization of flourescence. The relaxation times of the various substructures were only slightly smaller than that of the native molecule. This indicates that the substructures have rotational independence in the native molecule. (Drs. Edelhoch and Metzger)

Further studies have been done on the fragmentation of antibovine serum albumin rabbit immunoglobulin by cyanogen bromide. The 5.3S fragment was purified, and found to have a molecular weight of 94,800. A study of its immunological properties showed that, unlike the native antibody, it failed to produce passive cutaneous anaphylaxis and had a reduced ability to fix complement. A comparison of its properties with those of the papain and pepsin fragments of immune globulin indicated that the cleavage with CNBr occur within that region of the heavy chains which is between the critical points of cleavage by the two enzymes. (Dr. Cahnmann and Drs. Arnon and Sela-Weizmann Institute)

PEDIATRIC METABOLISM BRANCH

This branch has concentrated its activity primarily on efforts to elucidate the pathogenesis of the inborn error of metabolism, cystic fibrosis of the pancreas (CF). Four principal areas of study have been followed:

Establishing the nature of the sweat gland defect in CF by the use of structure-function studies with the electron-microscope.

Attempts to define further the immunological and biochemical defect of mucopolysaccharide metabolism considered to be responsible for the protean manifestations of the disease.

Observations on the relationship between salt retaining steroids and the striking and unique abnormality of sweat and other body fluids in cystic fibrosis.

Clinical and pathologic studies designed to improve the knowledge of the pathogenesis, course and complications of this disease, and its treatment.

Immunological and Biochemical Investigations

Evaluation of the inborn error of mucopolysaccharide metabolism in cystic fibrosis

The search for a unique antigenic mucoprotein as a genetic marker for the generalized mucosubstance abnormality in cystic fibrosis has been continued. A systematic immunologic study comparing body fluids and tissue extracts of patients with CF and normal subjects was initiated in preceding years. Rabbit serum antibodies and donkey serum antibodies prepared by inoculating macromolecular precipitates from urine, saliva, pancreatic cyst fluid, and tissue homogenates from lung, pancreas, and salivary glands, have been used, as well as specific antisera to urinary mucoprotein of Tamm and Horsfall.

This year special attention was paid to the urinary glycoprotein of Tamm and Horsfall as it represented a separate fraction whose immunologic and chemical activity could be analyzed in detail. The following conclusions can be drawn from these observations:

The present methods have not been able to detect any antigenic or component sugar con-

tent differences between Tamm and Horsfall urinary mucoprotein or other glycoprotein from urine of patients with cystic fibrosis and that of normal subjects.

The antigenic determinants of the Tamm and Horsfall urinary glycoprotein are not found in the organs and tissues which are involved in the pathology of cystic fibrosis, except for the kidney where it is also found in the normal subject.

The glycoprotein fraction consists of at least two antigenic components and easily breaks down into subunits of different sizes. There is no evidence to indicate the glycoprotein is a single substance in vivo.

From these investigations we can conclude that immunological and chemical differences between cystic fibrosis and normal urinary Tamm and Horsfall glycoprotein do not exist. However, one cannot make the statement that physical differences do not exist, and studies in this regard are being carried out.

The possible genetic significance of this glycoprotein was further pointed out by German investigators, who claimed the Tamm-Horsfall fraction was absent in urines of Negroes, in whom cystic fibrosis is rare. Urines obtained from American Negro children with and without cystic fibrosis as well as from native African Negroes shows no difference in the content of this urinary glycoprotein. (Drs. Schwartz, Pallavicini, and di Sant'Agnese)

Serologic Reactions in Patients with CF

The importance of this study lies in the evaluation of host defense and host response to antigenic challenges because of the chronic nature of the pulmonary disease in CF and because of the finding of *Staphylococcus aureus* in the sputum of almost every patient and the presence of Pseudomonas aeruginosa as well in approximately 70% of the patients with this disease. Investigations of the immunoglobulin groups involved in serologic antibody responses to bacterial insult and studies of antibodies secreted by the respiratory tract have given information regarding most defense of the respiratory epithelium. Using immunoelectrophoresis and immunodiffusion techniques, serum immunoglobulin levels in patients with this disease were found to be elevated. The hypergammaglobulinemia is reflecte primarily in elevations of IgG and IgA. IgM becomes elevated in the most severe cases of pulmonary disease, several months before significant changes appear in clinical or roentgenographic symptoms. Mixed saliva from these patients contains IgG and IgA. Serum IgD levels are in the normal range. (Dr. Schwartz)

Study of Pseudomonas Aeruginosa *and its Slime in Relation to the Pathogenesis of Cystic Fibrosis*

Pseudomonas aeroguinosa isolated from the nasopharyngeal flora of patients with cystic fibrosis produces usually large quantities of viscous slime. Because of the frequent and almost unique association of cystic fibrosis and this mucoid type of organism, there was reason to believe that this change might be induced by a chemical compound produced by fibrocystic patients which acts as a substrate. In addition, the role, if any, of Pseudomonas aeruginosa in making worse the chronic inflammatory and obstructive pulmonary disease of cystic fibrosis needed to be clarified.

A variety of studies have been performed including the demonstration of precipitin antibodies in the serum of most patients with cystic fibrosis from whom Pseudomonas could be isolated from the nasopharynx. A large number of mucoid and non-mucoid strains of this organism were typed, and many different strains were found to be present in the noses and throats of patients with cystic fibrosis. A change from a non-mucoid to a mucoid type of organism could be produced by varying the composition of the growth media. The major component of the mucoid material of Pseudomonas aeruginosa was found by paper chromatography to be mannuronic acid. Mannuronic acid, however, could not be detected in 25 sputum samples from fibrocystic patients carrying this organism. No deoxyribonuclease or collagenase activity could be demonstrated from isolates of Pseudomonas, but elastase activity was consistently found. Pseudomonas

elastase was shown to differ from elastase of pancreatic origin by its electrophoretic mobility in agar-gel and by its lack of inhibition by human serum. (Drs. Schwartz, Pallavicini, and di Sant'Agnese)

Structure-Function Studies With the Electron-Microscope

A morphologic abnormality which would account for the functional defect of sweat glands in CF was looked for. Using skin punch biopsies from both normal subjects and patients with CF, numerous techniques were utilized for the characterization of mucopolysaccharide structures, both intra- and extra-cellular. Metabolic activity as reflected in sweat gland lysozomes were also studied, as well as sites of electrolyte transport.

No distinct differences between the staining for mucopolysaccharide material could be found between CF patients and normal subjects using a variety of histochemical methods. However, dense granules were present in the sweat glands of patients with CF, but not in sweat glands of normal patients when the preparations were stained with permanganate-aldehyde-fuchsin. Similar localization of stain was present when this material was studied with acid phosphatase stains; and, in the electron microscope, staining of these sites also occurred with dialyzed iron. However, the association of acid mucosubstance and acid phosphatase has not been previously seen in lysozomes in any tissue and represents an important finding for the evaluation of cell function.

In preliminary studies further electron microscopic evaluation of transport sites in sweat glands revealed that patients with CF do not deposit as much reactive material at expected transport sites as do normal subjects. (Dr. Grand and Dr. Spicer, LEP)

The Effect of Aldosterone on Sweat Gland and Renal Function in Normal Subjects, Patients with Cystic Fibrosis, and Carriers of the CF Gene

Investigations of the effects of sodium retaining steroids on sodium transport in sweat glands and kidneys have been continued and expanded. These studies originally were initiated because of suggestions in the literature that the peculiar sweat abnormality in cystic fibrosis is due to alteration in the relationship between sodium retaining steroids and the sweat gland as an end organ. Patients in this study were selected either from the normal volunteer patient program or from the cystic fibrosis clinic with an age range of 6 to 26 years. Groups of patients below and above the age of 13 were divided as children and adults respectively. Similar study protocols were followed for all patients and included sweat tests by iontophoresis of pilocarpine nitrate twice daily, constant diet, environment, activity, and suitable control periods before and after steroid administration. In adults aldosterone was given intramuscularly at a dose of 1.0 mg./24 hours, and in children 0.5 mg/10 kg. of body weight per 24 hours.

Normal Adults and Children

These subjects had comparable sweat gland results with aldosterone producing sweat sodium retention, sweat potassium excretion and with a fall in sodium to potassium ratio of approximately the same degree. Age differences were not seen. In adults the renal responses to aldosterone were as expected from the literature. Normal children, however, showed a surprising inability to reduce urinary sodium, did not exhibit the escape expected during the remainder of the treatment period, and reacted by only a small rebound when the steroid was stopped. The results suggested a peculiar lack of responsiveness of the juvenile kidney to the sodium retaining effects of exogenous aldosterone. The oldest child gave an adult type response. The findings are quite different from those seen in normal children subjected to sodium restriction with the production of endogenous aldosterone.

Patients with Cystic Fibrosis

In this group, the responses of renal sodium and potassium excretion were in all ways comparable to those of normal subjects. Sweat sodium retention was significantly less than in

normals. The sweat potassium response in cystic fibrosis patients was in the normal range as was the fall of sodium to potassium ratio, there being no statistically significant difference in the two groups for the latter two parameters. Mothers of CF patients showed kidney and sweat responses identical to those of normal adults.

This ability of sweat glands of patients with CF to respond to large doses of sodium retaining steroids makes it impossible to define the sweat defect in this disease on the basis purely of an end-organ unresponsiveness to normally secreted steroids. (Drs. di Sant'Agnese, Grand, Schwartz, and Pallavicini)

Other Investigations in Cystic Fibrosis

Pregnance in Cystic Fibrosis

Because of the longer survival and increasing life span of subjects with cystic fibrosis new problems arise; such patients present serious and often unique problems in management, not readily comparable to those of other disease entities. This study represents the first attempt to collect information on pregnant women with cystic fibrosis and their offspring. Two patients were followed on the wards of this Institute and data on eight others was obtained from the literature and from a national survey. It was found that the toll of morbidity and mortality as well as the high risk of fetal prematurity made the pregnant women with this disorder a significant challenge in management.

In addition, significant genetic information was obtained from this survey. The fact that all of the 11 children of mothers with cystic fibrosis who survived long enough for observations to be made were normal is further evidence that cystic fibrosis is transmitted as an autosomal recessive disorder. It was calculated that, assuming the incidence of carriers for the cystic fibrosis gene in the United States to be approximately 2 to 5 percent of the general population, the overall risk of having an affected child in a random mating of a fibrocystic mother with a male of unknown genetic status would be 1 to 2 percent. (Drs. di Sant'-Agnese, Grand, and Schartz)

New Syndromes of Pancreatic deficiency Simulating Cystic Fibrosis

The studies initiated last year on patients thought to have cystic fibrosis on the basis of pancreatic insufficiency and at times chronic pulmonary disease, but with normal sweat electrolytes, were continued and extended. Further metabolic, endocrinologic, and pathologic studies were performed on the 24-year-old white male with pancreatic deficiency, microcephaly, dwarfism, deafness, hypothyroidism, chronic lung disease and a chromosome abnormality. Additional studies were also carried out on a 16-year-old white female with dwarfism, pancreatic insufficiency and bone marrow dysfunction who required splenectomy for hypersplenism.

Several other patients with similar conditions are being studied at the present time in the hope of elucidating these previously unrecognized, interesting new disease entities.

Albumin Turnover Studies in Cystic Fibrosis

Hypoproteinemia is a rare, but known complication of cystic fibrosis. Previous investigators have suggested decreased synthesis on the basis of malabsorption and poor nutrition as the main etiological factor. However, loss of protein by gastrointestinal protein leakage or through the respiratory tract and hemodilution are also possibilities.

^{125}I albumin turnover studies were performed in 10 patients with cystic fibrosis and 51 Cr albumin tests for gastrointestinal protein leakage were performed on 10. Albumin survival was studied in the whole body counter using ^{131}I labeled human albumin in 5 patients with cystic fibrosis.

The results indicated that the hypolbuminemia in patients with cystic fibrosis may be due to a variety of causes. Examples of low serum albumin due to an expanded plasma volume, but with normal total circulating albumin were seen as well as patients in whom the hypoalbuminemia could be explained, at least in part, by some increased loss of albumin into the gut. In one patient with cystic fibrosis and multilobular biliary cirrhosis secondary to cystic fibrosis, failure of synthesis appeared to play a role.

These studies are being continued and it is expected that they will clarify the etiology of the hypoalbuminemia in this disorder and point to methods for more effective therapy. (Drs. Schwartz and di Sant'Agnese, and Dr. Strober, NCI)

Studies of Growth, Development and Sexual Maturation in Patients with cystic fibrosis

Retarded growth has been considered up to the present time as a consistent feature of the clinical picture of cystic fibrosis. It is now becoming apparent that the eventual height achieved by these patients as young adults is normal or close to normal for the age group, even though the adolescent growth spurt may at time be delayed. This appears to be especially true of patients diagnosed later in life, presumably indicating that the lesser degree of pulmonary involvement has interfered only slightly or not at all with the growth process. A program of intensive clinical and metabolic studies on young adults with cystic fibrosis is being initiated to study further this question.

Sexual maturation has proceeded normally in patients with cystic fibrosis in both males and females even in the presence of severe lung disease. Unpublished histologic observations indicate extensive periurethral gland and prostatic concretions in post-pubertal males with cystic fibrosis. Evaluation of these various parameters is being undertaken. (Drs. O. SantAgnese, Schwartz, and Grand)

CLINICAL HEMATOLOGY BRANCH

Study of the Immunology of Blood Cell Deficiencies

Idiopathic Thrombocytopenic Purpura

Following identification of the ITP factor as a 7S gamma globulin, a quantitative *in vivo* assay system, using passive transfer of ITP plasma, was developed to evaluate the effectiveness of different forms of therapy with respect to levels of circulating ITP factor. The titer of ITP factor has been compared with clinically demonstrable response to adrenocorticosteroid therapy and splenectomy in a total of 23 cases. Patients who benefited from therap all had very low plasma titers of ITP factor (less than 1:4), whereas all but two patients who did not respond to therapy had high titers of ITP factor (greater than 1:20). The two exceptions were in a patient who had received the antimetabolite 6-mercaptopurine in attempts to suppress immunolgobulin formation and in a patient who had been exposed to benzene and other organic solvents over a period of years in the course of his work.

The method of *in vivo* titration of ITP factor used not only appears to be helpful in predicting the outcome of therapy, particularly splenectomy, but also helps differentiate instances of isolated megakaryocyte dysfunction from otherwise apparently classical ITP. (Drs. Shulman, Watkins, Libre and Cowan)

The Effects of Reticuloendothelial Blockade in Sequestration of Immunologically Altered Cells

Continued evaluation of the use of intravenous red cell stroma as a reticuloendothelial blocking agent has confirmed the effectiveness of this material in preventing platelets sensitized by minimal concentrations of antibodies from being sequested. It was hoped that reticuloendothelial blockade would provide effective therapy for patients with a variety of immunologic blood cell deficiency states who were refractory to more conventional forms of treatment. Use of intravenous red cell stroma in two cases of severe ITP, however, proved to be ineffective, no doubt because cells highly sensitized by immunoglobulins compete effectively with stroma for sites of sequestration, whereas lightly sensitized cells do not. (Drs. Shulman, Cowan, Libre and Watkins)

The Etiology of Transfusion Anuria

There is a body of literature concerning the role of hemoglobin in production of anuria, the most serious consequence of mismatched transfusion. There has been no evalution of the effects of stroma as a possible toxic agent in the hemolytic transfusion reaction. In evaluating intravenous red cell stroma as a possible

therapeutic agent (see above), it was found that renal function, as measured by creatinine and PSP clearance, total urinary output, pH and specific gravity, etc., remained entirely normal when stroma from 9 to 10 units of blood per day were given for a week or more. However, on one occasion when a unit of stroma containing a minor blood group antigen was inadvertently given to a patient who had formed an antibody against it, the mismatched" stroma produced a mild febrile reaction and consequent anuria typical of that following a hemolytic transfusion reaction, despite the fact that no hemoglobin was involved. The evolution of the reneal lesion was typical of that following intravascular hemolysis and fortunately renal function returned completely to normal. Renal failure following intravascular hemolysis is therefore clearly due to the antigen-antibody reaction, and if anything, only secondarily contributed to by precipitation of hemoglobin in renal tubules. There are no other known instances of antigen-antibody reactions per se in which the kidney is the target organ, although much experimental work has been done in attempts to relate antigen-antibody reactions to lesions such as glomerulonephritis and nephrosis. Work on stroma infusions is continuing in association with Drs. Holland and Schmidt. (Drs. Shulman, Libre. Schmidt (CC) and Holland (CC))

Post-Transfusion Purpura

Four additional cases of the rare syndrome of post-transfusion purpura were studied through referrals of patients from London, New Jersey, Baltimore and St. Louis. The syndrome represents the only example of a self-destructive lesion induced by sensitization to an isoantigen (see previous summary). The ten cases that have now been observed are providing clues to factors leading to this type of sensitization. For instance, it is apparent that prior exposure to the antigen is necessary some years before the second exposure that intiates thrombocytopenia. All but one case were in women who had had children, and all of the children tested had the causative $P1^{A_1}$ antigen on their platelets (inherited from the father). The one male patient had had a previous transfusion of antigenic blood ten years before the provocative transfusion. Response to therapy in all instances was that predictable from the experimental evaluation of therapy in all instances was that predictable from the experimental evaluation of thrombocytopenia induced by passively transferred antibody in normal individuals, splenectomized individuals and individuals receiving steroids. This syndrome provides insight into the types of sensitization to foreign antigens that may lead to apparent "autoimmune" states. (Dr. Shulman and Miss Hiller)

Isoimmune neonatal purpura

An additional 24 families have been studied in which neonatal purpura occurred as a result of isoimmunization of the mother by fetal platelets and leukocytes. The data on frequency of immunization to specific antigens in the accumulated 175 cases continues to provide important information on the relative antigenicity of specific platelet and white cell antigens which are potentially important in transplantation immunity (see below). Effectiveness of steroid therapy to the mother antenatally, the child postnatally, and of exchange transfusion, continues to be evaluated. (Dr. Shulman and Miss Hiller)

Transplantation Immunity

Three additional platelet and leukocyte blood group antigens were identified and characterized during the past year, to bring the total identified by C.H.B. to 12 specific platelet or leukocyte groups that can be used in unequivocal immunologic tests for phenotyping cells. These continue to be the only antisera available for clear-cut typing of individuals in platelet and leukocyte antigen systems by complement fixation and cytotoxicity techniques. Typing sera for leukocytes in other laboratories that are dependent on agglutination or antiglobulin consumption tests have been found to be totally inadequate for obtaining reproducible results.

Despite the indications in lower animals that some white cell, platelet, and even red cell

antigens are the same as general tissue transplantation antigens, the massive amount of work done in attempts to correlate blood cell antigens with transplantation immunity in man has not been conclusive. In attempts to find an animal system for directly evaluating human transplantation antigens, the antigenic content of blood cells from lower animals and non-human primates have been tested, using the monospecific antisera mentioned above, as well as multispecific sera derived from patients receiving numerous transfusions. The following findings resulted from continued work done under a contract with Dr. Moor-Jankowski of New York University: Human isoantigens were present more frequently in primates as they ascend the evolutionary scale from monkeys to anthropoid apes, Rhesus monkeys being most dissimilar and chimpanzees most similar to man. Animal cells were found to be effective in characterizing the specificity of human isoantibodies and in fractionating sera containing mixtures of isoantibodies. Chimpanzees, baboons and Rhesus monkeys were readily immunized against human leukocytes and platelets, but only chimpanzees produced antisera that distinguished differences between individual human beings. Heteroimmunization of this type did not appear to be as practical as use of sera from multitransfused human beings, for the human sera are readily available from patients who willingly donate the required amount, and the mixtures of antibodies formed in human beings are not as complex as those formed in non-human primates. In addition, the availability of chimpanzees is too limited to justify use of these animals simply to produce typing sera or to perform blind histocompatibility tests. Anthropoid apes appear to be best utilized in transplantation research for crucial experiments to evaluate some shared human antigens once analysis of the survival of human grafts indicates which antigens should be evaluated in this way (see below). (Dr. Shulman and Miss Hiller)

Evaluation of Histocompatibility in Human Homograft pairs

In collaboration with Dr. Marchioro, Dr. Starzel, Dr. Goldsmith and Dr. Haglin, who are surgeons performing renal homotransplantation, C. H. B. has been able to collect 38 human transplant pairs and phenotype them with the large number of platelet and white cell antibodies available in the laboratory. The outcome of transplantation with respect to survival, the nature of rejection if it occurs, and the histologic picture of biopsies taken at intervals up to two years after transplantation, has been correlated with the number of incompatibilities between recipient and donor in white cell and platelet antigen systems. Although the literature contains reports implicating white cell antigens as being histocompatibility antigens, and suggests that the number of incompatibilities of white cell antigens is very significant with respect to renal transplant rejections, C.H.B. has not found this to be the case. No one specific antigen can be implicated as a transplantation antigen and as a matter of fact, there appears to be no correlation between the number of mismatched white cell antigens as identified by complement fixation and cell toxicity tests and graft survival. Since this series is as large as any studied, these data indicate the difficulties faced in attempting to evaluate transplantation antigens in patients, using white cell and platelet antigens as indicators of compatibility.

Although cultured human blood and lymph cells were found to contain leukocyte isoantigens, cultured human somatic cells did not, by criteria of adsorption as well as direct complement fixation tests. This suggests that leukocyte antigens may not predict incompatilities in organ transplantation other than bone marrow. Mixed agglutination techniques have been used by other laboratories and reportedly detect antigens in common between white cells and other organs. Attempts to confirm this possibility, using numerous antisera, have so far suggested that the shared antigens do not exist. (Drs. Shulman and Libre and Miss Hiller)

Immunologic Therapy of Malignancies

The possibility that some somatic tissues share antigens with blood cells and that malignant blood cells or other tissues may be de-

stroyed by isoantibodies against normal leukocytes or platelets provides a good starting point for evaluating possible immunologic therapy of tumors. In current work, cultured Burkitt lymphoma and leukemia cells have been tested for their antigenic content in collaboration with Drs. Rabson and Shohet and found to contain the usual distribution of antigens that normal leukocytes contain. This, combined with the fact that cells from patients with chronic and acute leukemia also contain the normal complement of laukocyte antigens, suggests that these antigens are a relatively invariable component of the cell and that antibodies against them will be effective in producing cytotoxicity. So far, *in vivo* tests have been carried out on only three laukemics, one chronic lymphocytic, one chronic myelocytic and one acute myelocytic form. The first two patients had a dramatic decrease in circulating leukemic cells with much less antibody than expected from *in vivo* tests with the same antibodies in individuals with normal cells. The patient with acute leukemia had a marked febrile reaction to relatively small amounts of antibody, but evidence of only relatively small numbers of peripheral cells destroyed. None of the patients had sustained effects from the infusions. Studies are continuing to elucidate reasons for increased susceptibility of some leukemic cells and to determine possible effects of these isoantibodies on marrow precursors. The fact that cultured leukemic and lymphomatous cells contain all of the recognized isoantigens suggests that homologous immunization for production of specific anti-tumor antisera will be difficult, if not impossible, unless these antigenic factors are taken into account. (Drs. Shulman and Watkins)

Immunological Considerations Attending Platelet Transfusions

Platelet transfusions in aplastic anemia, idiopathic thrombocytopenic purpura and leukemia were evaluated with respect to the effects of isoimmunization on the survival of platelets and the effects of circulating levels on therapeutic effectiveness. It was found that isoimmunization against platelets occurs infrequently with less than 10 transfusions, but that the frequency of immunization rises steadily with the number of transfusions until all patients eventually become immunized. Isoimmunization against common platelet antigens prevents transfused platelets from circulating and from being hemostatically effective. More than half of the anti-platelet isoantibodies that arose after transfusion were "incomplete" and could not be detected by conventional serologic tests. Some of these antibodies were identified by 'blocking" tests or by passive transfer. These findings explained why refractoriness to platelet transfusions has not always been accompanied by serologic evidence of immunization.

Isoimmune destruction of platelets was found to be a relatively innocuous occurrence and did not contraindicate further trials of platelet transfusions. Although platelets could be matched under some circumstances when isoimmunization took place, at the present time empirical selection of donors based on survival of transfused platelets appears to be the best approach to platelet transfusion therapy in sensitized individuals. (Dr. Shulman and Miss Hiller)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

Identification of the Spleen as an Organ Controlling the Level of Anti-Hemophilic Factor

Little is known about the physiologic variations in Factor VIII and the site of production of this factor has not yet been identified. In recent years it has been found that Factor VIII is elevated in stressful situations and some pathologic conditions, but reasons for these changes have been obscure. Using adrenalin infusions as a means of studying changes in Factor VIII in stressful situations, it was found that Factor VIII levels could be elevated as much as two times normal judged by *in vitro* assays and that plasma obtained from individuals receiving adrenalin was as effective in elevating the Factor VIII levels in

hemophiliacs as indicated by *in vitro* tests. A surprising finding was that Factor VIII did not become elevated in asplenic individuals who were given adrenalin. The spleen is not the organ that produces Factor VIII, for asplenic individuals have a normal concentration of the factor. It appears that the spleen is able to function as a storehouse of Factor VIII and release it under stress, including the stress of severe exercise. Since platelet levels also increase under similar stimuli, there appeared to be a possible relationship between platelet count and Factor VIII level. There was, however, no correlation in the timing of the platelet and Factor VIII responses, and in chronic diseases, in contrast to acutely stressful situations, it was found that thrombocytosis was associated with relatively low levels of Factor VIII and thrombocytopenia with relatively high levels. The reason for the inverse relationship between platelet levels and Factor VIII in chronic diseases is not yet apparent, for numerous *in vitro* studies on the effects of platelets on Factor VIII have failed to reproduce the *in vivo* phenomenon. (Drs. Libre, Shulman and Cowan)

Evaluation of a new Plasma Fraction in the Treatment of Classical Hemophilia

For many years it has been known that Factor VIII travels with fibrinogen in most physicochemical fractionation procedures. A very simple method of precipitating fibrinogen from plasma that has been known for about 20 years, involves slow thawing to produce a cryoprecipitate. This fraction of plasma has been found to contain on the order of 60% of plasma Factor VIII. When resuspended in small volumes, it was effective in producing *in vivo* Factor VIII levels high enough to perform major surgery in three patients without causing vascular overload or any other untoward effect. It was superior to very expensive commercial preparations of Fraction I in that it contained a higher ratio of Factor VIII to fibrinogen and other proteins, contained less calcium binding agents which produce toxic symptomatology, and did not contain anti-red cell antibodies that are coprecipitated with fibrinogen in the commercial Fraction I preparation.

Of special importance is the fact that the cryoprecipitate is a by-product of other Blood Banking procedures. It can be removed from a unit of blood without contamination, using appropriate plastic equipment, and the unit can then be reconstituted for usual transfusion purposes. In the Clinical Center, a waste product of platelet transfusions, the supernatant plasma, has been found to be an excellent source of Factor VIII for treating our hemophiliac patients undergoing major surgery.

The cryofibrinogen precipatate appears to be the best product available for treatment of hemophiliacs at present and can be made in any small hospital that has minimum Blood Banking equipment, can be stored in the deep freeze at home by hemophiliacs, and can be administered without special equipment. (Drs. Cooke, Holland (CC), and Shulman)

The Basis for Thrombocytopenia in Malaria

Using malaria as a disease for studying the pathophysiology of thrombocytopenia associated with infections, we had up until last year found that initiation of intravascular coagulation could not account for the phenomenon, despite numerous proponents of this mechanism in the literature. By using different strains of malaria parasites, and determining platelet survival and localization with chromium-labeled cells, as well as quantitative measurements of all known clotting factors and antimalaria antibodies and complement levels, it was found that thrombocytopenia developed independently of parasitemia, was not due to bone marrow inhibition, and was not associated with significant changes in any of the clotting factors. It was always associated with development of anti-malarial antibodies and fall in plasma complement. This therefore appears to be an immunologically induced thrombocytopenia, probably a reflection of the general type of thrombocytopenic states documented in human beings in association with circulating antigen-antibody complexes. (Drs. Shulman, Sheagren and Jeffrey)

Diseases of Megakaryocyte Dysfunction

Following last year's demonstration that the thrombocytopenia of Aldrich syndrome is due to a peculiarity in the maturation of megakaryocytes rather than peripheral destruction of platelets as previously supposed, several pediatric and adult patients with thrombocytopenia and adequate megakaryocytes have been found to have normal platelet survival. Generally it has been assumed that adequate megakaryocytes in the bone marrow indicate peripheral destruction. Continuing studies are aimed at differentiating abnormal from normal megakaryocytes. (Drs. Shulman and Watkins)

Platelet Physiology

Very little is known about the nature of the physiologic control of platelet levels and the organs responsible for removing these cells normally. Using plasmapheresis and labeled cell techniques in normal volunteers, it has been found that human beings have no platelet reserve that can be called forth under an acute thrombocytopenic stimulus and that newly released platelets from the bone marrow do not appear directly in the circulation. By studying the splenic content of platelets in individuals splenectomized for a variety of diseases and comparing results obtained in normal individuals and splenectomized individuals, it appears that normally platelets reside in the spleen during a 2 to 3 day maturation phase after release from the bone marrow. This temporary sequestration, combined in diseased states with any additional insult by such factors as isoantibodies or ITP plasma, acts synergistically to destroy platelets. Adrenalin infusions appear to be able to release physiologically sequestered platelets and have been a useful tool in evaluating normal platelet production. Continued use of the total body counter appropriately calibrated to take into account all body areas of platelet sequestration has provided additional information on the sites of abnormal and normal platelet sequestration. (Drs. Shulman, Watkins, Cowan and Libre)

Studies of Unusual Hemorrhagic Disorders

Studies of acquired hemophilia due to abnormal gammaglobulins have continued in attempts to elucidate the nature of the immunization leading to this unusual "autoimmune" manifestation. The number of patients now studied with this rare abnormality is nine. Studies also continue on an unusual form of painful purpura, autoerythrocyte sensitization, in attempts to establish the basis for abnormal hemorrhage in which all known vascular and coagulation factors are normal. (Ds. Shulman, Cowan and Watkins)

NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

INTRODUCTION

In fiscal year 1966, the NICHD was reorganized into three major operating arms: The Scientific Programs under a Scientific Director, Communications Activities under an Associate Director, and Program Services under an Associate Director. The Central concept of the reorganization was to place the principle scientific focus on four substantive program areas rather than on administrative mechanisms or scientific disciplines. These program areas, Reproduction, Growth and Development, Aging, and Mental Retardation, are under the general direction of a Scientific Director.

This organization enables the Institute to accomplish its mission more effectively by focusing on the goals of research rather than the means. The substantive programs are responsible in the respective areas for both the conduct and support of research and training. In furtherance of this concept, broad direction and planning of intramural and extramural efforts are appropriately interrelated by an echelon of senior staff.

REPRODUCTION PROGRAM

The Laboratory of Biology and the Endorcinology and Metabolism Branch were created in December 1965 as a result of a transfer of a number of former members of the Endocrinology Branch, NCI, to the newly formed Reproduction Program of NICHD. The transition was not difficult because of the avid interest in reproductive biology developed earlier under the leadership of Dr. Roy Hertz, who became NICHDN'S first Scientific Director.

The research activities of the Laboratory of Biology concentrate on problems relating to con ception, gestation, interrelationships of the anterior pituitary-adrenal-reproductive systems, and the role of biotin and related factors in the physiology of reproduction. Primate studies by this group have been directed toward systematic, quantitative studies of hormone production and excretion through pregnancy and the menstrual cycle in the rhesus monkey. They are also conducting related studies of ovarian function in the pregnant animal.

The staff of the clinical Endocrinology and Metabolism Branch have united as an effective team for the practice of clinical medicine and the conduct of clinical and laboratory research. Efforts begun by this group while affiliated with the Endocrinology Branch of the NCI have been continued, largely without interruption. Areas of investigative interest include the biological and radioimmunological assays of protein and polypeptide hormones, cytogenetic aspects of endocrine disorders, hormonal induction of ovulation, and follow-up of patients successfully treated for gestational trophoblastic neoplasms.

In collaboration with the NINDB, construction of facilities for investigations on primates has been planned. Laboratories and a primate facility will be constructed at El Centro Medico and Sebana Seca in Puerto Rico. NICHD's activities, established in conjunction with the NINDB's program of research in the development of the central nervous system, will focus on the areas of basic reproductive biology and reproductive behavior.

The Program reorganization within the Institute resulted in the creation of the Reproduction Program by the combination of the former Reproductive Biology, Perinatal Biology, Congenital Malformations, and Developmentl Pharmacology Programs. In terms of extramural research and research training, the new Program constitutes about one-half of the NICHD grants. During the year, there was a marked in-

crease in interest in research on population problems. The number of such applications is increasing, but the total investment should be larger. With the advice and encouragement of of the Council, the Reproduction Program continues its active programming of foreign research and training grants.

GROWTH AND DEVELOPMENT PROGRAM

The reorganization of the Institute created a Biological Sciences Branch and a Behavioral Sciences Branch in the Growth and Development Program. In the Nutrition Section of the Biological Sciences Branch, studies being conducted include investigations on the regulation of hepatic protein metabolism and the regulation of carbohydrate metabolism in the isolated rat liver.

In the Personality Development Section of the Behavioral Sciences Branch, research has recently been initiated in certain Washington, D.C., Head Start populations. This project is designed to analyze the components of learning environments of preschool children; to delineate the characteristics of children from deprived environments; to study relationships between specific environmental variables and changes in intellectual and personality characteristics; and to study the interrelationships between cognitive and personality functions of preschool children.

The Program's interest in the sudden death syndrome has contined. The proceedings of the Conference on the subject, held in 1964, have been published and are being distributed. The MEDLARS printout on the sudden death syndrome continues to go to interested investigators. A review article prepared on contract is before the Editorial Board of a national journal.

AGING PROGRAM

During the Fiscal Year 1966, the Gerontology Branch in Batlimore, Md., was transferred from the National Heart Institute to the NICHD. This activity is currently being carried out in laboratories and wards in the Baltimore City hospitals. A building to house this Branch is currently being constructed and should be ready for occupancy about October 1967.

The goals of the Gerontology Branch are to describe in quantitative terms the changes that take place with aging and to investigate the basic biological mechanisms of these changes with a view to reducing the impairments and disabilities of older people.

Highlights of findings from the Gerontology Branch which have extended knowledge of aging include the following: (1) Older subjects show an impaired response with respect to blood sugar levels to the intravenous administration of tolbutamide. (2) The impairment of glucoye tolerance in the aged cannot be scribed to an increase in free fatty acids in the plasma. (3) Total caloric, vitamin, and mineral intakes diminish with age even in subjects whose diets are not limited by economic factors. (4) A mathematical method has been devised which may make it possible to assess individual differences in non-uniformity of lung ventilation. (5) A double isotope derivative assay for angiotonin has been developed which permits the quantitative estimation of nanogram amounts of the hormone. (6) In rats the ability to make fine discriminations in taste diminishes significantly with age. (7) Age changes in collagen may be due to a reoganization of existing intra-molecular crosslinks rather than the addition of new crosslinks.

The Extramural Program supports research and training for research that bears on the aging process and the developmental and retrogressive changes that occur during the adult years. As of March 31, 1966, there were 63 research grants, nine training grants, seven fellowships, four research career developmental awards, and one research career award being supported by the Aging Program. The total expenditure by the Aging Program was in excess of $6 million. The Program supports a wide range of disciplines that are concerned with various aspects of aging. These diciplines can be divided into four major areas of interest: molecular and cellular biology, comparative and human biology, behavioral sciences, and social gerontology.

The Aging Program has collabcrated with the Scientific Information Centers Branch in developing a system that will permit rapid and comprehensive storage and retrieval of infor-

mation bearing upon aging. This will be achieved by the use of both abstract journals and completely computerized techniques. A wide range of subjects, from cellular biology to social gerontology, will be covered. It is believed that the ability to retrieve information rapidly and comprehensively would be a major aid to scientists in the field of aging.

MENTAL RETARDATION PROGRAM

Three Branches and one Laboratory have been organized to carry out direct research activities in mental retardation. These include a Clinical Research Branch, a Laboratory of Biomedical Sciences, a Behavioral Sciences Branch, and an Epidemiology Branch. The Clinical Research Branch and the Laboratory of Biomedical Sciences are operational, but the Behavioral Sciences Branch will not be functional until fiscal year 1967, and the Epidemiology Branch in fiscal year 1968.

The Children's Diagnostic and Study Unit is a Section in the Clinical Research Branch. This outpatient clinic is located at the National Naval Medical Center. The first patient was in December 1965, and by the end of the fiscal year, approximately 45 to 50 children will have been evaluated. The purpose of the Children's Diagnostic and Study Unit is to provide a program of clinical research and a source of case material for basic research in the biomedical and behavioral aspects of mental retardation.

Studies are just being started and include investigations on improving attending behavior in the preschool ratardate, behaviorl assessment of neonates at risk for mental handicaps, and informational discrepancies by parents about their mentally retarded children.

The mission of the Laboratory of Biomedical Sciences is to contribute to the Program's multidisciplinary investigation of mental retardation. Initially, the Laboratory will emphasize biochemsitry, cytogenetics, and neurophysiology.

Projects conducted by personnel in the Laboratory of Biomedical Sciences include pregnancy-associated plasma proteins, electrophoretic variants of leucocyte alkaline phosphatase, studies on hypercalcemia, effects of nutirition on myelinization, effects of amino acids on myelinization, and evaluation of methods of therapy in homocystinuria.

INTRAMURAL PROGRAMS

The principal population research carried out by the Reproduction Program of NICHD is conducted by Dr. Roy Hertz and his associates who for a number of years have carried out important research on the endocrinological aspects of reproduction.

Dr. Henry Vaillant, an NICHD Research Associate, was assigned through the Population Council to a research project designed to measure the efficacy and safety of the intrauterine device with particular emphasis to its possible relationship to cervical neoplasia. This project is being conducted in Barbados, an area with a high population density and a government favorably disposed to family planning.

Our interest in population research has become menifest through the use of contracts. The largest current contract supports the Third Growth of American Family Study. The principal investigator, Dr. Charles Westoff of Princeton, is analyzing the data from the survey conducted last fall and will give a report of his findings in the near future.

Another contract (reimbursable agreement) is held by Dr. Harold Hawk of the Department of Agriculture Research State in Beltsville. Dr. Hawk is carrying out an intensive study of the effect of intrauterine contraceptive devices on the reproductive function of various domestic animals. His findings are significant and extremely interesting. The action of these devices appears to be markedly different in different species. The data from this study not only has direct relevance to fertility control but also indicates that intrauterine foreign bodies may prove to be a useful biological tool.

There are several smaller contracts also under way. Of particular interest is one (reimbursable agreement) with the District of Columbia Department of Public Health to determine if it is possible to carry out in the District a long-term study of the safety and efficacy of various contraceptive methods. We hope that the District will prove to be one of several locations where studies of this type may be carried out.

The staff of the clinical Endrocrinology and Metabolism Branch, consisting of two senior physicians, four clinical associates, two biochemists, four technicians and one secretary have united as an effective team for the practice of clinical medicine and the conduct of clinical and laboratory research. Efforts begun by this group while affiliated with the Endocrinology Branch of NCI have been continued, largely without interruption.

Areas of investigative interest include the following:

Biological and radioimmunological assays of protein and polypeptide hormones
Isolation and purification of protein and polypeptide hormones
Cytogenic aspects of endocrine disorders
Hormonal induction of ovulation
Follow-up of patients successfully trated for gestational trophoblastic neoplasms
Application of computer technology to problems in clinical and laboratory investigation

The inter-relations of our individual and joint efforts will be apparent from the discussion of results obtained as the several interest areas are considered in detail. This integration is the spontaneous derivative of a community of interests rather than the result of administratively-imposed, goal-oriented or directed research.

Thyroid-stimuating Hormone (TSH)

Estimations of secretion rates of TSH in euthyroid and hypothyroid human subjects have been completed. Reasonably reliable estimates of degradation time and secretion rates of TSH in man have been made for the first time. Results show that in hypothyroidism, secretion rates are increased and degradation time prolonged, accounting for the high levels of TSH observed in the plasma of patients with primary myxedema.

Similar studies have been performed in cattle using a radioimmunoassay for bovine TSH.

Luteinizing Hormone (LH)

A radioimmunoassay for luteinizing hormone in man (HLH) has been developed. The method is capable of detecting and quantifying this hormone in volumes of 0.3 ml. of plasma or less and is about 500 times as sensitive as the most sensitive biologic assays.

For the first time levels of luteinizing hormone have been followed in plasma of normal women daily throughout the menstrual cycle. A single sharp peak of activity has been found, occurring about mid-cycle, lasting for 24 hours, and associated with presumptive evidence of ovulation (elevation of basal body temperature and increased urinary pregnanediol excretion). The height of these peaks equals or exceeds levels regularly found in post- menopausal women. In women with ovaries and irregular menses, minor day-to-day variations have been observed for periods of up to 50 days of amenorrhea. Levels in normal males show minor daily variations and no significant variation has been shown diurnally or in relationship to bed rest or ambulation. In contrast, levels are high in castrate males.

Clomiphene, an agent used in attempts to induce ovulation in anovulatory or oligo-ovulatory women, has been shown to cause an elevation of plasma luteinizing hormone in both men and women. Studies are currently under way to determine the effects of the oral contraceptive agents on plasma leuteinizing hormone activity.

Parallel studies, including immunoassays of blood and biologic and immunologic assays in urine, are being carried out in a population of prepubertal children ranging in age from 6 to 9 years. These investigations, in addition to providing information of clinical usefulness in evaluating urinary gonadotropin excretion, should provide some information about the hormonal changes incident to puberty.

The assay methods offer promise in the clinical evaluation of disorders of pituitary and gonadal function.

Melanocyte-stimulating Hormone (MSH)

Considerable progress has been made in the development of a radioimmunassay for alpha melanocyte-stimulating hormone. The assay can be carried out with excellent sensitivity and precision in the absence of serum. So far, it has not been possible to demonstrate any

alpha MSH in either blcod or pituitary tissue of humans but it appears to be present in pituitary tissue of albino rats. The method is not sufficiently sensitive to permit detection of this hormone in the blood of normal rats.

The half-time of disappearance of alpha MSH injected into hypophysectomized rats has been studied by three methods:

Following disappearance by biologic assay using hypophysectomized frogs

Following disappearance of radioimmunoassay

Following disappearance of tracer amounts of radioactively labeled alpha MSH

Data obtained thus far show a half-life of 1.6 minutes by bioassay and of 2.1 minutes by radioimmunoassay. A much longer half-life of 7.8 minutes has been observed in following the disappearance of the radioactively labeled hormone. Studies of the basis for this disparity indicate that the process of labeling alpha MSH results in alterations in the molecule so that biologically it is handled differently than unlabeled hormone. This important observation has considerable pertinence to studies using tracer amounts of isotopically labled hormones in determination of plasma half-life, secretion rates, etc.

Growth Hormone

A clinical test has been devised for the evaluation of pituitary growth hormone secretory capacity by measuring plasma levels of this hormone before and after the administration of pyrogens. The test has the advantage that pituitary ACTH reserve can also be tested simultaneously by measurment of plasma cortisol levels. This test has been utilized in evaluating patients suspected of having pituitary dysfunction in order to ascertain whether the dysfunction relates to one or more pituitary hormones.

LABORATORY OF BIOLOGY

The research activities of the Laboratory of Biology are being concentrated on problems relating to conception, gestation, interrelationships of the anterior pituitary-adrenal-reproductive systems and the role of biotin and related factors in the physiology of reproduction.

Primate studies by this group have been directed toward systematic, quantitative studies of hormone production and excretion throughout pregnancy and the menstrual cycle in the rhesus monkey (Macaca mulatta) as well as related studies of ovarian function in the pregnant animal. In parallel studies in monkeys and rats, we have observed that chemical stimuli for decidua formation in the rodent appear to have no effect in the rhesus monkey. In earlier experiments by this group, it was shown that ovarian hormones are not necessary for normal gestation after the third week of pregnancy, a finding in keeping with observations in man. These marked differences in the reproductive physiology of rodents make it clear the more intensive primate research is necessary.

Steroid hormone metabolism in pregnant monkeys is being actively investigated. As a result of improved analytical procedures, especially isotopic tracer methods, thin-layer and gas chromotography, we are now beginning to obtain quantitatively reliable information on steroid hormone excretion. Estrone, estradiol 17–β, and estriol have been identified in monkey pregnancy urine and quantitative methods are now being applied to determine alterations in estrogen excretion during normal pregnancy. It is expected that this data will prove useful in understanding normal and pathological changes during pregnancy.

Studies on the nature and effects of fetal hormones are continuing. We have found that the fetal rabbit testis has the capacity to synthesize hormones prior to the time of differentiation of the embryonal reproductive tract. Current efforts are directed at identification of hormones in the gonads of the fetal pig. Knowledge of the actions of fetal hormones should lead to a better understanding of the mechanisms regulating fetal tissue differentiation in both normal and abnormal development.

Our investigations on the effects of anterior pituitary hormones on sex steroid production mediated by the adrenal cortex have demonstrated that ACTH in high doses stimulates es-

trogen production in the ovariectomized monkey. Earlier findings on the adrenal-mediated androgenic action of exogenous ACTH and prolactin in the castrated male rat have been extended by means of hormone-producing pituitary tumors which reproduce the androgenic effects reported earlier. Thus we can stimulate certain aspects of steroid function found in pathological states.

The study of biotin distribution in maternal and fetal tissues has demonstrated a marked elevation of biotin in fetal muscle and brain in contrast to the corresponding maternal tissues. Study of cofactor metabolism opens new areas of research in reproduction physiology at the molecular level.

It is to be anticipated that in the coming year we shall continue current research in addition to initiating studies relating to implantation and placenta formation in monkeys and the basic processes of reproduction as our goal.

It is a pleasure to express our appreciation for the moral and material support provided by the NICHD staff.

GROWTH AND DEVELOPMENT PROGRAM

The final approval of the organizational pattern for the entire Institute of Child Health and Human Development in December 1965 brought formal structure to the Growth and Development Program. This area represents one-fourth of the Institute's scientific program. One-third of the Institute's extramural activities are in the Growth and Development Program.

The Growth and Development staff is working with the Information Center staff in the final phases of the preparation of a working vocabulary for indexing the literature in this field. One working conference was held with several of the same consultants used a year ago.

The proceedings of the Conference on Sudden Death in Infants held in Seattle, Washington, September 1964, were published in May 1966. Distribution of the proceedings upon request to physicians, investigators, and organizations supporting research on this subject has been initiated.

The Institute staff has continued to make the MEDLARS sudden death bimonthly printout available to investigators working in the field.

The Growth and Development Program has assumed administrative responsibility for six of the Institute's clinical or research associates. Each physician has been placed with research laboratories outside NICHD. The activities are briefly summarized as follows:

Clinical nuerology combined with a research experience-study of various aspects of cortical maturation in the cat. Staining techniques for the immature cortex have been a major problem in the development of the research. One immediate goal is to quantify the pattern of dendritic growth of the cortical neurons.

In immunochemistry—a study to elaborate and refine a new method to quantitatively estimate immune cytolysis of nucleated cells; differentiation of various murine leukemias by complement fixation and application of this test to studies of rabbit antibody to mouse leukemia; the ontogeny of sheep complement; the evaluation of complement in neonatal cord blood and amniotic fluid and correlation with respect to hemolytic disease of the newborn.

In a biochemistry laboratory—a study of the biological properties of rodent mammary tumors of varying functional differentiation. Tumors have been produced in rats by injection with nitrosourea. The initial problem has been the development of methods for the quantitation of milk production by tumors.

In surgery, clinical care of patients; preparation of a manuscript on hypernephromas; developing plans to study the effect of Dextran infusions in reducing the incidence of metastases.

In oro-pharyngeal development—Took part in a review and analysis of dysphagia in infants; then initiated an anatomical, physiological, and orthodontic study which includes cineradiography. A spirometer has been adapted to measure palatal-pharyngeal insufficiency. Sucking pressure and sucking behavior is under study in cleft palate infants.

In biochemistry—various mechanisms of growth, particularly recovery growth is under study in children with cardiac defects, endocrine abnormalities and malnutrition along

with their normal siblings. Also, rats are being studied, observing the effect of starvation, hormonal deficiency and stress. Work on caloric expenditure and dietary intake under various conditions for children has been undertaken.

AGING PROGRAM

Summary

The Aging Program supports research and training for research that bears on the aging process and the developmental and retrogressive changes that occur during the adult years. It is thus concerned with problems of the entire adult lifespan as well as problems of the elderly. The program supports a wide range of disciplines that are concerned with various aspects of aging. These disciplines can be divided into four major areas of interest; Molecular and Cellular Biology, Comparative and Human Biology, Bahvioral Sciences, and Social Gerontology.

During the fiscal year 1966 the Gerontology branch in Baltimore, Maryland was transferred from the National Heart Institute to the National Institute of Child Health and Human Development. This activity is currently being carried on in laboratories and wards in the Baltimore City Hospitals. A building to house this activity is currently being constructed and should be ready for occupancy about October 1967.

One vital area of the research carried out under the auspices of the Aging Program deals with the basic biology of aging. In the past two decades there have been developments that augur well for an increased understanding of the basic biology of aging. There has been a transformation of our knowledge of the extremely minute structure of the cell brought about by electron microscopy and its ancillary techniques. Our knowledge of the genetic mechanisms through which the daily activities of cells are controlled has increased greatly. The entire body of information that deals with chemical changes in cells and their bases in cellular structure has expanded greatly. It may be that an understanding of the basic biology of aging may be found in this area involving cellular biology and now under investigation by morphologists, geneticists, biochemists, and biophysicists. It is extremely important to an understanding of the basic biology of aging that work in these fundamental areas continue and that the concepts and techniques of these fields be applied to the study of the aging.

One possible mechanism of aging deals with the changes in macromolecular structures that are replaced very slowly. One such protein is collagen. Evidence has accumulated that chemical cross-links in collagen increase with increasing age. This may have some importance from the standpoint of the function of collagen. In addition similar changes may take place in proteins less accessible to study than collagen. The Aging Program is supporting the work of several investigators interested in collagen and in changes in collagen with age. One project has shown that an aldehyde forms one of the cross-links that occur in collagen. Work is being carried on with several agents that reduce cross-linking to see if such a reduction has any effect on other manifestations of aging.

It is possible that during aging errors develop in the genetic deoxyribunucleic acid (DNA) which contains the information that directs the metabolic activities of the cell. One current theory is that this is the fundamental mechanism of aging. If errors do occur in DNA with increasing age, then one might expect these errors to lead to the formation of defective ribinucleic acid (RNA) which in turn would lead to defective enzyme function. These defective enzymes might not suppress the rate of formation of RNA as normal ones do. Thus there might be a greatly accelerated production of RNA. This possibility is being invesigated in several laboratories.

As cells grow old they may fail to excrete all the metabolic products that they produce. One line of evidence suggesting that this is the case is the accumulation of insoluble, brown material in some of the cells of older animals. Work by several investigators on these pigments is being supported. The pigments are being studied from the standpoint of their chemical composition and their mode of production. One theory that is emerging as a re-

sult of these studies is that the cellular lysosomes release enzymes that digest cellular constituents and leave an indigestible residue of lipids and other products which form the pigment.

Still another approach to the basic biology of aging suggests that with increasing age there is an increased production of antibodies against the bodies own cells. It is known that the autoantibodies in the plasma increase with age and that the incidence of amyloidosis increases with age. The Aging Program is supporting research bearing on this approach.

A study on the rats of oxidation by young and senescent rats of various compounds labeled with radioactive carbon has been supported. The overall results and conclusions from the study may be summarized as follows: Acetate-2-^{14}C is oxidized more rapidly in senescent rats than in adult rats. No significant age-associated differences in oxidative rats were observed with several other compounds including acetate-1-^{14}C, octanote-1-^{14}C, propionate-2-^{14}C, D-glucose-1-^{14}C, succinate-2-^{14}C. DL-alanine-3-^{14}C, and DL-glutamate-3-4-^{14}C. The results have suggested that there may be an alteration with advancing age in the metabolic control mechanisms involving mitochondrial respiration and the extra-mitochondrial biosynthetic mechanisms. Studies on the regulatory mechanisms, possibly involving changes in concentrations of intermediates, allosteric alterations of enzyme activity, and hormonal effects will be considered in future research.

One of the recurrent theories in aging research deals with the alterations in tissue permeability with age. There has been much speculation but few direct data on this topic. Recent work under the auspices of the Aging Program have shown that aortic permeability increases greatly with age up to the age of about 45 years. Further studies on the permeability of connective tissue as a function of age will be carried out.

The survival of labeled erythorcytes from young and old rats has been studied in young and old rats in work supported by the Aging Program. It has been found that neither the age of the donor nor the age of the recipient has any effect on the survival of the erythrocytes.

There are many biologic problems that arise as a result of the aging process and that manifest themselves at the level of the organ systems that comprise the body and at the level of the intact organism. These problems in the human can be effectively investigated by cross-sectional and logitudinal studies. The Aging Program is supporting several such studies. One of these is the study being conducted by the Gerontology Branch in Baltimore. About 600 participants have been involved in this study. They have been examined from 1 to 6 times. Measurements of biochemical, physiological, and psychological variables have been made. One of the very interesting findings of this study is that carbohydrate tolerance decreases progressively with age and that beyond the age of 45 years half of the subjects studied have glucose tolerance curves that by the standards applied to young adults would be considered diabetic. In the past year these subjects have been shown to have a smaller decrease in plasma glucose following intravenous tolbutamide than young subjects. This represents further evidence of impairment of carbohydrate metabolism with age.

In addition to the above and other studies which include cross-sectional and longitudinal components, some purely cross-sectional studies are being carried out in men and experimental animals. Purely cross-sectional studies have the advantage of permitting a relatively rapid accumulation of information though they will not answer certain types of questions.

One of these studies has shown that older subjects are unable to correct an induced acidosis as rapidly as young subjects. This is due to an impairment of excretion of titratable acidity and ammonia in the urine.

Studies on the ability of the isolated rat heart to perform work have unequivocally confirmed the previous findings by the same investigators of a significant decrease in cardiac capability with age. Studies to determine the mechanism of this decrease in capability are continuing.

The Aging Program sponsors a number of analyses of age-related differences in behavior

and of relationships between age and psychological, sociological, physiological, and medical variables. Work is being carried out both on humans and on rodents. Work sponsored by the Aging Program and elsewhere shows a welcome trend away from a mere cataloging of age-related differences in behavior and toward analyses of the nature of these differences and the development of techniques to optimize psychological capacities in the older organism.

An abundant literature indicates that older humans usually learn verbal associations much less rapidly than young adult humans. Recent experiments supported by the Aging Program indicate that much of this apparent deficit is due to a reluctance to respond which in turn appears to be related to physiological overarousal or "anxiety" as defined by autonomic measures. Present work on these variables indicates that verbal-associative learning in older humans can be improved greatly by allowing more time in which to respond, perhaps by blocking autonomic activity by drugs, and perhaps by manipulating the older learner's expectancies of the time available for responding.

Older people also have been reported to perform more poorly than young on certain kinds of logical problems which require logical inferences and the discovery of concepts or rules and require a considerable short-term memory load. Studies on the qualitative work patterns and "strategies" of young and old humans are being supported.

Several investigations are dealing with the "information processing" rates and techniques of younger and older humans in a variety of laboratory tasks. Although older people are usually found to be slower, and sometimes poorer than the young in most such laboratory tasks, one of the Aging Program's grantees is testing the hypothesis that the human's ability to learn the patterns in a flow of information increases with age throughout most of the working life. Other investigators are dealing with a variety of other relevant variables, such as short-term memory load.

Several projects concerned with psychological task performances and concurrent psychophysiological indices of arousal and stress, such as electroencephalographic patterns, autonomic changes and free fatty acid mobilization are being supported. The effects on behavior of manipulating autonomic activity by chemical means, in young and old humans are also being studied. Other longitudinal studies in humans relate changes in intelligence test scores and personality measures across time with changes in health status, electroencephalogram, chromosomal changes, and other physiological and biological measures.

It has been demonstrated that a sudden change in environment—such as being moved from one institution to another—produces a substantial increase in death or the development of depression and other psychiatric conditions in elderly humans. A large group of elderly people scheduled to enter institutions will be studied from one year before to one year after institutionalization, with emphasis on personality structure and psychiatric status, in an effort to mitigate this unfortunate effect. A scale of "attitudes toward the elderly" is being developed in order to counsel those on whom the elderly person is dependent.

A less well-developed area for research carried out under the auspices of the Aging Program is that of social gerontology. Investigations in this area focus upon developmental or retrogressive reactions of whole persons rather than upon alterations in abstracted processes such as verbal association, visual acuity, or a particular motor response. Investigators tend to view persons within their existing environmental contexts. Of particular relevance are the effects of economic, physical, physiological and social background factors, and changes in them which are usually concomitant with advancing chronological age in present-day society. Such investigations will help to clarify knowledge of aging through differentiating those changes determined by chronological age from those consequent upon alterations in environmental influences, which are commonly concurrent with advancing age in our culture.

One NICHD-supported investigator analyzed some effects of the well-documented low income level of older people. He studied the ways in which older persons used their more limited incomes, whether the amounts they spent for various categories of consumptions were ade-

quate for their needs, and ways in which the patterns of expenditure of the aged differed from those of younger segments of the population.

Though aging and illness are not synonymous, the incidence of chronic ailments is much higher for people over 65 than for those younger. An investigator found that, while older people were generally aware of the increased likelihood of their incurring such illness, only a small percent expressed concern. Concern about illness was not significantly related to objective or subjective health evaluations, but probably was influenced by the security of these old persons in family life, religion, a stable environment, and financial resources.

Results of another study are consistent in showing that socioeconomic variables are related to reported health status. Tendency to report chronic health conditions was related to labor force status, financial position, and education. More specifically, blue collar workers tended to report illness more frequently than white collar workers, the higher education groups reported less illness than persons at lower educational levels, and higher income groups report less than the lower income groups.

Great emphasis has been placed on the training area in the last year. The amount of money approved for training in aging by the National Advisory Council has tripled in the last year. The total number of training grants approved has increased from 5 to 15.

The Aging Program has collaborated with the Information Center of the Communications Branch to help in developing a system that will permit the storage and retrieval by abstract journals and computerized techniques of information bearing on aging. All areas from cellular biology to social gerontology will be covered. It is believed that the ability to retrieve information rapidly and comprehensively will be a major aid to scientists in the field of aging.

Research at Bethesda

Two research projects are being conducted by the staff of the Aging Program at Bethesda. One of these is concerned with the changes in connective tissue with age. Studies carried out under this project have shown that there is a striking increase in the permeability of the aortic wall to albumin with increasing age. Studies directed toward detecting changes in the permeability of other connective tissues are being started. Such changes in permeability of various connective tissues might have considerable significance with regard to changes in the functioning of those tissues with age.

The second research project is concerned with the reaction of elderly people to their housing conditions. Previous studies by the investigator concerned have shown that housing has a great psychological impact on the elderly. The present studies are designed to confirm and extend the previous studies.

GERONTOLOGY BRANCH

The goals of the Gerontology Branch are to describe in quantitative terms the changes that take place with aging and to investigate the basic biological mechanisms of these changes with a view to reducing the impairments and disabilities of older people. The research program falls into two major categories: (a) the description of biochemical, physiological, and psychological changes that take place with aging, and (b) investigations of the mechanisms of age changes at the social, psychological, clinical, physiological, biochemical, cellular, and molecular levels.

During the past year, members of the professional staff have contributed considerable time and effort to working out the design of many special features of the Gerontology Research Building to be constructed in Baltimore. Construction of this 6.9 million dollar research building started in September 1965. Completion of the building is scheduled for October 1967.

Highlights of findings from the Gerontology Branch which have extended knowledge of aging include the following.

Age differences in interference effects in learning are significantly reduced when anticipation time during the original learning is long.

Older subjects show an impaired response with respect to blood sugar levels to the intravenous administration of tolbutamide. These results offer further evidence of impairment of carbohydrate metabolism with advancing age.

The impairment of glucose tolerance in the aged cannot be ascribed to an increase in free fatty acids in the plasma.

Total caloric, vitamin, and mineral intakes diminish with age even in subjects whose diets are not limited by economic factors. In contrast, the intake of vitamin C increased after age 70. In about half of the middle aged and elderly subjects the dietary intake of Ca was less than the NRC recommended intake.

A mathematical method has been devised which may make it possible to assess individual differences in non-uniformity of lung ventilation.

The impairment in the ability of the aged kidney to form ammonia is not due to inadequate amounts of substrate (glutamine) for its production.

A double isotope derivative assay for angiotonin has been developed which permits the quantitative estimation of nanogram amounts of the hormone.

A method has been developed which permits the achievement of a steady state of blood glucose levels at any concentration desired by the investigator. This method will be of great value in investigating many problems of glucose metabolism in humans, such as the mechanisms of insulin synthesis and release in response to hyperglycemia.

In rats the ability to make fine discriminations in taste diminishes significantly with age.

Although the experimental introduction of "errors" by feeding ethionine to rats increases protein and RNA metabolism, no age differences in the response were observed.

In rats, enzymes involved in neural transmission and conduction failed to show significant differences between young and old rats.

The reduction of food intake in maternal rats has been shown to produce young which grow at a slower rate than normal and fail to attain normal adult size even when fed *ad libitum* after weaning. This method will be of great value in projected studies of the life lengthening effects of starvation in rats.

Under appropriate conditions the degradation of RNA by metal ions such as zinc can be made specific and this specificity can be changed by inhibition of the degradation by another metal ion. Thus non-enzymatic reactions may be utilized for base-sequence determinations of nucleic acids.

An additional coupling factor associated with oxidative phosphorylation has been isolated from cardiac mitochondria.

Age changes in collagen may be due to a reorientation of existing intra-molecular cross-links rather than the addition of new cross-links.

Aging in the Human

The longitudinal multi-disciplinary study of subjects aged 17 to 103 years has been successfully continued in the past year. The total subject group now numbers 587 self-recruited community-dwelling men. It is 8 years since the study began and we are thus just beginning to examine the earliest volunteers for the sixth time. Half of the group has now had at least 3 visits (at approximately 18-month intervals) and one fourth of the group has had 4 visits. Considering the life span of man and the anticipated rates of change in the various physiological and psychological functions being measured, the study is reaching a stage of longevity in which it will begin to be appropriate to analyze the data for longitudinal trends and for intercorrelations of various related functions. In anticipation of this complex task the rate of conversion of data to a punch card system is being increased and preliminary tests of the data retrieval system have been conducted by the *Human Performance Section.*

These subjects, the Longitudinal Group, continue to provide a valuable cross-sectional population for a variety of studies by several sections of the Gerontology Branch.

Because of their high level of education, these subjects are of special value in studies of psychological functions. The *Section on Experimental Psychology* has been especially interested in determining the factors which lie

behind the impaired learning ability which has been observed even in these subjects. Although the elderly subjects can learn material to the same criterion as the young, it takes them longer to do so. It is now apparent that the extent of the impairment observed depends on the conditions imposed in the learning experiment. In fact, when old subjects are allowed as much time as they want to respond they can reach the criterion of learning with the same number of trials as the young, but the time will be longer. The number of errors (as well as the number of trials required) increase more with age when the time given to respond is short than when it is long. In fact, the relationship between errors and age is curvilinear when the anticipation time is short, whereas it is linear with a smaller slope when the anticipation interval is long. It has also been shown that old subjects are more susceptible to interference (increase in errors) from extraneous material introduced between trials than are the young. However, it has now been found that interference is greater in old subjects than in young only if the time available to respond when learning the original task is short; if that time is long, there is no age difference in the degree of interference from an interpolated task.

These studies lead to the hypothesis that short-term memory storage may be a primary factor in the difficulties older people experience in learning. The experiments have shown, however, that alterations in the conditions of learning can compensate for this difficulty. It is further hypothesized that active responding is conducive to learning, particularly for an old person and lengthening the anticipation interval increases the probability of a correct response. It is also possible that reenforcement by presentation of simultaneous cues from different sense modalities will enhance learning in the adult. Further studies to test these hypotheses will be conducted.

Unravelling of the complex relation between (1) the decline in performance of a physiological function with increasing age, and (2) the increasing prevalence with age of a disease associated with that function is one of the major areas of investigation in clinical gerontology. A prototype of this problem is under study in the *Metabolism Section*: (1) the physiological decline with age in the ability to metabolize administered glucose loads, and (2) the age-associated increase in the prevalence of overt diabetes mellitus. The immediate goals of this program have been (*a*) to delineate the underlying mechanism (s) of the decline in function with age, and (*b*) to develop the first realistic age-adjusted standards for the several diagnostic tests for diabetes mellitus used in clinical medicine.

Studies on the Longitudinal Group had previously shown that the standards in common use for interpretation of the intravenous glucose tolerance test and the cortisone glucose tolerance test were appropriate for young adults only since their application to middle-aged and older adults resulted in a diagnosis of diabetes in 50% of the group. In the past year an extensive study was done on the Intravenous Tolbutamide Response Test as well. The test is an interesting addition to the diabetes diagnostic armamentarium of the physician since it is not simply a variant of glucose tolerance testing and reputedly has higher specificity than other diagnostic procedures. Results show that just as diabetics respond deficiently in the test, the presumably nondiabetic middle-aged and older longitudinal subjects also have deficient responses when compared to young adults. The rigid application of the currently recommended standards for normality to subjects over age 40 results in the classification of almost half of them as "diabetic". These unrealistic standards are probably the result of an inappropriate technique for selection of the subjects used to establish the normal standards for the test. From the results on the Longitudinal Subjects new standards for the test have been developed. These are presented in the form of a nomogram which permits the determination of the percentile rank of each individual among his age peers.

The Longitudinal Subjects have also been used as a group for the testing of one of the current hypotheses concerning the cause of decreased glucose tolerance. The hypothesis states that free fatty acids (FFA) in plasma

inhibit glucose utilization. The decline in tolerance to glucose with increasing age then could be explained by an increasing concentration in FFA with age. Results in Longitudinal Subjects who were given oral glucose tolerance tests, however, show no correlation between FFA concentration and age or between FFA concentration and tolerance to glucose.

Results of a computer analysis of detailed 7-day food intake diaries on 252 Longitudinal Subjects have been analyzed under the supervision of the *Nutritional Biochemistry Section.* This again is purely a cross-sectional study at this time. The conversion of dietary food portions of the nutrient elements (calories, carbohydrates, protein, fat, minerals, and vitamins) was accomplished with a computer program devised by members of the Heart Disease Control Program. At the moment, it is possible only to characterize the nutrient intake of the Longitudinal Group as a function of age; important correlations with other parameters (anthropometrical, physiological, and biochemical) will be the next step in the analysis of the data. Thus far it is clear that in the upper middle-class highly educated group of men, total caloric intake, vitamin and mineral intake tend to decrease with age with the main exception that intake of vitamin C does not follow this trend and, in fact, its intake is increased in the diet after age 70. Of interest is the fact that National Research Council recomended allowances were exceeded for all nutrients except Ca in the great majority of subjects. Calcium intake was below NRC allowances in about half of the middle-aged and older subjects. The pattern of food intake shows distinct differences with increasing age in that while the percentage of total calories derived from protein remains constant, more of the calories are derived from carbohydrate while fat plays a decreasing role as a source of calories. Whether these differences in caloric source in individual subjects on spontaneous diets will correlate with such parameters as serum lipid levels, carbohydrate tolerance, or the prevalence of atherosclerotic disease at the time of the study or its incidence in the future are some of the problems that remain to be determined.

Studies on the Longitudinal Group by the *Pulmonary Physiology Section* have begun to be examined for longitudinal trends in individual subjects. Preliminary analyses on 70 subjects who have had 4 visits show that with regard to vital capacity and maximal breathing capacity, only the older subjects show a downward trend in function while younger subjects show either no change or an increase in function. The ability to detect age differences in time trends in the relatively brief period of 6 years (4 visits) is encouraging.

A subsample of the Longitudinal Group, 20 subjects aged 39–83 years, has been studied in a set of experiments using techniques too complex to apply to the entire group. The characteristics of the barrier in the lungs across which gases must diffuse have been thought to change with increasing age; pulmonary diffusing capacity presumably decreases. However, these measured changes could be artefactual due to an increasing degree of non-uniformity of lung ventilation associated with aging. This hypothesis was tested using a technique for measuring pulmonary diffusing capacity by sequential steady state and washout methods. The extreme complexity of the equations generated by a multi-compartment system with non-uniform ventilation volume/diffusion was handled with the aid of a computer program (IBM 7094, University of Chicago). Thus more accurate measurements of diffusing capacity could be made in the non-uniform lung. These measurements show that part if not all of the age changes in diffusing capacity previously reported may be the result of an age differential in the non-uniformity error of the methods employed to make the measurement.

Future studies from the *Section on Pulmonary Physiology* will capitalize further on the advantages of the high speed digital computer in terms of accuracy, ease, and rapidity of the necessarily complex calculations in this field. In addition, pulmonary evaluations will be continued with measurements of lung volumes, pulmonary gas distribution, diffusion and compliance measurements. The addition

of a body plethysmograph will also permit more direct estimates of the effects of deep breathing on thoracic gas volume and distribution.

New tests introduced into the longitudinal testing program in the past year included (1) detailed blood typing in order to characterize the participants immunogenetically, (2) "tapping-in-place" tests in order to investigate the possibility that the extra time required by older subjects in the two target tapping test is due to uncertainty in aim, and (3) a self-administered activity survey in place of the time-consuming interview.

Renal function in the Longitudinal Group has been evaluated with the 24-hour creatinine clearance test. In the past year the number of subjects with 3 consecutive tests has increased to 192, a group large enough to permit a preliminary examination for cross-sectional data, individual longitudinal trends, and within-subject variance. The cross-sectional data were best fitted with a second order regression; this development is interesting since, unlike most linear physiological age differences, in this test the decrements with age are small in early adult life, but become much more pronounced as age advances. The within-subject coefficients of variation at all ages are about 11–13 percent. The importance of this type of analysis is that it permits us to plan the most efficient testing schedule in terms of number and frequency of re-tests for the detection of longitudinal trends. Such information should also prove of value to other prospective studies on man now in progress or planned for the future. We plan to use similar anlytical techniques for the other tested physiological variables in our program.

A number of clinical investigations in the Branch are being conducted on subjects other than the Longitudinal Group. In some of these studies the stimulus to the study again was the apparent similarity between changes occurring with advancing age on the one hand and in certain disease states on the other.

The *Renal Section* has continued its studies on the factors controlling urinary cation excretion. It had previously been noted that under conditions of a water diuresis during the basal state older subjects commonly (7 of 15 studied) had an inappropriately high loss of sodium in the urine. Unchecked, this defect could lead to hyponatremia. A detailed investigation has now been made of the mechanism of this defect and, in a related study, of the mechanism of the development of hyponatremia seen in certain acute and chronic illnesses (tuberculosis, malignancies, febrile states). For these comparative studies relatively healthy older subjects on the Gerontology Ward at the Baltimore City Hospitals composed the first group and all of the verified cases of hyponatremia (sodium concentration less than 125 mEq./L.) seen on the wards of the Baltimore City Hospitals during an 18-month period comprised the second group. The 16 hyponatremics were supplemented by another equal sized group of patients with similar illnesses, patients who might be expected to develop hyponatremia but whose serum sodium concentration had not yet reached abnormal levels. Distinct differences in these groups were found. The defect in the old but healthy group with inappropriate urine sodium loss was correctible by administration of glucose or related metabolites. Since the renal medulla is totally dependent upon glycolysis, this result suggests that an overnight fast leads to inadequate substrate supply to renal tubules with resultant inadequate sodium reabsorption; glucose availability then corrects this defect. In contrast, the acutely and chronically ill patients and hyponatremics rarely showed this defect. In 4 carefully studied patients and syndrome of inappropriate anti-diuretic hormone secretion could be shown to be responsible for the hyponatremia. These subjects had not only balance studies, but also measurements of aldosterone secretion rates, steroid levels, blood volume determinations, and erythrocyte electrolyte levels.

In the past year another defect of the renal function in the aged has been reinvestigated. The observation had been made in the Gerontology Branch a number of years ago that recovery of serum pH following an administered acid load (ammonium chloride) was slower in older than in younger subjects. The mechanism of this defect has now been investigated by

measuring the urinary excretion of "titratable acid" and of ammonium ion. Both of these avenues of H^+ loss in the urine were decreased in the older subjects. The decrease in titratable acidity was commensurate with the lowered glomerular filtration rate in the aged, but the diminished ability to excrete ammonium was proportionately greater than the GFR decrease. A further study was therefore conducted to test the hypothesis that the defective ability to excrete ammonia is due either to inadequate substrate (glutamine) or enzyme (glutaminase) for its production. Glutamine administration to old and young subjects resulted in equal increases in NH_3 production during an acid load and therefore the hypothesis seems unlikely.

An extension of these acid-base studies conducted by the *Renal Section* concerned the regulation of hydrogen ion concentration with the mitochondria of rat liver. Mitochondrial preparations met the criteria of respiratory control and their pH was measured with the C^{14} DMO technique. The pH was found to be linearly related to the extramitochondrial pH when the latter was set at levels of 6.90 to 7.60, but intramitochondrial fluid always remained somewhat more alkaline by about 0.3 pH unit.

The *Endocrinology Section* has also conducted studies in an area in which the change in a physiological variable (blood pressure) with increasing age resembles the change occurring in a disease syndrome (hypertension). Understanding of the relation between aging and disease in this instance again requires knowledge of the basic mechanisms underlying the changes. An intensive effort has therefore continued to develop a physico-chemical method for the measurement of plasma renin and angiotensin. Progress in this area of research has been long hampered by lack of such methods; heretofore, all analytical techniques depended on bioassays, most of which are of questionable specificity and precision. Over the past year we have succeeded in developing a double isotope derivative assay for angiotensin. This assay permits quantitative determination of nanogram amounts of the hormone. This degree of sensitivity is required for the measurement of the small quantities of angiotensin generated during *in vitro* assays of plasma renin. It should soon be possible to apply this new technique to the determination of renin. The importance of this project is along both theoretical and practical lines. Along theoretical lines, it is the first isotope derivative assay to be developed for a small polypeptide hormone and could lead to the development of other assays of similar important peptides. A practical renin assay, on the other hand, would be a major step in the everyday hospital differential diagnosis of hypertensive disease, since it would almost certainly permit recognition of unilateral ("ischemic") renovascular hypertension. Recently the determination of plasma renin has assumed great importance in the diagnosis of primary aldosteronism, a disease which may account for as much as 10-25 percent of cases of hypertension heretofore considered to be of the "essential" variety. The problem has, therefore, a place in the attack on hypertension, a major age related disease complex. In addition, renin and angiotensin are intimately related disease complex. In addition, renin and angiotensin are intimately related to the secretion of the adrenal glands' mineralocorticoid hormone, aldosterone, and adequate methodology is badly needed in this area of physiology and pathophysiology. An additional aspect of the program is an interrelated study of renin-angiotensin-aldosterone in normal old persons. The data obtained in this study will establish normal standards for aldosterone secretion rate under conditions of salt loading and salt depletion. This information is unavailable but is badly needed in evaluating the role of aldosterone in the elderly hypertensive subject.

Another area of major interest to the *Endocrinology Section* is the field of thyroid hormone metabolism. The control of the rate of thyroid hormone degradation is age-dependent, as we have shown previously, but factors which determine this rate are largely unknown. By studying, as we presently are doing, the situation in acute febrile illness (bacterial infection) where rapid changes in hormone metabolism are known to occur, we hope to learn more of the normal control mechanisms for

thyroid hormone metabolism and ultimately those involved in the changes with age. Of current interest are the interrelationships of hormone degradation rate, plasma thyroxine-binding proteins and plasma "free" (non-protein-bound) thyroxine. Preliminary results in human subjects experimentally infected with the organism of tularemia (Pasteurella tularensis) indicate that in this particular febrile illness thyroxine degradation rate is *not* accelerated, in contrast to the situation in other bacterial pneumonias. Nonetheless, thyroxine binding pre-albumin falls to low levels and, presumably, "free" thyroxine rises as a consequence. These results would appear to exclude "free" thyroxine and febrile illness, *per se*, as important physiological determinants of thyroxine degradation rate and will require extensive revision of current thinking in this area.

The remarkable but unexplained decrease in glucose tolerance with increasing age has provided the stimulus to the development of a new experimental technique for the study of the physiology of the glucose-insulin system by the *Metabolism Section*. The feed-back principle is used to adjust periodically the rate of infusion of glucose intravenously in order to maintain a predetermined new steady-state arterial glucose concentration. In these studies the hyperglycemic stimulus to the beta cells of the islets of Langerhans is constant and nearly identical in all subjects, in contrast to the marked variability in glucose levels that occur during the usual glucose tolerance tests. The ability to achieve a steady state with this technique is shown by the low coefficients of variation in glucose concentration over a two-hour period. In 32 subjects the mean CV was 3.9 percent. The time course of insulin release in response to sustained hyperglucemia is complex and cannot be described by a simple dose-response curve; in young adult subjects plasma concentrations of insulin continue to increase over the two-hour period. This changing insulin level is reflected by the progressively increasing glucose infusion rate needed simply to maintain the arterial glucose concentration at its predetermined level. Older subjects differ from younger subjects in that (1) they require a lower glucose infusion rate and (2) they show less change in infusion-rate over the two-hour period. The implication of these age differences is that there is a diminished ability to respond to hyperglycemic stress in old age. The ability to increase plasma insulin concentrations progressively over a two-hour period which young people possess seems to be deficient in older subjects. These age differences raise questions concerning the mechanism of insulin synthesis and release in response to hyperglycemia; the servo-infusion technique should help to answer these.

Biology of Aging

Although the quantitative description of age changes is of great importance, the development of methods for mimizing the deleterious ecects of these changes depends on understanding the basic mechanisms of aging. This requires studies on an animal species in which genetic and environmental factors can be controlled and experimental techniques can be applied to isolated systems. Studies on basic cellular and molecular mechanisms must be pursued agressively. It is for this reason that part of the resources of the Branch have been devoted to studies of cellular biochemistry and the structural characteristics of molecules involved in biological processes.

Recent gerontological theory has attempted to explain age differences in motivation on the basis of physiological factors and in terms of differences in response to various environmental factors. A general factor termed "rigidity" has also been proposed as an explanatory construct. The program of the *Section on Animal Behavior* is concerned with (*a*) determining basic behavioral age differences, and (*b*) finding environmental factors which reduce age differences. The use of lower organisms such as the rat is necessary for such a program.

It has been found that environmental variables such as systematic handling by the investigator or testing during the dark portion of the dark-light cycle which reduced fear in young rats as evidenced by increased exploratory behavior generally had no effect on old animals. However, restriction of food intake

increased exploratory behavior in both young and old animals. Thus, an increase in motivation (food deprivation) has resulted in behaviorally less rigid groups of animals, which are similar to animals younger in age.

An initial experimental study of taste discrimination was concerned with ingestion of very low concentrations of sucrose or quinine and the effects of past experience with quinine upon sucrose ingestion and vice versa for male rats 1, 5, 15, and 25 months old. Old animals did not discriminate the solutions as well as younger groups, and preceding ingestion experience was important (e.g., rats initially tested with sucrose drank *more* quinine and rats initially tested with quinine drank *less* sucrose). However the effects of past experience were clearly not a function of age, but rather of reduced sensory discrimination with increasing age. Changes in the plasticity of the nervous system could be responsible for the reduction of sensory discriminability with increasing age.

A second experiment examined the ability of rats 1, 5, 15 and 25 months old to discriminate between two above threshold sucrose solutions. Normally rats prefer the more highly concentrated of two sucrose solutions. By making the discrimination more difficult, i.e., pairing very similar solutions, it was possible to examine age differences in the ability to detect the more highly concentrated solution. If the neural system of the aged rat was more rigid (e.g., reverberating cell assemblies were less flexible in form or resistant to change) than in the young rat, such discriminations should be more difficult for the older rats than for young. The data were consistent with such a hypothesis. In addition, when presented with palatable solutions young rats markedly increased their fluid intake, while senescent (25-month-old) rats were unable to alter ingestion rates. The fluid intake increase for young rats was greater as the discrimination became more difficult, suggesting that younger rats may also make more comparisons between tube pairs, i.e., have a higher general behavioral plasticity, than senescent rats.

Other experiments are planned to test further the hypothesis that aging is associated with greater rigidity of behavior. In this connection conditioning studies will be carried out on old and young animals with various schedules of training and the introduction of multiple responses.

Rats have also been used for studies on age differences in the performance of the isolated heart by the *Cardiovascular Section.* Additional experiments have fully confirmed the preliminary report that the heart of the senescent rat shows a significant impairment in its capacity to do work. The left ventricular work index expressed in gm-M/100 mg dry heart weight for the isolated heart-lung preparation was 34 $\pm$ 4 for 24-month-old animals and 166 $\pm$ 14 for 12-month-old animals ($P<$ 0.01). Similar differences (15 $\pm$ 5 and 77 $\pm$ 13) were found for the isolated heart. It has also been found that direct electrical stimulation of the sino-auricular node fails to revive hearts that have worked to exhaustion. Hence it is concluded that the failure to perform is related to biochemical factors in the myocardium. These studies will continue to explore the biochemical mechanism of failure of the senescent hearts to perform as well as adult hearts. Studies of specific enzyme systems and concentrations of substrates essential for energy production will be conducted.

One of the current theories of aging proposes that with senescence alterations occur in the structure of the DNA molecule. This error is transmitted to messenger RNA and ultimately to newly synthesized enzymes. These defective enzymes may be inactive and therefore an accumulation of substrates within the cell may take place.

Data obtained during the past year in the *Section on Nutritional Biochemistry* have failed to support this theory of aging, which proposes an accumulation of structural errors in DNA which are reflected in RNA and protein, as well as increased turnover of these compounds to compensate for such errors. For example, it may be postulated that structurally altered proteins should be detected by alterations in the mobility of enzymes during gel electrophoresis. However, no changes with age were found in the patterns of isoenzymes of seven dehydrogenases in various tissues of

rats. Furthermore, although marked increases were found in the metabolism of RNA and protein when animals were fed ethionine to experimentally introduce errors into protein, a comparison of 40 old (24–31 months old) and 40 young (12-month-old) rats failed to demonstrate differences in metabolism of RNA and protein between the two age groups.

It has been proposed that age changes may be most easily detected in enzymes which control specialized functions of highly differentiated cells. In order to test this proposal, two enzymes which are involved in neural conduction and transmission were measured in cerebrum, cerebellum and brain stem of young and old rats. However, no age-associated alterations were observed in the concentrations of DNA, RNA or protein or in the activities of acetyl cholinesterase or sodium-potassium activated ATPase.

Efforts made to identify the factors responsible for increased longevity in rats subjected to dietary restriction have required time consuming measurement of daily food allotments for large numbers of animals. During the past year, nutritional methods were sought which would avoid this necessity but would yield animals with similar characteristics and longevity. Preliminary data indicate that restriction of nutrients prior to weaning by (1) reduction of maternal intake or (2) employing, during lactation, mothers who had just nursed another litter, produced animals which exhibited low growth rates and which were unable to attain body weights equal to normal animals in spite of unlimited access to food following weaning. Although data on longevity are not yet available, these procedures may offer means of obtaining restricted animals without the laborious task of daily food rationing.

The experiments conducted thus far to test the error theory of aging based on the experimental introduction of structural alterations into protein were complicated by the influence of the ethyl group of ethionine on RNA and fat as well as protein metabolism. Therefore, studies will be conducted in which a different analog, viz., p-fluorophenylalanine, will be fed to rats and changes in RNA and protein metabolism will be measured in liver slices. In addition, the *in vivo* incorporation of radioactively labelled p-fluorophenylalanine and ethionine into liver proteins will be carried out to demonstrate that the analogs are incorporated into proteins. Finally, techniques are being perfected so that age-associated structural alterations in enzymes, as proposed by the error theory, can be detected by the quantitative determination of the isozymes of a variety of dehydrogenases in rat tissues.

The studies in progress to test the proposal that age changes may be most easily detected in those enzymes which control specialized functions of highly differentiated cells will be continued. Similar data to that already reported for the rat will be obtained on mice, via., the activities of acetyl cholinesterase and sodium-potassium activated ATPase will be measured in cerebrum, cerebellum and brain stem and samples of these same sections will be examined for morphological alterations with age.

Efforts will be continued to obtain restricted rats without the laborious task of daily food rationing by restriction of nutrients prior to weaning. Studies will be conducted to produce large numbers of animals in order that data on longevity as well as information on the biochemical characteristics of such animals throughout the life span will be obtained.

In view of the key role of DNA and RNA in relation to "error" theories of aging the Gerontology Branch has placed special emphasis on studies of the molecular structure of these and related compounds.

The principal activity in the *Section on Molecular Biology* in the past few years has been the elucidation of the interaction of metal ions with the nucleic acids. There have been two main objectives in these studies: (1) to provide chemical techniques for the determination of the sequence of the nucleotides in the nucleic acid chains and (2) to understand the biological role of such metal ion-nucleic acid interaction. The importance of such fundamental studies to an ultimate understanding of the aging phenomenon is obvious.

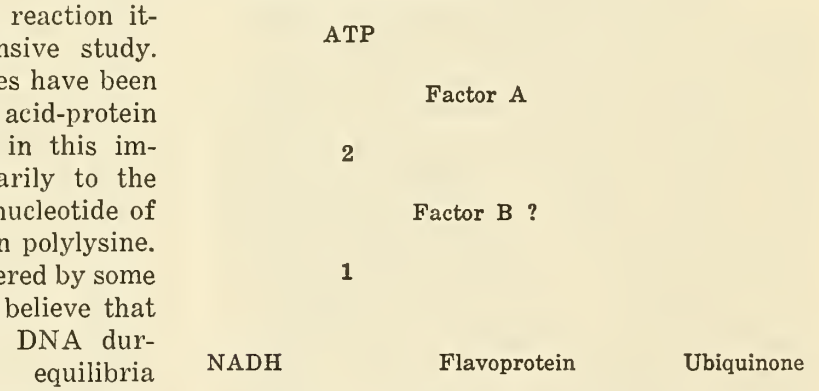
During the past year the important discovery has been made that under appropriate conditions the degradation of RNA by metal ions such as zinc can be made specific, and this specificity can be changed by inhibition of the degradation by other metal ions. Thus the way has been paved for the use of nonenzymatic reactions in the specific cleavage of nucleic acids for sequence determination.

In the effort to determine the biological role of the metal-nucleic acid interaction we have carried out extensive investigations on the effect of metals on the reaction of DNA with polysine, and the DNA-polylysine reaction itself has been subjected to intensive study. Several physical chemical techniques have been pioneered for the study of nucleic acid-protein interaction; the lack of progress in this important field has been due primarily to the absence of such techniques. Each nucleotide of DNA is bound to one amino acid in polylysine. The DNA-polylysine complex is severed by some metal ions and not by others. We believe that the unwinding and rewinding of DNA during replication involves such equilibria between nucleic acid, protein (histone) and metal ions. These studies do not prove this hypothesis, but represent the beginning of an attempt to unravel these complicated relationships.

During the coming year we intend to exploit the findings on the specific cleavage of RNA by metals. The reaction of metals with nucleic acids and proteins will lead to studies on the DNA-histone interaction, and, hopefully, to an understanding of the role of metals and histones in DNA regulation and the implication of these roles to the aging process. A new project on immunological studies on age-induced changes in tissue composition will be begun.

Previous work from the *Section on Comparative Biochemistry* had demonstrated that the oxidation of acetate-2-^{14}C to $^{14}CO_2$ occurred more rapidly in senescent than in adult rats. The findings have been explained on the basis of a change with age in the metabolic relationship between respiratory reactions of mitochondria and the extra-mitochondrial biosynthetic reactions. In order to devise rational experiments to understand the molecular basis for the above observation, a detailed knowledge of cellular energy-linked reactions and their metabolic control is necessary.

The studies on the mechanism of oxidative phosphorylation in beef heart mitochondria have been extended. The work has centered on the first site of phosphorylation; viz., that occurring during the oxidation of NADH by ubiquinone catalyzed by the ubiquinone reductase flavoprotein. The experimental approach and findings are summarized in the following formal scheme.



A coupling factor, an enzyme tentatively called Factor A, has been purified further. Over 90 of the protein appears as a single band in electrophoresis on cellulose acetate or polyacrylamide gel. It restores P/O and related partial reactions in urea-treated-phosphorylation-deficient particles. These particles have only electron transport capacity in the absence of added Factor A. The purified factor has little ATPase activity. which can be stimulated over 20-fold by exposure to pH 8.5 and 43° C. The appearance of the ATPase activity is believed to be due to distortion of the active site concerned with the normal phosphate transfer function. The distortion could make the enzymephosphate complex susceptible to hydrolysis by increasing the accessibility of water.

The major accomplishment in the laboratory has been the isolation of a second coupling factor. The new factor, tentatively designated Factor B, is another heat-labile, non-dialyzable protein. In particles depleted of phosphorylation coupling factors by urea-treatment, Fac-

tor B above gave little stimulation of energy-linked reactions. In the presence of saturation levels of Factor A, however, Factor B gave large additional stimulation. In ammonia-treated particles, which respond but little to Factor A, Factor B produced extensive stimulation of ATP-driven NAD reduction and P/O. The results clearly establish that the two factors are distinct from each other. Factor B did not affect oxidation rates in any of the particles. Its role, very likely in the energy transfer sequence, remains to be investigated.

The flavoprotein in the above scheme is probably NADH-uniquinone reductase. This enzyme has been isolated as two separate components with quite similar properties by chromatography on hydroxylapatite. It is conceivable that the two forms are derived from the same original mitochondrial segment by cleavage in two different ways. Both reductaste preparations (QR1 and QR2) catalyze the oxidation of NADH by suspensions of uniquinone-10 at high rates. They appear essentialy as single, but separate, bands in electrophoresis on cellulose acetate strips and polyacrylamide gel. In sedimentation velocity experiments (carried out in collaboration with Dr. H. Edelhoch, NIAMD) better than 90% of the protein migrated under one peak. The molecular weights of QR1 and QR2 have been determined by sedimentation equilibrium to be 69,000 and 87,000 respectively. The reductases have bound FMN, nonheme iron and acid-labile sulfur which can be liberated as H_2S.

Thus, three of the components of the first site of mitochondrial oxidative phosphorylation system have been isolated. Indirect evidence has suggested the involvement of an additional undiscovered factor, structural protein and phospholipid in the over-all reaction.

Among the demonstrated changes occurring during aging is the loss of cells from a number of tissues containing irreplacable cell types (e.g., nerve cells). However, the events leading to cell death and loss during aging are not well understood and therefore during the past years fundamental studies on histochemical and chemical changes during cell death in model systems, under controlled conditions, have been carried out. Earlier histochemical studies on the ischemic death of cardiac cells carried out in the *Section on Cellular and Comparative Physiology* had indicated that irreversible injury occurs when heart muscle is kept under anaerobic conditions for sufficient periods to exhaust glycogen reserves and suggested that the irreversible phase is reached as the adenosine triphosphate concentration drops in consequence of the depletion of suitable glycolytic substrates. This thesis was tested using the cellular level of adenosine triphosphate as a function of the duration of anaerobiosis. It was found that ATP concentration drops to 50% of the intial level within 7 minutes after the imposition of anaerobic conditions and to about 5% of the normal level by 15 minutes. This decrease in ATP level was paralleled, surprisingly, by the loss of ability by isolated mitochondria to carry out phosphorylation reactions *in vitro* using the firefly continuous oxidative phosphorylation assay developed in this laboratory as the monitoring system. A study of the properties of these mitochondria revealed that they, as well as all mammalian and avian mitochondria tested, are extremely sensitive to dilution. Dilution below about 0.1 mg. mitochondrial protein/ml completely abolishes the capacity to phosphorylate adenine nucleotides. However, oxidative phosphorylation can be completely and instantaneously restored by the addition of about 0.5 mg/ml of serum albumin to the reaction mixture. With the exception of lactalbumin, which is about 15% as effective as serum albumin, no other proteins tested show this recoupling effect. This powerful influence of a serum protein on the efficiency of substrate utilization in the production of usable energy carrying intermediates suggests that these substances play a regulatory role *in vivo*. The level of serum albumin intracellularly, which in turn may be under hormonal influence (thyroxin, etc.), may thus moderate the oxygen consumption of tissue cells and the organism, and decreases during aging in the intrcellular concentration of serium albumin or analogous factors may lead to a decreased efficiency of production of usable energy intermediates such as ATP. The mechanisms of serum albumin action *in vitro* will be examined, particularly its effect on mi-

tochondrial myokinase (which is preliminary studies appears to be stimulated by SA addition). The intracellular level of serum albumin vs. age as well as other studies suggested by the above will be pursued further.

A variety of lines of evidence indicate that cessation of cell division as well as related differentiation processes play a central role in the aging of higher forms of life. For example, animals with indeterminate life spans (e.g., *plaice, Testudo*); cells in tissue culture appear to have a limited growth potential (about 50 cell divisions); underfeeding of experimental animals slows down both the growth rate and the rate of aging as measured by age specific mortality; and finally, a general inhibition of cell division is observable when differentiation takes place which results in many cases in cells which can no longer be replaced (e.g., nerve, muscle), which cells are also the locus of many functional decreases during aging.

In order to evaluate the role of cell division, arrest of mitosis and differentiation in the aging process, a deeper understanding of related cell biology is desirable and the research activities of cell biologists in the Branch and in the collaborating VA Aging RResearch Laboratory in Baltimore are directed toward questions bearing on the relationships between aging and these cellular properties.

Cell division in metazoa is influenced both by intracellular and extracellular factors. The finding by cell culturists (e.g., Hayflick) that there is a limited division potential in human cells in artificial media suggests a model of aging in which important cell lines may exhaust their division potential during the life of an individual and directly cause senescence. That this is a general property of cells capable of growing in culture has been shown during the past year. Chick myoblast cultures are capable of undergoing about 20 divisions and the number of divisions obtained decreases with increasing age of the donor embryo. Using human epidermis labeled with tritiated thymidine, supplied by Dr. Gerald Weinstein of the University of Miami, work in this laboratory has shown that this tissue *in vivo* is *not* subject to the limitation of fibroblasts in culture. This was established by radioautographically following the fate in serial sections of daughter cells of individual cells pulse labeled with tritiated thymidine. If the system follows the Hayflick rule, all daughter cells should move upward into the spinous layer coherently in groups of 2^n. Since single labeled spinous cells as well as triplets and other unpredicted groupings were observed in this human material, it appears that human epidermis is not limited to 50 divisions. Whether other important cell types are limited by this rule is the subject of present and projected studies.

Environmental factors affecting cell division and differentiation under controlled conditions constitute another facet of the research of the Cell Biology Group within the Branch. It has been shown that slight variations in the pH of the medium inhibit cell division and promote differentiation into striated multinucleate muscle fibers. Below pH 7.2 cell division predominates; above pH 7.4 differentiation is favored. Interestingly, another factor which affects cell division is the level of serum albumin in the medium. Although some albumin is necessary for growth, somewhat higher levels are inhibitory. Related studies on the effect of sera of different ages, a la Carrell, are also under way.

Finally, the hypothesis that the process of differentiation leads to senescence by shutting off the synthesis of certain important cell components as a by-product of the specialized synthesis implicit in differentiation is being tested. These limiting components are supposedly long lived and possess a low turnover rate or no turnover at all.

The specific hypothesis being examined is that the pattern of synthesis is controlled by the species of s-RNA present and/or activated in specific differentiating cells. Since each amino acid is specified on the average by any one of 3 different codon triplets, the selection during differentiation of an appropriate group of triplets (codon set) for synthesis and/or amino acid activation would impose severe restrictions on the kinds of messenger that can be translated, since only those messengers can be read for which the corresponding species of s-RNA are available.

This proposition is being tested inter alia by comparing the pattern of s-RNA activation produced by activating enzymes from a variety of tissues from the same species. Identical s-RNA's are breifly labeled with tritiated amino acid in the presence of one enzyme and C^{14} labeled amino acid by another enzyme. The labeled s/RNA's are combined, chromatographically resolved and the ratio of tritium to C^{14} in each fraction determined. Concurrently the concentration of various s-RNA species present in different tissues is being examined.

Connective tissue is a fibrous matrix in which cells, water, mineral, vasculature, etc. are suspended. This idealized separation facilitates measurement of certain biophysical properties, viz., mechanics of deformation, diffusion-permeation and binding of water, the synthes's and depletion of macromolecular components in the matrix. Aging of connective tissue will be understood in terms of these properties, and others, as well as molecular and regulatory systems of fibroblasts and other tissue cells. Until there is a unified concept of the integrated connective tissue systems, this artificial separation serves a useful purpose.

Present work in the *Biophysics Section* is concerned with the fibrous macromolecules of the connective tissue matrix, viz., collagen, elastin, and mucopolysaccharide. Since these substances perform a mechanical function in the physiological systems, the main research effort so far has been concerned with deformation analysis. The mechanics of tissue deformation was investigated in aging tendon, skin, and blood vessels. Experimental data on tendons suggests that longitudinal elasticity depends upon waviness of collagen fibers. A model of connective tissue elasticity was proposed in which the longitudinal extensibility is expressed as a function of the number of curled fibers which are transformed into uncurled fibers. Mathematical representation of this model results in equations which are in agreement with experimental data. From this it appears that stiffness of large fibers increases during growth-development, but there is no further increase throughout the later period of life. On the other hand, small tendons show a concomitant reduction in stiffness; at all ages there is a negative relationship between stiffness and size of tendon (cross-sectional area). Since the original data were corrected for cross-sectional area, this negative dependence is a second-order effect. Presumably it represents an accumulation of imperfections during the build-up of large fibers. Aging, therefore, reduces the imperfections in large tendons, but it increases them in small tendons.

Data on aging dermis showed an initial linear extensibility, but this became non-linear at a critical strain. Morphological evidence suggests that the initial linear portion is due to elastin, whereas the non-linear data result from collagen fibers which become uncurled with stretch. Mathematical description of these delineated segments yields parameters of elasticity for aging dermis. It was noted that indices changed rapidly only during the early growth-development period of life. Comparison of these measurements with data in the literature also showed that composition of dermis, i.e., mucopolysaccharide, water, collagen, and elastin do not change rapidly during aging, but only during growth and development.

When the elasticity data of blood vessels were treated like that of dermis, it was seen that human arterial segments changed markedly during aging, but only slightly during the period of growth and development. Rat tail arteries and veins, in contra-distinction to human arteries, behaved more like refractile tendons, i.e., they did not change greatly during the later months of the life span.

Thus, one sees that connective tissue has both sensitivity and selectivity toward factors governing transformations during growth development, and aging.

Small quantities of aged human skin can be obtained by dermatologic biopsy, but there are restrictions on the types of measurements possible because of the small size of the sample. Compression tests can be done on these small samples (biopsy), and data can be used to study molecular transformations of the fibrous matrix. It was observed that compressibility (stress-strain) data are similar to extensibility (stress-strain) data for calf and rat skin. This testing procedure will be expanded and used to study the biophysical properties of connec-

tive tissue in organs from which it might be possible to get only microscopic size tissue samples.

The above summary minimizes the importance of some observations which purport to show aging of connective tissue. This is true only to the extent that one recognizes the physical state of collagen in these samples. All of the above studies pertain to non-melted connective tissue, i.e., to collagen fibers which are in a state of high crystallinity. Biophysical analysis of elasticity of amorphous collagen in melted tendon or skin shows, in contradistinction with the crystalline state, that extensive changes are introduced at the molecular level. It is generally concluded that melted collagen chains show an increase in the extent of interchain crosslinking, but recent evidence in the literature suggests a modification of this interpretation. It is now seen that perhaps these crosslinks are reoriented from intra- to intermolecular tropocollagen, thus not requiring *a priori* the addition of many new crosslinks. This would provide a rational explanation for the limited increase in stiffness of crystalline collagen fibers in tendon and dermis. Vascular collagen, admittedly, would undergo more extensive crosslinking than tendon or dermis collagen, a case difference which will be investigated more thoroughly.

Endocrine systems appear to control or influence some parts of the deposition-polymerization mechanism of connective tissue. The extent to which this is modified by aging, or the possibility that it is the basis of changes in connective tissue with aging, now is the subject of speculation. A series of experiments now underway will delineate certain aspects of the control mechanism. Primary emphasis will be placed on the effect of age on the rate or response of connective tissue to an endocrine disturbance. This design is "longitudinal" in concept in that endorcine disturbances will be induced at various ages then followed by serial observation. The first series of measurements will determine the rate of response to hypophysectomy of rats which are hypophysectomized at various ages. Tests will be conducted on elasticity of dermis, tendon, and blood vessels to establish the generality, or specificity, of their response. Various organs will be weighed while the femur length and whole animal weight will be used to follow growth. Ultimately, the project will explore the longitudinal response to disturbances of pituitary, thyroid, and adrenal functions.

MENTAL RETARDATION PROGRAM

The Mental Retardation Program has primary responsibility for research in the biological, behavioral and social aspects of mental retardation within the context of child health and human development. This responsibility embraces all factors—biological, social and behavioral—that influence subnormal development.

Direct Research

Three Branches and one Laboratory have been organized to carry out direct research activities in mental retardation. These include a Clinical Research Branch, a Laboratory of Biomedical Sciences, a Behavioral Sciences Branch, and an Epidemiology Branch. The last two Branches are not operational yet.

Two Sections will be included in the Clinical Research Branch—the Children's Diagnostic and Study Unit, and an Inpatient Diagnostic and Study Unit. Plans are now being formulated for the Inpatient Unit. The Children's Diagnostic and Study Unit is operational and is located at the National Naval Medical Center.

The purpose of this Outpatient Clinic is to provide a program of clinical research and source of case material for basic research in the biomedical and behavioral aspects of mental retardation. Currently two new patients are being evaluated each week by a multidisciplinary team. The Clinic team is interested in studies on learning. A small nursery school is associated with the Clinic and will provide an ideal setting for studies of learning, motivation, and teaching procedures.

Specific studies are being carried out on improving attending behavior in preschool retardates; behavioral assessment of neonates; informational discrepancies by parents; behav-

ioral assessment in a free-play situation; and operant conditioning audometric techniques.

A Laboratory of Biomedical Sciences is using borrowed space on and off the Reservation and has been collaborating with other Institutes on specific projects. Architects are now developing plans to renovate space for biomedical and behavioral research laboratories at the National Naval Medical Center. These laboratories will be located adjacent to the Children's Diagnostic and Study Unit to facilitate collaboration between clinicians and laboratory investigators. This facility will be ready for occupancy early in 1967.

LABORATORY OF BIOMEDICAL SCIENCES

The mission of the Laboratory of Biomedical Sciences is to contribute to the Program's multidisciplinary investigation of mental retardation. Initially the Laboratory will emphasize biochemistry, cytogenetics, and neurophysiology. To this end, investigators from each of these disciplines have been recruited. The staff of the Laboratory now consists of 15 individuals (9 professional, 4 technical, and 2 clerical).

During the past fiscal year, some of the biochemical research has been located in Room 308, Building 4, NIAMD. This module has been occupied by Dr. Robinson since his transfer from NIAMD to NICHD in July, 1965, and will be vacated on July 1, 1966. The Cytogenetics Unit, headed by Dr. Matti Al-Aish, is located in the REEL Building of the National Naval Medical Center (NMMC). Dr. Frederick Maire joined the Laboratory in March and has begun his research work in neurophysiology in the Children's Diagnostic and Study Unit.

Plans for additional laboratory facilities are in the design phase. These laboratories will be located adjacent to the Children's Diagnostic and Study Unit at NMNC and will be ready for occupancy before the end of the next fiscal year.

To avoid a delay in staffing after completion of the laboratories, personnel have been recruited and are now engaged in collaborative investigation in other Institutes. This expedient also should help to increase our inter-Institute collaborative work in the future and thereby considerably augment our research program.

General Review of the Activities of the Laboratory

Projects in progress during the past year are enumerated below. They have been classified according to the major discipline involved, but many aspects of the work are interdisciplinary in nature.

Biochemical Investigations

1. Pregnancy-associated plasma proteins.
2. Electrophoretic variants of leucocyte alkaline phosphatase.
3. Inhibition studies on serum cholinesterase.
4. Development of a general method for dedetecting peroxide-producing enzymes on starch gels.
5. Hypercalcemia study.
6. Effects of nutrition on myelinization.
7. Effects of amino acids on myelinization.
8. Evaluation of methods of therapy in homocystinuria.

Cytogenetic Investigations

1. Cytogenetic aspects of mental retardation.
2. Studies on human chromosome abnormalities.
3. Alteration of metaphase chromosome morphology and DNA synthesis during mitotic cycle.
4. The role of viruses in chromosome abnormalities in spontaneous abortions.
5. Cytogenetic studies of viral-transformed hamster kidney cells.
6. Clinical cytogenetic studies.
7. Cytogenetic studies of human abortuses.
8. Chromosome morphology in the vitamin E deficient rat.

Neurophysiology Investigation

Serial neurological and neurophysiological testing in the diagnosis of childhood disorders: The thesis being advanced by the Laboratory, as is evident in the foregoing list of individual

projects, is that an exhaustive effort should be made to describe as many of the mental retardation syndromes as possible in terms of modern molecular biology. This approach leads naturally to an examination of the sequence of events through which the genetic information of the chromosomes is utilized for biosynthesis of enzymes and structural macromolecules. The effect of an abnormal enzyme is frequently manifest as an accumulation of its substrate resulting in profound derangement of the normal pathways of intermediary metabolism. Two important aspects of these derangements must then be considered: (1) the effects of excess or deficiency of normal intermediary metabolites on cellular differentiation and organogenesis (particularly of the brain); and (2) the pharmacologic consequences of these excesses or deficiencies on nervous and mental function. Pursuant to the objective of describing mental retardation at the molecular, cellular, and organ levels of organization, the Laboratory is emphasizing genetics, developmental enzymology, intermediary metabolism, and neurophysiology.

In addition to its research activities, the Cytogenetics Unit has been responsible for the training of four individuals in the latest cytogenetic techniques during the past year.

CHILDREN'S DIAGNOSTIC AND STUDY UNIT

The Children's Diagnostic and Study Unit (CD&SU) is an outpatient facility where clinical research in biology, medicine, behavior, learning, speech and hearing, epidemiology, genetics as well as other types of investigations will be carried out on mentally retarded children. In addition, the Clinic will be used to train clinical and research associates as well as staff fellows. It will also be a source of clinical material for basic research. Collaborative studies will be carried out with the biochemstry, neurophysiology and behavioral sciences laboratories of the Mental Retardation Program.

A multidisciplinary team approach will be maintained in the clinical evaluation of patients. The Clinic population will be composed of children, six years old or younger, who fall under one of the following categories:

Those who appear or have been said to be developing slowly;

Those who appear or have been diagnosed as mentally retarded;

Those who appear or have been diagnosed as having learning handicaps or significant communication disorders; and

Those who appear or have been said to have birth trauma, neurological impairments or other injuries or symptoms which may be related to learning difficulties.

Normal controls, if indicated, will also be used in our studies.

On November 29, 1965, the Clinic staff moved to its present location in Building 125 at the National Naval Medical Center. Although the Clinic was officially opened on January 19, 1966, the first patient was evaluated on December 20, 1965.

In order to determine the proportion of mentally retarded children who are seen in the Pediatric Outpatient Clinic at the U.S. Naval Hospital, the Children's Diagnostic and Study Unit staff developed a case finding questionnaire which was mailed to families whose children were seen in the Outpatient Clinic between January 1, 1965, and July 31, 1965. Children who have been labeled as mentally retarded or developing slowly constitute the main source of the CD&SU population. In addition, referrals are being received from the Pediatric Departments of the different military hospitals in the area, including Fort Belvoir, Patuxent Naval Hospital, and Andrews Air Force Base. Each referral, including self-referral from parents, is made through the Department of Pediatrics of the U.S. Naval Hospital.

Each child is seen by a social worker, public health nurse, clinical psychologist, speech and hearing pathologist, education specialist, pediatrician, child neurologist, child psychiatrist and cytogeneticist. Consultation, if necessary, is obtained from various specialists from the U.S. Naval Hospital.

A minimum of two days is needed to evaluate a child. Consequently, the Clinic has been able

to evaluate two new patients a week. Case conferences and parent conferences are held on each case. Referrals, if indicated, are made to various community agencies since the Clinic is not designed, administratively and functionally, to provide service or therapeutic functions to the patients. As of April 12, 1966, 25 patients have been evaluated. By the end of the current fiscal year, we expect to see 21 more patients.

No individual research projects are being carried out as of April 13, 1966. However, we expect to initiate and pursue the attached descriptions of research projects before the end of the current fiscal year.

THE NATIONAL INSTITUTE OF DENTAL RESEARCH

INTRODUCTION

An essential underpinning for effective attack on the major oral health problems of today is the interdisciplinary crossfeeding provided by the Institute's several laboratories and branches. Coupled with high visibility of disease oriented mission is the maintenance of a research environment of excellence that continues to attract young basic scientists of superior quality.

During the past year the Institute's intramural professional staff of 84 members contributed over 100 papers to the scientific literature. Other avenues of communication, such as abstracts and attendance at meetings, provided further opportunity to contribute new knowledge and information to the dental and related sciences. Such scientific reports reflect a breadth of activity varying from developmental, clinical, and applied research to observations and findings in fundamental sciences having no immediately identifiable or applicable relationship to problems of oral and dental health.

During the year, for example, the isolation for the first time from gingival tissues of a collagen-destroying enzyme believed active in periodontal disease led to collaborative studies which have demonstrated this cellular factor in neuromuscular diseases. Similarly, the refinement of demineralization technics to conserve enzymatic activity in tissue specimens prepared for cytochemical study has led to new knowledge of the pathogenesis of skeletal and tooth defects in ascorbic acid deficiency.

Further illustrative of the Institute's broadening range of activities are its basic physiological studies on (1) elicitation of varied patterns of cry and related arousal response by electrodes placed stereotaxically in the brain stem of the cat, and the demonstration of action details by correlated pressure recording sound recording, laryngeal photography, and regional cineradiography; (2) neurophysiological patterns of representation of sensation in the trigeminal nucleus; (3) mechanisms of integration of afferent information in the cerebral cortex; and (4) patterns of respiratory motor response to stimulation in the pharyngeal area.

In cytological research, combined application of electron microscopy, antigenic fractionation, labeled specific antibody, and pharmacological testing have demonstrated recently that the endotoxic somatic lipopolysaccharide antigen in *Veillonella* is localized in a distinctive three-layered outer membrane. This can be stripped off by phenolic extraction, leaving the rest of the cell morphologically intact. An inner rigid wall, which maintains cellular integrity was demonstrated by its digestion with lysozyme, a mucopolysaccharase.

While the laboratory science base of the Institute's program has been enriched, there is also under way a concomitant acceleration of activity in the clinical component. For example, a new topical application procedure for caries control, utilizing mouth adaptors with a fluoride gel, has been tested for the second year on school children. As a result of promising preliminary findings, the Peace Corps has adopted the procedure for use in the field. Also, community interest stimulated by this study has been largely responsible for a proposal to fluoridate the township's water supply.

In order to further advance the Institute's programs, there was created this year an Immunology Section with a view to coordination, more efficient planning and performance, and eventual extension of current immunologic projects. This move not only recognizes the natural place of immunology as a major component in the Institute's Laboratory of Micro-

biology, but signalizes also the trend of basic investigations relating to periodontal disease.

In similar fashion, there was constituted in the Laboratory of Biochemistry a new section on Pharmacology which seeks to bring greater emphasis and focus on the teratogenic mechanisms of drug action and other environmental factors in oral-facial malformations.

Alert to the manpower needs of dental research and the opportunity to provide unique postdoctoral educational opportunities for promising young investigators, the Institute during the past year continued its support of trainees and fellows. These included four research associates, four clinical associates, one staff fellow, one visiting fellow, and one graduate student in out-of-service training.

In its engagement in international activities, the Institute has adhered carefully to the important purposes of advancing its categorical mission. Visiting associates are currently participating in studies with collaborators in our Laboratories of Microbiology, and Histology and Pathology. In reverse fashion, one of our own professional staff is on a one-year foreign assignment at the Royal Dental College in Malmo, Sweden, where he is pursuing continuing studies on microbiological factors in dental caries.

A particularly rewarding association was again experienced during the past year between Institute staff and the Board of Scientific Counselors. Giving their attention to a wide range of issues including recruitment of key personnel, furtherance of our epidemiological activities, and advice on computer needs, the Board members were indeed a valuable part of Institute planning.

In keeping with the format of previous annual presentations of the Scientific Director, the current report will again emphasize program areas of research rather than activities contained within the science discipline orientation of laboratories and branches.

DENTAL CARIES

Rampant dental caries is a very severe form of disease in which practically all of the teeth are attacked by decay in a relatively short period of time. It is found chiefly in young children, but may develop in adults who previously had little or no caries experience. Under suitable experimental conditions, comparable forms of rampant caries can be developed in laboratory animals such as rats and hamsters. From a research standpoint, rampant caries offers a most favorable opportunity to study the basic factors which activate or control the caries process because the usually prolonged time element in the development of carious lesions is reduced to a minimum, and the determination of caries activity can be much more certain than in caries of usual severity. In order to evaluate the many factors which may be important in rampant caries, a series of clinical and laboratory observations were made under well controlled conditions. Perhaps the most significant condition underlying the development of rampant caries in young patients was frequent nibbling of sweets and other fermentable foodstuffs.

The animal experiments testing these foods showed that some are very cariogenic, many are moderately cariogenic, and a few are noncariogenic to rats. During the past year, experiments also were conducted to determine the extent to which the very cariogenic foods would be made less cariogenic and the noncariogenic foods made cariogenic by procedures such as cooking, adding water, grinding, adding sugar versus adding fluorides, and other modifications of the food. In one example it was found that certain noncariogenic foods such as "dog biscuit" were not made very cariogenic by even large additions of sugar, and contrariwise, some highly cariogenic foods were not made non-cariogenic by the addition of fluoride.

These experiments indicate that cariogenicity is affected by several factors including chemical properties, physical properties, taste of the food, and the time it takes for it to be eaten.

Previous experimental work on the etiology of dental caries and periodontal disease has clearly demonstrated the importance of bacterial plaque deposits in both these infections. Consequently, this year's studies have concen-

trated on defining various factors involved in plaque formation and on testing the efficacy of different drugs and proprietary formulations in the control of plaque formation. The results indicate that many microorganisms, and among these several streptococci of human origin, are capable of inducing plaque and active caries in hamsters. The influence of diet, especially the effect of different sugars, has demonstrated that while sucrose in the diet is associated with rapid plaque formation, sucrose substitutes are much less plaque-conducive.

The host factor has been investigated in studies aimed at determining the effect of age on tooth resistance to plaque associated lesions. The findings suggest that in the absence of fluoride, tooth age is not a factor in caries susceptibility. While fluoride enhances tooth resistance, some fluoride-containing preparations also appear to be active in controlling plaque formation. None of the commercially available, fluoride-containing dentrifrices, however, have been effective in plaque control when tested in the hamster. The continued testing of anticariogenic drugs and methods in the experimental animal system and in *in vitro* studies will serve as an important step in the evaluation of agents suitable for human clinical trials.

For a number of years considerable emphasis has been placed by Institute scientists on the study of enamel structure, normal as well as pathological, and valuable new data accumulated which have advanced our understanding of the morphology of sound and carious human enamel, especially with regard to the prismatic structures and their participation in the spread of the carious process. While these earlier studies dealt with subsurface enamel, current efforts are being directed toward the surface layer. This layer is of particular interest because of its apparent greater resistance to decalcification as seen in early enamel caries, the so-called white spot lesions, where a seemingly intact surface layer covers an area of extensive subsurface demineralization. Although the differences in reactivity may be attributed to chemical differences such as high fluoride contents, the present study demonstrates clearly that morphological differences also exist. The lack of prism structure, the orientation of the crystals perpendicular to the surface, and the higher mineral content, coupled with a possible increase in crystal size, are all factors which might contribute to a decreased solubility. The influence and role of this "prismless" surface layer in the onset and spread of the carious lesion, especially relative to morphological differences from one tooth to another, are some of the important questions for which answers are being sought.

A contributory factor to the success of these studies has been the new microradiographic equipment which was constructed and put into service last year. The availability of an X-ray tube with exchangeable targets to vary wave lengths now makes it possible to detect very minute differences in mineral content which could not be recorded by previous methods.

Another related objective of this broad program in caries research has been to identify and classify human oral streptococci and to determine whether or not specific strains can be identified as etiologic agents. Utilizing serologic screening of plaque smears and fluorescent antibody technique for identification of strains in histologic sections of carious teeth and in mixed cultures of dental plaque, it was found that certain serologic groups of streptococci could be excluded as etiological factors. These observations are making it possible to focus, and ultimately identify, the suspect serologic groups.

Related experiments with gnotobiotic rats indicate that some adaption of the rodent mouth, sometimes involving antigenic shift, is necessary in order for these human oral streptococci to establish themselves and produce caries in the animal model system.

Evidence continues to accumulate that the level of dental caries activity in experimental animals is determined by very intricately balanced relationships between host, a limited variety of oral microorganisms, and dietary factors, notably sucrose. A considerable range of microbial species has now been tested in gnotobiotic rats and in hamsters deficient in cariogenic flora. Except for a single atypical strain of Lactobacillus acidophilus, only certain

strains of a particular cultural and immunological type of oral streptococcus have been found capable of initiating caries in such animals. Significantly, Institute scientists and collaborators at the National Children's Cardiac Hospital, Miami, Florida, and the Royal Detanl School, Malmo, Sweden, have isolated during the past two years a number of "rat-type" and "hamster-type" streptococci from human caries lesions and saliva. Many of these have proved to be cariogenic in the respective species of rodent. Recently, streptococcal strains have been isolated from humans, which are cariogenic in both rats and hamsters. In view of the long history of unsuccessful attempts to transmit caries activity from humans to animals, and from one species of animal to another, these results have been very surprising. Most importantly, they have encouraged renewed search for a specific bacterial element in human caries. Sufficiently distinctive cultural characteristics of these types of streptococci have been ascertained to make possible beginning human epidemiological studies. It would be especially meaningful, for example, to determine whether these organisms are natural inhabiants of the oral cavity in population groups with low caries experience, or whether the organisms must be transmitted to an individual before the disease can be established.

The reasons why some strains of these streptococci are cariogenic, while others are not, seem to relate to their behavior with sucrose. Thus, it has been demonstrated that cariogenic strains produce far more dental plaque, *in vivo* and *in vitro*, in the presence of sucrose as compared to equivalent amounts of other carbohydrates. On the other hand, cariogenic and non-cariogenic streptococci do not differ in their rates of acid production *in vitro* from such sugars as glucose, fructose, maltose, sucrose or mixtures of glucose and fructose. Nevertheless, when any of these sugars or sorbitol, or a hydrogenated starch product are substituted for sucrose in a caries-conducive diet, the incidence and severity of caries decrease markedly. These results are entirely consistent with the large amount of epidemiological evidence that dietary sucrose is a major determinant of caries in man.

Recent evidence indicates that differences in the caries susceptibility of different strains of rats correlate with their ability to support a cariogenic flora. When different strains of rats are fed the same diet and given equal exposure to a source of cariogenic flora, the levels of caries activity and the animals' ability to transmit the flora differ approximately in parallel. On a different caries-conducive diet, however, the relative susceptibilities of two strains of rats can be reversed; that is, a normally "resistant" strain develops more caries than its counterpart "susceptible" strain when both are fed one of our standard caries-conducive diets. Other studies indicate that the numbers of cariogenic streptococci in the mouths of rats and hamsters parallel the levels of caries activity. These results suggest that the support of a cariogenic flora depends upon some host-diet relationship, rather than on host and dietary factors independently.

In previous years the Institute has participated actively in surveys conducted by the Interdepartmental Committee on Nutrition for National Defense (now the Nutrition Section, Office of International Research, NIH). Population groups represented an ethnic and geographic spread ranging from sites in Alaska, Central and South America, to Asia and Africa. During the past year data from the survey in Nigeria were analyzed and new surveys were initiated in Guatemala and El Salvador.

Studies of dental caries within the United States included investigations with tube-fed children, clinical trials with a phosphate-fluoride dentifrice and a fluoride gel carried in a mouth applicator, and further analysis of findings in a long-term study carried out with children during the first ten years of use of a fluoridated community water.

Currently, analysis is continuing with the objective of discovering specific population factors which are associated with relative susceptibility to disease, on the general hypothesis that any causative factor should be found associated with disease in a consistent and predictable manner, population after population. Such associations should have been demonstrable because the prevalence of oral disease

was found to vary, area by area, by factors of 30- to 60-fold. For example, optimum, or even excessive, intakes of fluoride were invariably associated with an inhibition of dental caries, and there was a general relation between caries prevalence and per capita consumption of refined sugar. On the other hand, other widely-held theories were not supported. Very low caries prevalences were found in populations subsisting principally on such foods as rice, cassava, or yam, which are highly cariogenic in laboratory animals. No support could be adduced for the theory of a "protective" food or food element other than fluoride. Leads for further studies came from such findings as the observation in Viet Nam, Lebanon, and Nigeria of children with deciduous teeth attacked by caries alongside permanent teeth that were seemingly immune. One ominous note emerged; there was clear evidence that caries prevalence is rising, sometimes swiftly, in most populations now relatively free from this disease.

Studies of dental caries in laboratory animals fed by stomach tube have yielded much basic information about the relative roles of substrate and disease. Hitherto, there have been no parallel studies in human beings. During the year an investigation was begun with tube-fed children in the Sunland Hospital, Orlando, Fla. This study should establish or refute basic inferences about the baterial nature of dental caries which have not heretofore been studied directly in human beings. Other laboratory investigations have demonstrated that dental caries activity can be controlled at will in hamsters through the application of fluoride gels or antibiotics, topically to the teeth, using a fitted vinyl mouthpiece as a carrier. A field trial of a fluoride gel applied in this manner is in its second year with children of Cheektowaga, New York. Preliminary findings indicate that (*a*) much higher concentrations of fluoride appear in the outer layers of tooth enamel after such treatment than after conventional methods of application, and (*b*) that a single hygienist can supervise many more children when this technic is followed. An interim examination showed that caries had been inhibited in the study children, but that too little time had elapsed for determination of the magnitude of the effect.

Since the mechanisms governing the deposition of fluoride in calcifying structures are not understood, it is apparent that the role of fluoride in inhibition of caries and its possible inhibitory effect on bone loss in resorptive bone disease will be empirical until the mechanisms by which these effects occur are elucidated. A number of studies, therefore, are being carried out on the effect of fluoride in resorptive bone disease in the human and in experimentally-induced bone loss in the rat. Most significant is that fluoride inhibits alveolar bone loss induced in the rat by either hydrocortisone or a low protein diet. However, maximum levels of fluoride tolerated by the rat do not reverse the decrease of bone protein and citrate synthesis as induced by hydrocortisone. In one individual having multiple myeloma, the administration of about 30 mgs of fluoride per day for about two years was found to reduce the mobilization of fluoride from the bones.

Research pertinent to the cariostatic effect of phosphates proceeded during the year with attempts to define the mechanism of its action. Additional evidence was obtained in support of the hypothesis that this cariostatic action is localized in the oral cavity. Thus, NaH_2Po_4 added to drinking water or diet of white rats for a period of two weeks prior to initiating a cariogenic diet reduced the ultimate cariogenic effect of the diet. Apparently the tooth surfaces and/or the oral milieu had been favorably conditioned against caries by this prior exposure to phosphate.

To resolve the possible effect of phosphate on the chemistry of both permanent and deciduous teeth, the Pitman-Moore miniature pig and standard Duroc swine were fed low- vs. high-phosphate diets for a period of one year, starting at weaning age. The animals were sacrificed when both permanent and deciduous teeth were erupted and still *in situ*. Ash, calcium phosphorus, magnesium, and carbon dioxide were determined in enamel and dentin of permanent and deciduous teeth. The low phosphorus diet did not effect the content of the above major constituents of the teeth, as compared with the teeth of swine fed a normal

and higher phosphate diet. Apparently the cariostatic effect of phosphate supplements as observed in experimental rats may not be related to a change in major components of the teeth.

Related to the demonstrated cariostatic effect of phosphates on dental caries in the rat and hamster is the reported increase of phosphate in the saliva of humans associated with a low caries experience. Since phosphate is involved in the glycolytic cycle, studies have been initiated on the relation of various glycolytic enzymes of saliva to dental caries. Some enzymes involved in the so-called "shunt" pathway have also been studied. Thus, hexokinase, aldolase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase, and phosphoglucomutase have been found in whole saliva, whereas in parotid saliva only hexokinase, aldolase, and phosphoglucose isomerase were present. Bacteria, cellular debris, or perhaps the submaxiliary saliva thus may serve as a source of enzymes not found in the pure parotid secretion. Study of the activity of these enzymes in caries-free, caries-normal, and caries-rampant individuals will be continued.

It can be concluded that the problem of caries control assumes quite different aspects depending on the microbiological status of the subject population. If relatively caries-free populations, such as those found in several African nations, enjoy this status because they are not infected with cariogenic microflora, caries activity should remain low regardless of dietary composition, particularly the consumption of sucrose. On the other hand, if potentially cariogenic microorganisms are already widely prevalent in such populations, then introduction of sucrose into the diet should automatically result in increased caries activity. In the United States and most Western nations, sucrose consumption and caries are so prevalent that we must assume that cariogenic microorganisms are ubiquitous. Probably the only way to eradicate dental caries would be to eliminate the causative microorganisms from a population. Until this becomes feasible, as by suitable use of antibiotics, other control measures of known efficacy such as fluoridation and dietary regulation should be rigorously applied.

In the past, two major factors (fluoride and refined carbohydrates) have been shown to consistently influence the amount of dental caries in a population. A third consistent relationship has been established between the inherited ability to taste phenylthiocarbamide and dental caries, such that tasters for this substance have from 28 to 40 percent lower def (decayed-extracted-filled deciduous teeth) rates than nontasters. This finding was verified in a number of studies. Investigation of the thiourea concentration in saliva indicates this was not the basis for the caries difference observed. Thus, a genetic factor in dental caries is well demonstrated for the first time in clinical studies.

PERIODONTAL DISEASE

Within the past few years periodontal disease has been recognized as a major public health problem in the United States, where it is a major cause of oral pain and tooth loss. Clinical management of the condition is difficult, expensive, and time-consuming, and the number of available specialists is quite inadequate to meet the need for treatment. On a world-wide basis, people of the United States are probably the most favored in this regard; elsewhere, the periodontal diseases tend to be more prevalent, more severe, and earlier in onset, and professional personnel are even less able to cope with the problems.

Studies, to date, have led to the clear inference that prevention of this disease entity must depend upon control of deposits of dental calculus, with little benefit to be gained by nutritional therapy. Tooth loss in many of the populations studied by Dental Institute epidemiologists was high from periodontal disease, which leads to deterioration and loss of the structures which support the teeth in the mouth. In such areas as Lebanon, Trinidad, Viet Nam, Thailand, Burma, and Nigeria, the onset of disease was early, and advanced destruction was common even in young individuals. Noma, a disease which has virtually disappeared in western civilizations, was common in children in Nigeria. It was usually associated with protein-calorie malnutrition, exposure to malaria,

recent history of rubella, and an overwhelming infection with Vincent's organisms.

As a general rule, high prevalences of periodontal disease were seen in those populations where children exhibited signs of malnutrition in calories, protein, and vitamin A and carotene. There was a consistent and positive relationship between deposits of oral debris and calculus and the severity of periodontal disease in every population studied. When these factors, and age, were held constant, no consistent or strong relation could be demonstrated between disease and nutritional status. Estimates of nutritional status were based on biochemichemical determinations, dietary studies, and the character of diets followed in the past. Factors brought under study included such items as vitamin A, beta carotene, ascorbic acid, thiamine, riboflavin, niacin, total proteins and protein fractions in serum, calcium, iodine, and iron. On the basis of the findings, the value of any of these agents in therapy may be questioned.

On the other hand, calculus formation in the rat was related to a high protein concentration in whole saliva and with an elevated acid and alkaline phosphatase activity. Also, rat-strain differences seemed to be of more importance than diet variables as a factor in calculus formation. It is pertinent to mention also the parallel demonstration of extracellular polysaccharide (levan) production from sucrose by *Odontomyces viscosus*. A variety of other common sugars showed no such formation. This provides a reasonable explanation for the accumulation of this organism as a gelatinous plaque causing a form of periodontal disease in hamsters fed a high-sucrose diet.

Very recently a study of autogenous reimplantation of human teeth was initiated. It is hoped that by observing the response of the periodontal tissues to tooth reimplantation some knowledge relative to the factors permitting the differentiation of fibroblasts into cementoblasts will be obtained. After reimplantation, the teeth become firmly attached with new alveolar bone formation. In the absence of continued cemental production, periodontal fibers lose their attachment to teeth with eventual exfoliation. New functioning cementoblasts have not been found earlier than 29 days after reimplantation.

In the treatment of the osseous defects in periodontosis, preliminary results indicate that the autogenous transplantation of developing third molars into the first molar sites can be an effective way of inducing healing of the alveolar bony lesions and in restoring periodontal health.

In another study, dental abnormalities were produced in Syrian hamsters by infections with H–1 (isolated from human tumors by H. W. Toolan—Bull. N.Y. Acad. Med., 37:305: 1961), OLV (isolated from rats by G. Dalldorf—Bull. N.Y. Acad. Med, 36:795:1960), Krisini (isolated from connective tissue of carcinogen-treated rats by V. M. Zhdanov and Z. I. Merekalove—Veprosy Virusologii, 3:339: 1963), and Rat Virus (originally isolated from laboratory rats by L. Kilham and L. J. Olivier; Oral Surg., Oral Med., and Oral Path., 15:756:-1962, 15:1302:1962, and 17:116:1964). The abnormalities produced were generally somewhat similar, and consisted of root resorptions, misshapen roots, bony ankylosis of roots, and microdontia of the third molars. Differences were noted only between the OLV and H–1 virus. These consisted of absence of mandibular third molars in the animals infected with OLV virus; and a severe inhibition of osteoblastic activity and bone formation of the mesial and mesiolingual aspects of both maxillary and mandibular first molars in animals infected with OLV and H–1 virus, enabling Stenson's gland, which is a normal component of the sinus, to come into close apposition with the mesial root of the maxillary first molar.

In a study of the effects of various viremias on healing wounds, including periodontium, Syrian hamsters were injected intravenously with OLV infected tissue culture fluid which was obtained from rat embryo. Inoculation was at time intervals of 0 to 6 days following fracture of the left forearm bones (Group I) or extraction of the maxillary right second molar tooth (Group II). The intravenous injections of OLV strain of rat virus delayed fracture and alveolar socket healing when administered prior to the third postoperative day. The de-

lay in healing was transitory; i.e., by 15 days postoperatively no alteration in healing could be discerned.

Extensive investigations by Institute scientists and others have established that the members of the oral microbiota most significantly implicated in the pathogenesis of chronic periodontitis possess sufficient pathogenic factors to account for the principal features of the periodontal lesion. These factors include lipopolysaccharide endotoxins and histolytic enzymes (mucopolysaccharases, proteases, peptidases, collagenase, and other hydrolases). The observation can now be added that ammonia and hydrogen sulfide, in concentrations that might reasonably be expected from bacterial activity in the gingival crevice, exert cytopathic effects on epithelium similar to that lining the gingival crevice. The toxicity of these agents was demonstrated in rabbits with istonic solution in neutral pH ranges similar to the ranges exhibited by human mouth fluids.

Other directions of research are giving increased attention to the immunological relationships between the host and the microbial products occurring in the gingival crevice and periodontal pocket, and to associated basic immunologic studies. Thus, it has been shown that repeated deposition of antigen in the normal gingival pocket of the rabbit results in appearance of homologous antibody in the blood serum and in chronic allergic inflammation of the gingiva, histologically very similar to that seen in chronic human gingivitis. Paradoxically, and despite many investigations, little evidence has been presented that humans form antibodies to their indigenous gingival crevice bacteria. This failure seems to be due, at least partially, to the use of insufficiently sensitive serological methods. Using indirect hemagglutination and bactericidal tests for antibody, significant levels have been found in human sera against *Leptotrichia buccalis*, *Veillonella alcalescens*, and *Fusobacterium polymorphum*. Subjects with advanced chronic periodontitis exhibit significantly higher titers to F. polymorphum that subjects with a clinically uninflamed periodontium. Parallel studies have shown that the concentrations of antibodies in parotid secretion are only about 1:1300 those of serum. Presumably the major part of antibodies in the oral cavity comes from the blood serium, via gingival crevice exudate.

The histochemical and chemical studies of enzymes involved in connective tissue formation and destruction alos have been continued during the past year. Special emphasis has been placed on determination of collagenase activity in a number of connective tissue sites under normal and pathological conditions following the discovery last year of the enzyme system in human gingival tissues. Of the normal tissues tested so far, only uterus, gingivae and, to a lesser extent, portions of the oral mucosa exhibit collagenolytic activity. Some of the most exciting results, however, have been the demonstration of the enzyme in cultures of dermis from individuals with certain neuromuscular disorders and collagen diseases. Isolation and purification of the collagenase is being attempted and when accomplished will allow investigation of its action on the collagen structure. The cellular origin of the collagenase also is being studied. Results already obtained represent an important extension of our basic knowledge and, when fully exploited, will undoubtedly contribute significantly to a better understanding of the metabolic processes in healthy and diseased connective tissues.

GROWTH AND DEVELOPMENT

A population of 4,200 Indians in North Carolina has been exhaustively examined for clefts of lip and palate, and 42 cases have been found which makes this frequency the highest known in man. Since a number of other congenital anomalies appear to be associated with this type of defect in the same population, some clue may be uncovered to help define the pathogenesis of the oral defect.

Studies of cleft lip and palate children and their families in selected population groups in the United States indicate that, among the relatives of children with oral clefts, 27 percent have a hearing loss of 20 db or more at 500, 1000, 2000, or 4000 cps, compared to only 13 percent among control proband relatives. Other parameters such as visual acuity and minor anomalies (possible indication of a cleft palate habitus) have not shown a significant differ-

ence between the two groups. This would indicate that there are familial factors (possibly genetic) operating to cause a local, first arch syndrome disturbance rather than a general disturbance in development.

One of the major efforts of the Institute during the past year has been the establishment of a data retrieval system for research on congenital malformations among all American Indian neonates born in PHS facilities. To date, approximately 10,000 birth records have been examined of Indians born during the last year and a half. These data have yielded some unexpected results. Foe example: (1) only 5.0 percent of all Indian births are premature compared to 7.0 percent for whites and 9.7 percent for non-whites; (2) the incidence of Indian stillbirths was 4 in 1,000 births compared to 13.7 in 1,000 U.S. white births and 26.7 in 1,000 U.S. non-white births; (3) Indian neonatal death rates are 13 per 1,000 births compared to 16.7 per 1,000 births for whites and 26.1 per 1,000 births for non-whites; (4) major congenital malformations were 15 per 1,000 births, and minor malformations were 34 per 1,000 births; (5) of the major malformations, 2.2 per 1,000 represented clefts of the lip or palate; (6) approximately 32 per 1,000 births or 3.2 percent Indian children either die in the neonatal period or have major malformations. When corrected for underreporting, this is about the same value obtained in Japanese infants (4.3 percent); and (7) the malformation pattern in American Indians is intermediate between Caucasian and Mongoloid patterns.

Investigations of biochemical genetic defects in families and populations indicated that American Indian infants do not metabolize bilirubin in the same manner as Caucasian neonates; i.e., they have higher and more prolonged neonatal levels of unconjugated bilirubin, which apparently is normal for children in this ethnic group. Thus, indications for exchange transfusion are different in Indian neonates than they are in Caucasian neonates.

In interesting contrast to findings in American Indians is the striking absence of malocclusion among primitive groups of Brazilian Indians. This study has shown a 5 percent incidence of such malformations compared with approximately 50 percent for a corresponding group derived from the same tribal stock who have been in contact with civilization for a period 20–30 years.

Orthodontic care claims a considerable share of the funds spent to improve dental health, but the natural history of occlusion has received little emphasis in epidemiological study. Thus, a program of study is currently being designed to determine the sequence of events, and the causative factors, which lead to undesirable states of occlusion, in the hope that some of these factors may be amenable to simple methods of control in early states of development. Principal focus is on a longitudinal study in which individual children are examined and reexamined over a period which includes critical periods of tooth emergence and jaw development.

The Institute's Oral Pharyngeal Development Section has continued its studies during the past year of the structure and the motor performance of the mouth and pharynx. Basic anatomical studies have included (1) vital staining demonstration of differential skeletal growth patterns in face and cranium of rats, rabbits, pigs, and sheep; (2) detailed description of intrinsic growth patterns of individual bones as observed when they are cultured separately *in vitro*; and (3) sequence of histological demonstration of responses of bone to deforming forces. In the human, changes have been described in spatial relation to nasal septum and base of cranium during postnatal development.

Analogous studies of facial and cranial skeleton have continued in subjects distorted by anomalies and/or neurological impairments. These have included standard methods of radiological cephalometry, and adaptation of laminography which demonstrate the mid-line structures. In clinical application, evaluations of the basic orthodontic methods of tooth displacement have been performed, using precision techniques of portrayal of spatial changes of teeth.

Studies of upper respiratory and feeding functions have continued in an increasing number and variety of subjects impaired by anomaly, such as the Pierre Robin syndrome. The

methods of cineradiography, cinephotography, respiratory displacement, sound recording, and spectrographic display have been utilized in the study of selected patients having cleft palate and other oral and pharyngeal anomalies. Transducer methods of observation of tongue contacts and margin pressures have been developed, and multiple-site inputs recorded in parallel with speech and swallow indications. by these methods, Institute investigators have been able to redefine disabilities in terms of their deficiences and compensations of pharyngeal actions, rather than the overt deficiency of palate and related structures. Such demonstration methods also have been extended to subjects with neurological impairments of the oral and pharyngeal area, and initial descriptions have been made of the particular distortions of feeding, pharyngeal airway maintenance, and vocalization in subjects having spastic dyskinesia, athetosis, lower motor neuron disorders, and regional kinesthetic sensory disorders.

Clinical experimental methods have been devised to evaluate oral sensation and perception, and the development of oral perception of form (oral stereognosis) has been calibrated in normal children. These approaches have made possible the description, to date, of syndromes of disability of perception in dysarthric and orally dysphagic subjects.

Analogously, studies of the chemosensations, smell and taste, have been adapted to children and to neurologically impaired persons; and first demonstrations have been made of disabilities of these special senses in facially deformed children.

In another study concerned with the interaction of sensory stimuli in association areas of the cerebral cortex in cats, it has been found that when peripheral stimuli are employed (light flash, auditory click, and forepaw shock), most cells in the anterior lateral gyrus exhibit preferential responsiveness to auditory click stimulation. This preferential responsiveness, however, was noted to be dependent upon the types of stimuli employed; i.e., when cells are activated by electrical stimulation of optic and auditory nerves and the dorsal column of the spinal cord, equal responses to the stimuli are most prevalent. A comparison of response characteristics in anterior lateral and anterior middle suprasylvian gyri have revealed that responses to sensory stimuli are not identical in these two association areas, irrespective of the types of stimuli.

In a study of the functional organization of the trigeminal brainstem in the cat, it was found that the chief sensory nucleus of the trigeminal nerve contains cells with different response characteristics. Almost all cells studied to date have had small receptive fields in the oro-facial area and exhibit a short latency (1–5 msec) response to electrical stimulation of this field. The modifying influences upon these cells by peripheral and central inputs can be separated into three groups: (1) cells whose activity is not modified by cortical, thalamic, or light flash stimulation; (2) cells whose activity is modified by cortical stimulation and orthodromic thalamic stimulation; and (3) cells whose activity is modified by both peripheral and central inputs. Very few cells were found to be activated antidromically by stimulation of the contralateral arcuate nucleus. Histological analysis of electrode track placements indicates a more medial location of cells whose activity is modified by peripheral or central input, or both.

In determining the genetic mode of inheritance of the developmental anomaly, taurodontism, radiographic surveys and clinical histories of four affected families were made. Although no parent possessing the taurodont tooth form was identified, the trait occurred in ratios of (1) one of three children, (2) one of two children, and (3) two of two children. Of further interest was that taurodontism is found in both deciduous and permanent teeth of males and females. Also, its mode of inheritance is apparently compatible with a recessive characteristic, and not a dominant one as suggested in the German literature. With respect to its histologic appearance, there is normal odontoblastic and periodontal membrane activity, thus lending further support to the original theory that the developmental site of the taurodont tooth form is Hertwig's epithelial sheath.

Among the major objectives of the Institute's experimental program in teratology have been (1) to study the occurrence of metabolites of chlorcyclizine in maternal and fetal tissue during organogenesis, (2) to specify the effects of certain drugs on implantation and fetal development, and (3) to compare the histology of benzhydrylpiperazine vs. Vitamin A-induced cleft palate. Studying principally the rat and mouse species, it was found that, qualitatively, chlorcyclizine and norchlorcyclizine are the only detectable metabolites with at least one tertiary amine, found to be present in maternal and fetal tissue. The ratio of norchlorcyclizine to chlorcyclizine in fetuses is about 15:1. When the demethylation inhibitor SKF 525 A is administered together with chlorcyclizine, the fetal norchlorcyclizine to chlorcyclizine ratio is approximately 2:1. However, under these conditions of inhibited demethylation, teratogenesis was still present at approximately the same incidence.

In attempts to identify the specific and minimal chemical structure responsible for a teratogenic drug effect, it was found that buclizine hydrochloride induces the same malformations as meclizine and chlorcyclizine. Three antihistamines; i.e., perphenazine, prochlorperazine, and trifluoroperazine, which do not have a piperazine moiety in their molecular structure, have demonstrated only a low (5.4%) teratogenic effect.

Histological observations of palatal clefts produced by agents of the benzhydrylpiperazine series of drugs have shown that the adhesion in the palatal area consists of a fusion of the overlying epithelium of the tongue with the palatal processes. Gross examination of rat fetuses whose mothers had been given excessive doses of Vitamin A disclosed microstomia, mandibular-maxillary ankylosis, and cleft palate. Histologically, the palatal processes of these fetuses were hypoplastic, and there was must heterotopic cartilage located throughout the maxillary areas.

Recently, studies of congenital malformamations in experimental animal model systems have been extended to species other than rodents. These have included the swine, in which oral-facial defects were produced with high dosages of chlorcyclizine hydrochloride, and the Rhesus monkey, in which metabolic pathways of teratogenic drug actions are currently being observed.

In another area of research, the effect of tetracycline hydrochloride on enamel development has been explored further, and a reproducible dose-response relationships has been established. Using these results as reference, the studies were extended to include the effect of comparable dosages of oxytetracycline. Although the latter antibiotic was capable of introducing mineralization disturbances at similar dose levels, gross hypoplastic lesions were not seen, suggesting that oxytetracycline is somewhat less toxic than tetracycline hydrochloride. The similarity of the tetracycline induced defects to those resulting from injections of a number of other chemicals indicates a common, non-specific response. Localization and identification of the cytological sites affected by the various toxic agents promise to yield considerable data on enamel formation in general.

COLLAGEN, ELASTIN, AND CALCIFICATION

Collagen is the major structural protein of vertebrates and many invertebrates. The protein, in the form of a rod-like monomer, undergoes aggregation into fibers which gain additional stability by a maturation process involving the introduction of covalent crosslinks. These are chemical links and are found both between polypeptide chains in the same molecule and between chains in adjacent molecules. Past studies have elucidated the chain structure. Current studies are related to a more detailed examination of the chemistry and biosynthesis of crosslinks. This is being accomplished by utilizing chemical and enzymatic cleavage to give peptides suitable for chemical studies that cannot be done on the whole molecule owing to its large size.

An enzyme from the tadpole cleaves the molecule into two pieces of unequal size. Isolation and characterization of these pieces has shown that intramolecular crosslinks occur only in one, indicating that crosslinks are specific in location. Limited cleavage of native collagen with chymotrypsin, and characterization of

the products, has demonstrated that this enzyme splits a few peptide-bonds at the N–terminal end of the molecule. The crosslink is also lost, demonstrating that it must be in this region of the molecule. Chemical cleavage with cyanogen bromide has permitted the isolation of peptides from the crosslink region. Studies of amino acid sequences in these peptides have shown that the crosslink is derived from a lysyl-residue, five amino acids from the N–terminal end. Preliminary to crosslinking, the lysyl side chain is converted to an aldehyde. Two of these aldehydes in adjacent chains form a crosslink probably by an aldol-type reaction.

In protein chemistry studies, it has become apparent that although elastin is not as well characterized as collagen, it is known to be crosslinked, and lysine is its precursor. However, rather than two lysyl residues forming a crosslink as in collagen, four are involved to give highly stable ring structures which are necessary for the elastic properties displayed by this protein. Chemical and histologic studies have shown that aldehydes are intermediates, again in parallel to the crosslinking in collagen.

In the toxic condition known as lathyrism, crosslinking of both elastin and collagen is inhibited. Recent findings suggest that this inhibition is in the enzymatic conversion of the lysyl side chain to an aldehyde. A similar inhibition is produced in copper deficiency, suggesting that a copper-containing enzyme may be involved.

Related studies of calcification of organic matrices have been under way using an *in vitro* system with elastin as the nucleating tissue. Results, to date, indicate that trace amounts of a heavy metal such as ferric ion are required for calcification. The metal is apparently associated with sulfhydryl and perhaps imidazole groups in the protein phase. The first stage of calcification may involve the deposition of octacalcium phosphate at nucleation sites containing the complexed metal, with a later conversion to hydroxyapatite.

A series of calcium phosphates have been made synthetically and have been examined by X-ray diffraction and by infrared spectrophotometry. Among the problems investigated have been definition of conditions under which large hydroxyapatite crystals may be grown routinely and a single crystal X-ray structure determination made of a selected, well-defined crystal. The availability of such crystalline material of known structural composition is of major importance to all investigators interested in studying the chemical reactivity of hydroxyapatite in both synthetic and biological systems. Other studies have dealt with the characterization of the infrared absorption spectra of pure synthetic apatites and the identification of band changes associated with the presence of fluoride and carbonate ions in the crystal lattice. The knowledge of the exact location of these ions within the lattice is important for our understanding of their effect on caries susceptibility.

The relationship between fluoride content and crystallinity of biological mineral also has been under investigation using X-ray diffraction techniques.

One aspect of this project has been the development of new methods for assessing the degree of crystallinity directly from measurements taken from the instrument instead of using the more cumbersome template approach developed previously in this laboratory. The data compiled through the direct methods can be fed into a computer, thus facilitating more extensive studies of age effects on bone crystallinity in high and low fluoride areas. A second facet of the work has involved assessing the effect of fluoride on crystallinity of enamel. As with bone it was found that the crystallinity of human enamel improved with increased fluoride. Yet in the enamel the changes in crystallinity were not restricted to the A-axes directions, but also included the C-axes. Since both size and strain factors influence the degree of cystallinity, the full meaning of the observed changes awaits a separation of the two factors.

CELL BIOLOGY AND ENZYME CHEMISTRY

Resolution of the mechanism of action and specificity of proteolytic enzymes can serve to advance our understanding of biochemical processes. Such knowledge also may be utilized

for protein and enzyme modification, for study of protein molecular structure, and to advance our knowledge of a particular function of a body tissue. At the present time, the immediate objectives of this research relate to the specificity and mechanisms of the enzymatic action of chymotrypsin-C, as well as guinea pig liver transglutaminase.

Whereas the amino acid sequence of an approximately twenty-member tryptic peptide of the chymotrypsin-C active site has certain similarities to the analogous peptide from chymotrypsin-A, an outstanding difference is the fact that the peptide from the C-enzyme has only one methionine residue. The active peptide site of the A-enzyme contains two methionine residues. Likewise, an enzyme inhibitor specific for chymotrypsin-A has little or no inhibiting activity toward the C-enzyme.

Purified guinea pig transglutaminase was found to be essentially homogeneous. It contains 21 thiol-groups and no disulfide bridges. Only one thiol-group is involved in the active center. From tryptic and chymotryptic digests of C^{14}-labeled transglutaminase, a labeled peptide was isolated and found to contain the following amino acids: 1 tyrosine, 1 asparagine, 1 C^{14}-labeled amide of carboxymethyl cysteine, 1 glycine, and 3 tryptophans. The tryptophan is present in the C-terminal position.

Studies of enzyme structure and mechanism have related principally to the physical-chemical properties of aldolase, and a correlation of its structure with catalytic function. Results, to date, have yielded significant information on the number and type of active sites possessed by this enzyme. Thus, when native fructose 1,6-diphosphate (FDP) is reduced to native FDP-aldolase, it becomes evident that three highly organized active sites are involved, each of which contains two nonidentical phosphate-binding sites.

Protein synthesis involves the polymerization of individual amino acids into high molecular weight polypeptides. Soluble ribonucleic acid (sRNA) plays an essential role in this process as a specific carrier of amino acids to the site of polymerization. The Institute's program in this area is designed, therefore, to elucidate the role of methylated and thiolated constituents of sRNA in its biological activity and specificity. Results have indicated that while yeast amino-acid-activating enzymes can esterify amino acids to sRNA which contains methylated bases, these enzymes are unable to carry out this reaction with methyl-deficient sRNA. Continuing studies are attempting to resolve the role and significance of the methylated base of sRNA in its acceptor function. Current results indicate that methylated bases of sRNA play a significant role (1) in the initial attachment of a specific amino acid to sRNA, (2) in a reaction catalyzed by specific amino-acid-enzymes, and (3) in the capacity of the sRNA molecule carrying an amino acid, to find its proper alignment on the polymerization template.

An investigation relative to thionucleotides (sulfur-containing) in sRNA have pertained to their functional significance. Thus, it has been shown that formation of minor bases in sRNA is controlled by the sulfur amino acids cysteine and methionine. This relationship forms a cyclic metabolic regulatory system. Evidence was obtained that under ordinary circumstances the leucine activating enzyme from yeast recognized *E. coli* sRNA about 75 percent of the extent recognized by the enzyme from *E. coli*.

Research concerned with the translation, transcription, and replication of genetic information at the cellular level also received appreciable attention during the past year. Particularly significant have been the studies of salivary enzyme polymorphism and the demonstration of such polymorphism in the salivary amylase isoenzymes of humans. That the composition of these isoenzymes is under general genetic control has been demonstrated by showing a generic difference in these isoamylases.

Studies relating to the transcription of genetic information and its regulation within the cell also have been advanced through the use of a lymphocyte-phytohemagglutinin system. In this system, non-dividing lymphocytes are stimulated to enlarge and divide *in vitro* by treatment with phytohemagglutinin extracted from kidney beans.

Collaborative studies with the National Cancer Institute and the National Institute of Child Health and Human Development of RNA metabolism of human lymphocytes stimulated with phytohemagglutinin (PHA) indicate that the effect of this extract is to produce a synthesis of non-ribosomal RNA, whereas stimulation by specific antigens (streptolysin-O or tuberculin) results in cell growth with predominant synthesis of ribosomal RNA. The characteristics of non-ribosomal RNA are like those of messenger RNA. If this is messenger RNA, it implies that PHA has an important action on the cellular mechanisms which regulate the synthesis of messenger RNA. This system, therefore, may provide a tool for understanding the regulatory mechanisms which govern transcription of genetic information within the mammalian cell.

Malignant lymphoma cells resemble antigen-stimulated lymphocytes more closely as regards RNA metabolism than they do PHA stimulated lymphocytes. This may reflect the fact that the lymphoma cells still have greater control over their messenger RNA synthesis than the PHA stimulated lymphocytes have. This information is important for an understanding not only of the control of cell growth but also of cell differentiation.

VIROLOGY, IMMUNOLOGY, AND MICROBIAL PHYSIOLOGY

Continuing study of persistent herpes simplex (HSV) infection in a cell culture system now warrants provisional conclusions as to its mechanism, and inferences as to recurrent herpes infection in humans. The fact that persistent cyclic HSV infections can be initiated and maintained in the presence or absence of homologous antibody indicates that the underlying mechanism is not dependent on antibody directly or its ability to reduce extracellular HSV. Even when antibody is present, virus transmission still occurs by cell-to-cell transfer and by the reattachment of infectious cellular material. The only effect of antibody that has been identified is its ability to reduce the severity of the infections to a low grade, comparable to a subclinical stage in the human disease. The maintenance of these persistent infections appears to depend on the establishment of a dynamic cell-virus equilibrium which is characterized by the continual selection of cells resistant to the major fraction of virus being produced at any one time. Selection of such resistant cells should ultimately result in a cell population completely resistant to HSV infection. This does not occur, however, due to virus variations which parallel the cellular changes. The result appears to be that the selection of resistant cells is offset by the production of either a new virus variant, to which the cells are still susceptible, or to changes in the virus population resulting in selection by the cells of a virus variant which was present as a minor fraction. Superimposed on this continual selection mechanism is the probable production of factors that temporarily enhance the cells' resistance to HSV infection. Such factors, which thus far have been found in only low titers, are probably synthesized and maintained in localized areas of the cell cultures, thus allowing the few surviving cells to proliferate and replenish the cell sheet. It has been found, in fact, that replenishment of the cell sheet in localized areas must occur before a new cycle of virus multiplication and cytopathic effect is initiated.

In the presence of antibody, the severity of the infections in cultures producing a non-proliferative virus variant is reduced to a low level. After varying lengths of time, a short-term exacerbation paralleled by the appearance of a syncytium-forming variant is evident, followed by the reestablishment of a low grade infection. The occurrence of these exacerbations *in vitro* is strikingly similar to the recurrent episodes seen in human herpes labialis and appears to be related to viral changes which temporarily alter the cell-virus equilibrium in favor of the virus. Ultimately, the cells are able to readjust to the new virus type being produced, and a subclinical type infection is reestablished.

Previously, it had been reported that HSV could be cultured from the saliva of rabbits many months after intraperitoneal injection. It has now been found that the HSV-antibody

response in such animals is strong and lasting, with cyclic variations very similar to the response to HSV infection in man. In contrast, the antibody titer induced by inactivated HSV is low and transitory. These results indicate that HSV persists in the tissues of animals receiving live virus, with intermittent immunizing bursts. However, no HSV could be cultured from testes, kidney, liver, spleen, lung, lacrimal glands, submaxillary glands, lymph nodes, and brain of such animals. Rather surprisingly, organ cultures of all these tissues from the immune animals, except lymph nodes and brain, were susceptible to infection with HSV. Provisionally, it has been concluded that HSV persists in some inapparent form in these animals or, quite possibly, in tissues not yet tested for this virus.

Since lactic dehydrogenase virus (LDV) induces no discernible cytopathic effect, its cellular location in infected mice has been a mystery. In collaborative studies with the National Cancer Institute, this has now been partially solved by the electronmicroscopic observation of particles having the fine structure of LDV, in peritoneal macrophages. Presumably, this virus is either phagocytized by and/or replicated in the macrophages.

Rat submaxillary gland (RSMG) virus, described in previous reports, has been shown to be associated with a specific cold hemagglutinin, whose titer in triturates of the glands is a simply determinable and sensitive indicator of the presence of the virus in its natural host. This indicator has shown that RMSG virus is absent or scant in both germfree and conventional rats until two months of age. After three months of age, it becomes ubiquitous, with high hemagglutinin titers. Concurrently, a specific immunoglobulin hemagglutination inhibitor appears in the animals' sera. Its titer also was shown to be low in the younger animals and to increase with age. In general, the inhibitor titers of serum parallel the titer of hemagglutinin in the glands. These tests will greatly facilitate study of the ecology of this virus.

Basic studies aimed at relating bacterial and macromolecular structure to an experimental situation also were emphasized during the past year. The initial electron microscopic investigations on endotoxin formation and the character of the endotoxin of one kind of organism have been extended to other endotoxin producing gram-negative bacteria. While all the organisms appear structurally similar, the isolated endotoxic particles vary in morphology. Work has also been directed at determining the bacterial killing due to complement dependent antigen-antibody reactions. The initial findings demonstrate a similarity in morphology between complement dependent bacteriolysis and haemolysis.

Studies on human amyloid have been continued with special emphasis on purification of the pathological protein in order to characterize it chemically. The degree of purification is assessed electronmicroscopically as in the effect of various chemical treatments. These investigations constitute additional steps toward widening our knowledge of the morphology of this protein.

Continuing studies of the relationships between lactic dehydrogenase virus (LDV) and its host animal (mouse) have paid unexpected dividends in the immunological field. LDV is unusual in several respects: (1) infected animals exhibit lifelong viremia but remain apparently healthy; (2) the only known consequence of LDV infection has been persistent elevation of a number of plasma enzyme activities, due at least partially to impaired clearance mechanisms; and (3) until now no antibody response to LDV has been discernible. That LDV does activate an immunological response to its host is indicated by the recent finding that infection of germfree mice results in an elevated level of plasma γ-globulin (not due to impaired clearance) and a great increase of germinal centers in spleen and lymph nodes. Both these parameters normally are very low in germfree animals, and their increase is usually interpreted as an immunological response. How much of this γ-globulin production represents antibody cannot be ascertained at this time. Extension of these experiments, however, has shown that LDV acts as an immunological adjuvant; that is, LDV-infected conventional mice have a greatly enhanced capacity to produce antibody to a

foreign protein. Further investigation of these phenomena, including other viruses and hosts, should provide a new approach to some aspects of immunological response.

Consistent with the foregoing results, it was possible to demonstrate neutralizing antibody in the sera of mice infected for three months or longer, after selectively inactivating the LDV in the sera. Such treated sera readily neutralize LDV obtained from early infections (early LDV), before antibody has been produced; however, LDV obtained from long-standing infections (late LDV) is rather resistant. This result suggests that late LDV exists as an infectious virus-antibody complex. Consistent with this interpretation, late LDV could be "neutralized" by antimouse sera prepared in goats. This surprising discovery led to the concept of "sensitization" of virus by antiviral antibody, without neutralization, and opens the way to a fresh interpretation and investigation of viral neutralization by antibodies, whose mechanism has never been satisfactorily elucidated.

The chemistry of the labile, enzyme-sensitive region linking the three stable domains of rabbit γG immunoglobulin has been studied further by analyzing the amino-acid composition of fragments prepared with pepsin, insoluble papain, mild reduction, and alkylation. In this way it has been possible to narrow the locale of the important disulfide bond linking the heavy chains of rabbit γG to a region between the respective peptide bonds hydrolyzed by pepsin and papain. These studies have been materially facilitated by improvements in the program for automatic digital data acquisition and computer calculation developed for the amino-acid analyzer.

Further study during the past year also was made of the paradoxical "lactate dehydrogenase" of *Butyribacterium rettgeri*, which cannot convert lactate to pyruvate, as such enzymes usually do. In effect, it functions as an irreversible pyruvate reductase. Using a 100-fold purified preparation of this enzyme, the reaction with pryuvate rate was a sigmoidal function of pyruvate concentration, not a hyperbolic one. This indicates that the enzyme is allosteric, requiring reactions at two sites on the molecule, and therefore might function in a regulatory capacity. An associated observation was that reduction of pyruvate is strongly inhibited by adenosine triphosphate (ATP) but not by adenosine monophosphate. Thus, under conditions where the cellular energy supply (ATP) derived from glycolysis is high, and the potential for biosynthesis is consequently great, the conversion of pyruvate to lactate might be inhibited. Pyruvate would then remain available for a variety of biosynthetic reaction sequences, such as amino acid and fatty acid synthesis. Such a regulatory mechanism would provide the cell with a novel means of modulating the carbon supply for biosynthesis through a linkage with the available energy supply.

It is known that the biochemical and nutritional process taking place in the oral microbiota are dynamic and complex. The microbiota must depend not only upon the exogenous supply of metabolites but also upon the nutritional climate contributed by the symbiotic nature of the oral microbiota. The latter implies that certain microorganisms are capable of synthesizing varieties of metabolites which may be favorable to other microorganisms. Hence, the knowledge of how microorganisms can synthesize these metabolites may contribute to a better understanding of the microbiota which influence the many problems of oral health. Continued investigation of the biosynthesis of the vitamin folic acid, by enzymes extracted from lactobacilli, have shown that the pathway from guanosine monophosphate involves elimination of the number 8 carbon atom as formate, and subsequent closure to form the pteridine ring. A novel nonenzymatic reaction was discovered whereby guanosine monophosphate, ferrous ion, and mercaptoethanol yield an intermediate compound that releases formate when treated with lactobacillus extracts. Knowledge of the nature of these reactions should help elucidate the biosynthesis of folic acid.

Evidence continues to accumulate that the rate of differentiation of the cellular slime mold, *Dictyostelium discoideum*, from ameboid to spore stage depends on intracellular accumulation of the monomeric constituents of

ribonucleic acid. Thus, the 5'-nucleotides of adenine, guanine, cytosine, and uracil stimulate differentiation with equal efficacy, when supplied exogenously. The whole molecule is required. Respective purines, pyrimidines, and nucleosides are inhibitory. Pointing in the same direction is the fact that a number of substances other than nucleotides can stimulate differentiation, while causing intracellular accumulation of ultraviolet-absorbing materials. The latter have now been identified as a mixture of the mononucleotides and nucleosides of ribonucleic acid. This analysis was made possible by development of an improved column chromatography procedure combining molecular sieve and on exchange chromatography.

ORAL SOFT TISSUE LESIONS AND CLINICAL DIAGNOSIS AND TREATMENT

As the Oral Medicine and Surgery Branch of the Institute has grown, efforts have been made to give more adequate attention to the major problem areas of dentistry.

Studies of the human dental pulp have emphasized a continuing evaluation of response to changes induced by dental drilling procedures and by various restorative and related materials, such as cavity liners. These investigations have furnished the dental profession with some very practical information on operative procedures, particularly in regard to optimal cutting speeds, the proper use of coolants, and modifications in technic necessary for the safe placement of experimental restorative materials.

Because of the reduced inflammatory response of the pulp following high speed cutting technics, the incidence of reparative dentin production has been greatly reduced. Thus, dentinal tubules remain open and permit the toxic or irritating products of sterilizing agents, cements and silicates to permeate to the pulp tissue and cause further damage. This lack of reparative dentin formation creates a formidable problem in restorative dentistry, especially in the field of full mouth rehabilitation where often the entire coronal dentin is exposed. Experimental drugs designed to produce sensitivity of teeth (i.e., corticosteroid compounds) and to more effectively seal the dentinal tubules are being sought, as well as drugs and technics to increase the incidence of reparative dentin formation.

When prepared cavities are washed with a steroid formula containing 1% prednisolone in a vehicle of parachlorophenol, cresatin and gum camphor, before restoration with zinc oxide and eugenol, it is found that the pupal response to the cavity preparation is minimized about 50%. When prednisolone is used without the vehicle, the inflammatory response is sustained only 12 days. Also, when the same formula is applied to the cavity preparation several days after the full potential of the response has occurred, the resolution period is still shortened.

In a study comparing the healing of surgically exposed dental pulps in germfree and conventional rats, the latter group showed an immediate, severe inflammatory response which quickly led to total pulpal necorsis, whereas the germfree animals, without exception, showed minimal inflammatory response (in spite of food impaction) with subsequent dentinal bridging.

In another area of clinical investigations, a collaborative study with the Anesthesiology Department of the Clinical Center on general anesthesia in ambulatory dental patients has been developing important information concerning the physiological effects of various anesthetic agents and oral surgical procedures. Since in some localities there are almost as many general anesthetics administered in dental offices as in local hospitals, such information should prove particularly important for the specialty of oral surgery.

Accumulated data are providing a continuing record of pulse, blood pressure, arterial O_2 saturation, respiratory phenomena, cortical brain activity, and the electrical activity of the heart. Among the more significant findings have been (1) consistent hypertension in all ambulatory anesthesias, which directly parallels the intensity of the surgical stimulation; (2) preoperative and operative tachycardias in almost 100 percent of the anesthetics (the preoperative changes in rate being apprehensive in nature whereas the operative changes

are due primarily to the pharmacologic action of the intravenous barbiturates and secondarily, to the surgical stimulation in extremely light anesthetic planes); and (3) depression of arterial oxygen saturation, which is a controllable factor related to anesthetic management and drug administration (i.e., avoidance of obstructions and drug overdosage).

Studies of soft tissue lesions continued to receive major attention during the past year. As described in earlier reports, a transitional L-form of an alpha steptococcus has been isolated from or allesions in patients with recurrent aphthae and periadentitis aphthae. This organism has now been consistently recovered from lesions in numerous patients on repeated examinations over a 12-month period. Blood obtained for culture during two exacerbations in one patient was found positive for the same organism, and a stable L-form was obtained from scar tissue at the site of previous lesions during a remission.

The injection of a licensed intravenous vaccine (Strep. indifferent, Lilly) into one patient with periadenitis aphthae, over a period of several months, reduced the severity of the ulcers but did not prevent their recurrence completely. Therapeutic investigation also is under way on these stubborn and resistant chronic debilitating diseases with a limited success observed with various forms of acromycin and topical steroids.

Parallel animal studies have suggested that hypersensitivity to the antigens of the alpha streptococcus isolated from recurrent aphthae is an important factor in the development of these lesions. Positive skin tests (delayed type hypersensitivity) to these antigens were obtained in patients with aphthous stomatitis but not in control individuals. The degree of the skin test reaction was found to be directly proportionate to the severity of the disease in the patients tested.

The injection of a vaccine intravenously for 5 days prior to skin challenge reduced the size and duration of the ulcers experimentally produced in the hypersensitized and control guinea pigs. The greatest protective effect was noted in the non-sensitized (controls) guinea pigs.

In studies of mucous membrane changes associated with age and certain diseases, it has become apparent that human buccal mucosa, although appearing clinically normal, may undergo various changes with advancing age. Since the buccal site is frequently biopsied, standards need to be established on normal mucosa to eliminate errors in diagnosis due to the age factor.

Other work is being carried out on a previously undescribed "focal epithelial hyperplasia" in Indian children. Special studies, including viral analyses and electromicroscopy, failed to reveal evidence of a viral agent. The present knowledge concerning this lesion indicates that either an environmental or a genetic factor is involved in its development.

Preliminary studies, using tritiated thymidine, on oral mucosa involved in verrucous carcinoma have revealed epithelial turnover rates remarkably similar to those believed to be found in normal oral mucosa. Similar investigations are currently under way to better define the oral mucosal alterations in patients with Darier's disease.

In a collaborative study with the Laboratory of Pathology, National Cancer Institute, the individual and/or combined roles that calcium hydroxide, tobacco, and gambier might play in causing betal quid induced carcinomas of oral mucosal tissues has been under examination. Principally utilizing the hamster cheek pouch as a model system, one or more of the following changes were noted: Deposits of calcium, inflammation, giant cell formation and fibroblastic proliferation in the lamina propria; and inflammation, ulceration, atrophy, hyperplasia, hyperkeratosis, parakeratosis, acanthosis, and cellular atypia in the epithelium. In no instances were squamous cell carcinomas produced, nor were any changes noted in those cheek pouches treated with snuff or starch powder alone.

Another study designed to classify and determine the tissue of origin of benign fibro-osseous lesions of the jaws drew heavily on material made available at the Armed Forces Institute of Pathology. Among the more significant observations were: (1) that ossifying fibroma, cementifying fibroma, and cemento-

ossifying fibroma appear to arise predominantly from the periodontal membrane but can also arise from the medullary bone; (2) that oxytalan fibers may occur in most benign fibro-osseous lesions of the jaws, regardless of their tissue of origin, provided that mature collagen fibers are also present in the lesion; (3) that since oxytalan fibers and pre-elastic fibers cannot be distinguished with present histochemical methods, the demonstration of fibrous elements stained with the oxytalan fiber method does not constitute conclusive evidence of odontogenic origin of the tumor; and (4) that the birefringence pattern under polarized light serves as an excellent differential for diagnosis. Fibrous dysplasia gives a random irregular birefringence, indicative of woven bone, whereas the other fibro-osseous lesions manifest birefringence as parallel light and dark bands, indicative of the varying degrees of lamellar bone formation.

In a related study to define the behavioral patterns of osteosarcoma and chondrosarcoma of the jaws, the following findings have been reported: (1) tumors in the mandibular symphysis area are most amenable to cure whereas those in the maxillary sinus are least amenable; (2) the average age of occurrence for osteosarcomas of the jaws is about a decade older than for osteochondroma of the jaws, but there is a quite wide range, and the range is wider for the mandible than for the maxilla; and (3) early findings are quite variable and often non-specific, and diagnosis depends upon correlation of clinical behavior, histologic appearance, and roentgenographic appearance, any one of which may be deceivingly benign appearing. An important early finding may be roentgenographic evidence of symmetrical widening of the periodontal membrane space, with maintenance of an undisturbed lamina dura radiopacity.

Investigation of Gardner's syndrome, which consists of multiple odontomas, multiple osteomas of the jaw, multiple polyps of the intestine, and subcutaneous tumors which are inherited, revealed that these patients are refractive to the effects of parathyroid hormone. Thus, it is possible that a group of diseases, including pseudohypoparathyroidism, basal cell nevus-jaw cysts syndrome, and Gardner's syndrome, share some defect in parathyroid hormone utilization. Other diseases with similar signs and symptoms are being investigated for this biochemical abnormality.

Genetic studies of inherited sensory traits indicate that the inability to smell potassium cyanide is a polymorphism and a recessively inherited trait.

In related activities, saliva studies have yielded a major finding concerning amylase isoenzymes in humans. A polymorphism in man has been defined in that different forms of the enzyme are consistently produced in a particular individual, and a generic difference exists in parotid amylase between man and rat.

A discrepancy in the classical method for determining secretor status was found and tested on 2,875 individuals, which indicates that approximately 6½ percent more aberrant secretor individuals can be detected by this new method.

The foregoing account of intramural research reflects a rather purposeful approach to the delineated problems and objectives encompassed in the Institute's broadly based program. Likewise, it makes abundantly clear the view that dentistry's attainments of tomorrow will be a direct consequence of the extension of the boundaries of knowledge, and that this can come about only coincident with the intimate bonding of the health professions and related sciences in the kind of environment exemplified at the National Institute of Health.

LABORATORY OF MICROBIOLOGY

Immunology

The principal organizational event in the Laboratory of Microbiology this year was the creation of the Immunology Section, with a view to co-ordination, more efficient planning and performance, and eventual extension of current immunologic projects. This move not only recognizes formally the natural place of immunology as a major component in this Laboratory, but signalizes also the trend of our basic investigations relating to periodontal disease. Extensive investigations in this Laboratory and elsewhere have established that the members of the oral microbiota most sig-

nificantly implicated in the pathogenesis of chronic periodontitis possess sufficient pathogenic factors to account for the principal features of the periodontal lesion. These factors include lipopolysaccharide endotoxins and histolytic enzymes (mucopolysaccharases, proteases, peptidases, collagenase, and other hydrolases). We can now add the observation that neutral solutions of ammonium ion and hydrogen sulfide, in concentrations that might reasonably be expected from known bacterial actions in the gingival crevice, exert a marked cytopathic effect on unkeratinized epithelium such as that lining the critical contact of gingiva with oral bacteria.

With this background largely filled in, it seemed timely to increase attention to the immunological relationships between the host and the microbial products occurring in the gingival crevice and periodontal pocket, and to associated basic immunologic studies. Thus, it was shown that repeated deposition of antigen in the normal gingival pocket of the rabbit resulted in appearance of homologous antibody in the blood serum and in chronic allergic inflammation of the gingival histologically very similar to that seen in chronic human gingivitis. Paradoxically and despite many investigations, little evidence has been presented that humans form antibodies to their indigenous gingival crevice bacteria. This failure seems to be due at least partially to the use of insufficiently sensitive serological methods. Using indirect hemagglutination and bactericidal tests for antibody, we have found regularly significant amounts of antibodies to *Leptotrichia buccalis*, *Veillonella alcalescens*, and *Fusobacterium polymorphum* in human sera. Subjects with advanced chronic periodontitis exhibited significantly higher titers to *F. polymorphum* than subjects with a clinically uninflamed periodontium. Parallel studies showed that the concentrations of antibodies in parotid secretion were about 1:1300 those of serum. Presumably the major part of antibodies in the oral cavity comes from the blood serum, via gingival crevice exudate.

Continuing studies of the relationships between lactic dehydrogenase virus (LDV) and its host animal (mouse) have paid unexpected dividends in the immunological field. LDV is unusual in several respects: LDV-infected animals exhibit lifelong viremia but remain apparently healthy; the only known consequence of LDV infection has been persistent elevation of a number of plasma enzyme activities, due at least partially to impaired clearance mechanisms; and until now no antibody response to LDV has been discernible. That LDV does activate an immunological response of its host is indicated by our recent finding that LDV infection of germfree mice results in an elevated level of plasma γ-globulin (not due to impaired clearance) and a great increase of germinal centers in spleen and lymph nodes. Both these parameters normally are very low in germfree animals, and their increase is usually interpreted as an immunological response. How much of this γ-globulin production represents antibody cannot be ascertained at this time. Extension of these experiments, however, showed that LDV acts as an immunological adjuvant; that is, LDV-infected conventional mice have greatly enhanced capacity to produce antibody to a foreign protein. Further investigation of these phenomena, including other viruses and hosts, should provide a new approach to some aspects of immunological response.

Consistent with the foregoing results, it was possible to demonstrate neutralizing antibody in the sera of mice infected for three months or longer, after selectively inactivating the LDV in the sera. Such treated sera readily neutralize LDV obtained from early infections (early LDV), before antibody has been produced; however, LDV obtained from long-standing infections (late LDV) is rather resistant. This result suggested that late LDV existed as an infectious virus-antibody complex. Consistent with this interpretation, late LDV could be "neutralized" by antimouse sera prepared in goats. This surprising discovery led to the concept of "sensitization" of virus by antiviral antibody, without neutralization. It opens the way to a fresh interpretation and investigation of viral neutralization by antibodies, whose mechanism has never been satisfactorily elucidated.

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Dental Caries

Evidence continues to accumulate that the level of dental caries activity in experimental animals is determined by very intricately balanced relationships between host, a limited variety of oral microorganisms, and dietary factors, notably sucrose. A considerable range of microbial species has now been tested in gnotobiotic rats and in hamsters deficient in cariogenic flora. Except for a single atypical strain of *Lactobacillus acidophilus*, only certain strains of a particular cultural and immunological type of oral streptococcus have been found capable of initiating caries in such animals. Significantly, this Laboratory and collaborators at National Children's Cardiac Hospital, Miami, Fla., and Royal Dental School, Malmö, Sweden, have isolated during the past two years a number of "rat-type" and "hamster-type" streptococci from human caries lesions and saliva. Many of these have proved to be cariogenic in the respective species of rodent. Recently we have isolated streptococcal strains from humans, which are cariogenic in both rats and hamsters. In view of the long history of unsuccessful attempts to transmit caries activity from humans to animals, and from one species of animal to another, these results have been very surprising. Most importantly, they have encouarged renewed search for a specific bacterial element in human caries. Sufficiently distinctive cultural characteristics of these types of streptococci have been ascertained to make possible beginning human epidemiological studies. It would be especially meaningful, for example, to determine whether these organisms are natural inhabitants of the oral cavity in population groups with low caries experience, or whether the organisms must be transmitted to an individual before the disease can be established.

The reasons why some strains of these streptococci are cariogenic, while others are not, seem to relate to their behavior with sucrose. Thus we have demonstrated that cariogenic strains produce far more dental plaque, *in vivo* and *in vitro* in the presence of sucrose as compared to equivalent amounts of other carbohydrates. On the other hand, cariogenic and noncariogenic streptococci did not differ in their rates of acid production, and fermented glucose, fructose, maltose, or mixtures of glucose and fructose as readily as sucrose. Nevertheless, when any of these sugars, or sorbitol, or a hydrogenated starch product was substituted for sucrose in a caries-conducive diet, the incidence and severity of caries decreased markedly. These results are of course entirely consistent with the large amount of epidemiological evidence that dietary sucrose is a major determinant of caries in man. (Concerning sucrose it is pertinent to mention also the parallel demonstration of extracellular polysaccharide (levan) production from sucrose, but not from a variety of other common sugars, by *Odontomyces viscosus*. This provides a reasonable explanation for the accumulation of this organism as a gelatinous plaque causing a form of periodontal disease in hamsters fed a high-sucrose diet.)

Recent evidence indicates that differences in the caries susceptibility of different strains of rats correlate with their ability to support a cariogenic flora. When different strains of rats have been fed the same diet and given equal exposure to a source of cariogenic flora, the levels of caries activity and the animals' ability to transmit the flora have differed, approximately in parallel. On a different caries-conducive diet, however, the relative susceptibilities of two strains of rats can be reversed; that is, a normally "resistant" strains has developed more caries than its counterpart "sus-

ceptible" strain when both were fed one of our standard caries-conducive diets. Other studies indicate that the numbers of cariogenic streptococci in the mouths of rats and hamsters parallel the levels of caries activity. These results suggest that the support of a cariogenic flora depends upon some host-diet relationship, rather than on host and dietary factors independently.

We conclude that the problem of caries control assumes quite different aspects depending on the microbiological status of the subject population. If relatively caries-free populations, such as those found in several African nations, enjoy this status because they are not infected with cariogenic microflora, caries activity should remain low regardless of dietary composition, particularly the consumption of sucrose. On the other hand, if potentially cariogenic microorganisms are already widely prevalent in such populations, then introduction of sucrose into the diet should automatically result in increased caries activity. In the United States and most Western nations, sucrose consumption and caries are so prevalent that we must assume that cariogenic microorganisms are ubiquitous. Probably the only way to eradicate dental caries would be to eliminate the causative microorganisms from a population. Until this becomes feasible, as by suitable use of antibiotics, other control measures of known efficacy such as fluoridation and dietary regulation should be rigorously applied.

Virology

Continuing study of persistent herpes simplex virus (HSV) infection in a cell culture system now warrants provisional conclusions as to its mechanism and inferences as to recurrent herpes infection in humans. The fact that persistent cyclic HSV infections can be initiated and maintained in the presence or absence of homologous antibody indicates that the underlying mechanism is not dependent on antibody directly or its ability to reduce extracellular HSV. Even when antibody is present, virus transmission still occurs by cell-to-cell transfer and by the reattachment of infectious cellular material. The only effect of antibody which we have been able to identify is its ability to reduce the severity of the infections to a low grade, comparable to a subclinical stage in the human disease. The maintenance of these persistent infections appears to depend on the establishment of a dynamic cell-virus equilibrium which is characterized by the continual selection of cells resistant to the major fraction of virus being produced at any one time. Selection of such resistant cells should ultimately result in a cell population completely resistant to HSV infection. This does not occur, however, due to virus variations which parallel the cellular changes. The result appears to be that the selection of resistant cells is offset by the production of either a new virus variant, to which the cells are still susceptible, or to changes in the virus population resulting in selection by the cells of a virus variant which was present as a minor fraction. Superimposed on this continual selection mechanism is the probable production of factors that temporarily enhance the cells resistance to HSV infection. Such factors, which thus far have been found in only low titers, are probably synthesized and maintained in localized areas of the cell cultures, thus allowing the few surviving cells to proliferate and replenish the cell sheet. It has been found, in fact, that replenishment of the cell sheet in localized areas must occur before a new cycle of virus multiplication and cytopathic effect is initiated. In the presence of antibody the severity of the infections in cultures producing a non-proliferative virus variant is reduced to a low level. After varying lengths of time, a short-term exacerbation paralleled by the appearance of a syncytium-forming variant is evident, followed by the re-establishment of a low grade infection. The occurrence of these exacerbations *in vitro* is strikingly similar to the recurrent episodes seen in human herpes labialis and appears to be related to viral changes which temporarily alter the cell-virus equilibrium in favor of the virus. Ultimately, the cells are able to readjust to the new virus type being produced, and a subclinical type infection is re-established.

Previously we have reported that HSV could be cultured from the salivas of rabbits many months after intraperitoneal injection. We

have now found that the HSV-antibody response in such animals is strong and lasting, with cyclic variations very similar to the response to HSV infection in man. In contrast, the antibody titer induced by inactivated HSV is low and transitory. These results indicate that HSV persists in the tissues of animals receiving live virus, with intermittent immunizing bursts. However, no HSV could be cultured from testes, kidney, liver, spleen, lung, lacrimal glands, submaxillary glands, lymph nodes, and brain of such animals. Rather surprisingly, organ cultures of all these tissues from the immune animals, except lymph nodes and brain, were susceptible to infection with HSV. Provisionally we conclude that HSV persists in some inapparent form in these animals, or quite possibly in tissues not yet tested for this virus.

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required. Respective purines, pyrimidines, and nucleosides are inhibitory. Pointing in the same direction is the fact that a number of substances other than nucleotides can stimulate differentiation, while causing intracellular accumulation of ultraviolet-absorbing materials. The latter have now been identified as a mixture of the nononucleotides and by nucleosides of ribonucleic acid. This analysis was made possible by development of an improved column chromatography procedure combining molecular sieve and ion exchange chromatography.

Systematic Microbiology

Two members of the staff continued to devote much effort to accumulation of necessary data and their collation by respective international committees engaged in clarifying definitions of *Lactobacilleae, Neisseriaceae, Propionibacteriaceae, Actinomyces,* and *Bifidobacterium.* Definitive redescription of the genus *Veillonella* was completed and published. In the cytological area, combined application of electron microscopy, antigenic fractionation, labeled specific antibody, and pharmacological testing demonstrated that the endotoxic somatic lipopolysaccharide antigen in *Veillonella* is localized in a distinctive three-layered outer membrane. This can be stripped off by phenolic extraction, leaving the rest of the cell morphologically intact. An inner rigid wall, which maintains cellular integrity, was demonstrated by its digestion with lysozyme, a mucopolysaccharase.

Basic studies of these kinds are of the first importance, particularly in the oral field, because the usefulness of large parts of previous work is vitiated by lack of adequate cytological, immunological, biochemical, and pathogenetic characterization of microorganisms.

LABORATORY OF HISTOLOGY AND PATHOLOGY

For the purposes of the present report the activities of the Laboratory of Histology and Pathology are summarized according to several areas of general interest. The projects from which the results have been gathered together are carried on by staff members alone or jointly, and often in collaboration with workers from other laboratories. The specialized fields represented include biophysics, histochemistry and experimental pathology.

Microstructural Characteristics of Normal and Abnormally Developed Enamel

For many years considerable emphasis has been placed on the study of enamel structures, normal as well as pathological. Valuable new data have been accumulated which have advanced our understanding of the morphology of sound and carious human enamel, especially with regard to the prismatic structures and their participation in the spread of the carious process. While the previous studies dealt with subsurface enamel this year's efforts have been directed toward the surface layer. This layer is of particular interest because of its apparent greater resistance to decalcification as seen in early enamel caries, the so-called white spot lesions, where a seemingly intact surface layer covers an area of extensive subsurface demineralization. Although the differences in reactivity may be attributed to chemical differences such as higher fluoride contents, the present study demonstrates clearly that morphological differences also exist. The lack of prism structure, the orientation of the crystals perpendicular to the surface, the higher mineral contents coupled with a possible increase in crystal size are all factors which might contribute to an increased resistance. The influence and role of this "prismless" surface layer in the onset and spread of the carious lesions especially relative to morphological differences from one tooth to another are some of the important questions which may find an answer in the continuing research.

A contributory factor to the success of these studies has been the new microradiographic equipment which was constructed and put into service last year. The availability of an X-ray tube with exchangeable targets has made it possible by selecting X-rays of suitable wave lengths to detect very minute

differences in mineral contents which could not be recorded by previous methods.

The effect of tetracycline hydrochloride on enamel development has been explored further and a reproducible dose-response relationship has been established. Using these results as reference, the studies were extended to include the effect of comparable dosages of oxytetracycline. Although the latter antibiotic was capable of introducing mineralization disturbances at similar dose levels, gross hypoplastic lesions were not seen, suggesting that oxytetracycline is somewhat less toxic than tetracycline hydrochloride. The similarity of the tetracycline induced defects to those resulting from injections of a number of other chemicals indicates a common non-specific response. Localization and identification of the cytological sites affected by the various toxic agents promise to yield considerable date on enamel formation in general.

Crystallographic Studies of Synthetic and Biological Phosphates

A series of calcium phosphates have been made synthetically and have been examined by X-ray diffraction and by infrared spectrophotometry. Among the problems investigated have been definition of conditions under which large hydroxy-apatite crystals may be grown routinely and a single crystal X-ray structure determination of a selected, well-defined crystal. The availability of such crystalline material of known structural composition is of major importance to all investigators interested in studying the chemical reactivity of hydroxyapatite in both synthetic and biological systems. Other studies have dealt with the characterization of the infrared absorption spectra of pure synthetic apatites and the identification of band changes associated with the presence of fluoride and carbonate ions in the crystal latice. The knowledge of the exact location of these ions within the lattice is important for our understanding of their effect on caries susceptibility.

The relationship between fluoride contents and crystallinity of biological mineral was investigated using X-ray diffraction techniques. One aspect of this project has been the development of new methods for assessing the degree of crystallinity directly from measurements taken from the instrument instead of using the more cumbersome template approach developed previously in this laboratory. The data compiled through the direct methods can be fed into a computer, thus facilitating more extensive studies of age effects on bone crystallinity in high and low fluoride areas. A second facet of the work has involved assessing the effect of fluoride on crystallinity of enamel. As with bone it was found that the crystallinity of human enamel improved with increased fluoride. Yet in the enamel the changes in crystallinity were not restricted to the a-axes directions, but also included the c-axis. Since both size and strain factors influence the degree of crystallinity, the full meaning of the observed changes awaits a separation of the two factors.

Histochemical and Chemical Studies of Connective Tissues

The histochemical and chemical studies of enzymes involved in connective tissue formation and destruction have been continued. Special emphasis has been placed on determination of collagenase activity in a number of connective tissue sites under normal and pathological conditions following the discovery last year of the enzyme system in human gingival tissues. Of the normal tissues tested so far, only uterus, gingivae and to a lesser extent portions of the oral mucosa have exhibited collagenolytic activity. Some of the most exciting results, however, have been the demonstration of the enzyme in cultures of dermis from individuals with certain neuromuscular disorders and collagen diseases. Isolation and purification of the collagenase is being attempted and when accomplished will allow investigation of its action on the collagen structure. The cellular origin of the collagenase is also being studied. The results already obtained represent an important extension of our basic knowledge and when fully exploited will undoubtedly contribute significantly to a better understanding of the met-

abolic processes in healthy and diseased connective tissues.

Experimental Pathology

Previous experimental work on the etiology of dental caries and periodontal disease has clearly demonstrated the importance of bacterial plaque deposits in both these infections. Consequently, this years studies have been concentrated in defining various factors involved in plaque formation and on testing the efficacy of different drugs and proprietary formulations in the control of plaque formation. The results indicate that many microorganisms and among these several streptococci of human origin are capable of inducing plaque and active caries in hamsters. The influence of diet, especially the effect of different sugars, has demonstrated that while sucrose in the diet is associated with rapid plaque formation, sucrose substitutes are much less plaque conducive. The host factor has been investigated in studies aimed at determining the effect of maturation on tooth resistance to plaque associated lesions. The findings suggest that in the absence of fluoride, tooth age is not a factor in caries susceptibility. While fluoride enhances tooth resistance, some fluoride containing preparations also appear to be active in controlling plaque formation. None of the commercially available, fluoride containing dentifrices, however, have been effective in plaque control when tested in the experimental hamster model. The continued testing of anticariogenic drugs and methods in the experimental animal system and in *in vitro* studies will serve as an important step in the evaluation of agents suitable for human clinical trials.

Bacterial and Macromolecular Structure

These are largely basic studies aimed at relating morphology to an experimental situation. The initial electron microscopic investigations on endotoxin formation and the character of the endotoxin of one kind of organism have been extended to other entoxin producing gram-negative bacteria. While all the organisms appear structurally similar, the isolated endotoxin particles vary in morphology. Work has also been directed at determining the bacterial killing due to complement dependent antigen-antibody reactions. The initial findings demonstrate a similarity in morphology between complement dependent bacteriolysis and hemolysis. Studies on human amyloid have been continued with special emphasis on purification of the pathological protein in order to characterize it chemically. The degree of purification is assessed electron microscopically as in the effect of various chemical treatments. These investigations constitute additional steps toward widening our knowledge of the morphology of this protein.

LABORATORY OF BIOCHEMISTRY

Research continued in special areas of biochemistry, pharmacology, calcification and the etiology of dental caries. The research in pharmacology now involved in the study of dental caries. The research in pharmacology now involved in the study of oral-facial malformations induced by teratogenic drugs was organized into a Pharmacology Section of the Laboratory.

Enzyme Chemistry

Resolution of the mechanism of action and specificity of proteolytic enzymes advances understanding of all biochemical processes. Likewise these may be utilized for protein and enzyme modification, for study of protein molecuar structure, to advance our knowledge of a particular function of a body tissue. At this time, the immediate objectives of this research relate to the specificity and mechanisms of the enzymatic action of chymotrypsin C, as well as guinea pig liver transglutaminase.

Whereas the amino acid sequence of an approximately twenty-member tryptic peptide of the chymotrypsin C enzyme has only one methionine residue. The active peptide site of the A enzyme contains two methionine residues. Likewise an enzyme inhibitor specific for chy–motrypsin A has little or no inhibiting activity toward the C enzyme.

Pruified guinea pig transglutaminase was found to be essentially homogeneous. It con-

tains 21 thiol-groups and no disulfide bridges. Only one thiol-group is involved in the active center. From tryptic and chymotryptic digests of C^{14}-labeled transglutaminase, a labeled peptide was isolated and found to contain the following amino acids, one tyrosine, one asparagine, one C^{14}-labeled amide of carboxymethyl cysteine, one glycine and three tryptophans. The tryptophan is present in the C-terminal position.

Enzyme Structure and Mechanism of Action

The studies related to enzyme structure and mechanism pertained to the physical-chemical properties of aldolase, and a correlation of its structure with catalytic function. Results have yielded information on the number and type of active sites possessed by this enzyme. Thus when native fructose-1, 6-diphosphate (FDP) was reduced to native FDP-aldolase there was evidence that three highly organized active sites were involved, each of which contained two nonidentical phosphate-binding sites.

Methyl Groups of sRNA

Protein synthesis involves the polymerization of individual amino acids into high molecular weight polypeptides, and soluble ribonucleic acid (sRNA) plays an essential role in this process as a specific carrier of amino acids to the site of polymerization. Our program in this area is designed therefore, to elucidate the role of methylated and thiolated constituents of sRNA in its biological activity and specificity. Results have indicated that while yeast amino acid activating enzymes can esterify amino acids to sRNA which contains methylated bases, these enzymes were unable to carry out this reaction with methyl-deficient sRNA. Continuing studies attempt to resolve the role and significance of the methylated base of sRNA, in its acceptor function. The results indicate that methylated base of sRNA, in its acceptor function. The results indicate that methylated bases of sRNA play a significant role (*a*) in the initial attachment of a specific amino acid to sRNA, (*b*) in a reaction catalyzed by specific amino acid activating enzymes and (*c*) in the capacity of the sRNA molecule carrying an amino acid, to find its proper alignment on the polymerization template.

The investigation relative to thionucleotides (sulfur-containing) in sRNA has pertained to their functional significance. It was shown that formation of minor bases in sRNA is controlled by the sulfur amino acids cysteine and methionine. This relationship forms a cyclic metabolic regulatory system. Evidence was obtained that under ordinary circumstances the leucine activating enzyme from yeast recognizes *E. coli* sRNA about 75% of the extent recognized by the enzyme from *E. coli.*

Phosphate and Dental Caries

The research pertinent to the cariostatic effect of phosphates proceeded with attempts to define the mechanism of its action. Additional evidence was obtained in support of the hypothesis that this cariostatic action is localized in the oral cavity. Thus, NaH_2PO_4 added to drinking water or diet of white rats for a period of two weeks prior to initiating a cariogenic diet reduced the ultimate cariogenic effect of the diet. Apparently the tooth surfaces and/or the oral milieu, had been favorably conditioned against caries by this prior exposure to phosphate.

To resolve the possible effect of phosphate on the chemistry of both permanent and deciduous teeth, the Pitman-Moore miniature pig and standard Duroc swine were fed low- *vs.* high-phosphate diets for a period of one year, starting at weaning age. The animals were sacrificed when both permanent and deciduous teeth were erupted and still *in situ.* Ash, calcium phosphorus, magnesium and carbon dioxide were determined in enamel and dentin of permanent and deciduous teeth. The low phosphorus diet did not affect the content of the above major constituents of the teeth, as compared with the teeth of swine fed a normal and higher phosphate diet. Apparently the cariostatic effect of phosphate supplements as observed in experimental rats, may not be related to a change in major components of the teeth.

Prenatal and Fetal Development Factors Influencing Oral Disease

The current objectives in this program of research are as follows: (*a*) to study the occurrence of metabolites of chlorcyclizine in maternal and fetal tissue during organogenesis, (*b*) to specify the effects of certain drugs on implantation and fetal development and (*c*) to compare the histology of benzhydrylpiperazine vs. Vitamin A induced cleft palate. Qualitatively chlorcyclizine and norchlorcyclizine were the only detectable metabolites with at least one tertiary amine, found to be present in maternal and fetal tissue. The ratio of norchlorcyclizine to chlorcyclizine in fetuses (normally about 15:1) could be reversed by administration of a demethylation inhibitor to a ratio of about 2:1. Under these conditions of inhibited demethylation, however, teratogenesis was still present at approximately the same incidence.

In attempts to identify the specific and minimal chemical structure responsible for a teratogenic drug effect, it was found that buclizine hydrochloride induced the same malformations as meclizine and chlorcyclizine. Three antihistamines, i.e. perphenazine, prochlorperazine and trifluoroperazine which do not have a piperazine moiety in their molecular structure demonstrated only a low (5.4%) teratogenic effect.

Histological observations on palatal clefts produced by agents of the benzhydrylpiperazine series of drugs, showed that the adhesion in the palatal area consisted of a fusion of the overlying epithelium of the tongue and the palatal processes. Gross examination of rat fetuses resulting from excessive doses of Vitamin A disclosed microstomia, mandibular-maxillary ankylosis, and cleft palate. Histologically the palatal processes of these fetuses were variable and vestigial in nature. Associated with these hypoplastic palatal processes was intramembranous bone formation. A large amount of heterotopic cartilage was located throughout the maxillary areas.

It is particularly important to explore animal species, in addition to the mouse and rat, which may be susceptible to teratogenic drugs. Thus, it was found that at high levels, chlorcyclizine hydrochloride induced abortion in swine. In reduced quantities this teratogen produced typical oral-facial malformations in the swine fetus. The Rhesus monkey is now under observation to determine the status of this species with respect to susceptibility to teratogenic drugs.

Protein Chemistry

Collagen is the major structural protein of vertebrates and many invertebrates. The protein, in the form of a rod-like monomer, undergoes aggregation into fibers which gain additional stability by a maturation process involving the introduction of covalent crosslinks. These are chemical links that are found both between polypeptide chains in the same molecule and between chains in adjacent molecules. Past studies have elucidated the chain structure. Current studies are related to a more detailed examination of the chemistry and biosynthesis of crosslinks. This is being accomplished by utilizing chemical and enzymatic cleavage to give peptides suitable for chemical studies that cannot be done on the whole molecule owing to its large size.

An enzyme from the tadpole cleaves the molecule into two pieces of unequal size. Isolation and characterization of these pieces have shown that intramolecular crosslinks occur only in one, indicating that crosslinks are specific in location. Limited cleavage of native collagen with chymotrypsin and characterization of the products have demonstrated that this enzyme splits a few peptide bonds at the N-terminal end of the molecule. The crosslink is also lost, demonstrating that it must be in this region of the molecule. Chemical cleavage with cyanogen bromide has permitted the isolation of peptides from the crosslink region. Studies of amino acid sequences in these peptides have shown that the crosslink is derived from a lysyl residue, five amino acids from the N-terminal end. Preliminary to crosslinking the lysyl side chain is converted to an aldehyde. Two of these aldehydes in adjacent chains form a crosslink probably by an aldol-type reaction.

Elastin is not as well characterized as a protein as collagen; but this connective tissue protein is also known to be crosslinked and lysine is the precursor. Rather than two lysyl residues forming a crosslink as in collagen, four are involved to give highly stable ring structures which are necessary for the elastic properties displayed by this protein. Chemical and histologic studies have shown that aldehydes are intermediates, again in parallel to crosslinking in collagen.

In the toxic condition known as lathyrism crosslinking of both elastin and collagen is inhibited. The inhibition appears to be in the enzymatic conversion of the lysyl side chain to an aldehyde. A similar inhibition is produced in copper deficiency, suggesting that a copper-containing enzyme may be involved.

The calcification of organic matrices is being studied using an *in vitro* system with elastin as the nucleating tissue. It has been found that trace amounts of a heavy metal such as ferric ion are required for calcification. The metal is apparently associated with sulfhydryl and perhaps imidazole groups in the protein phase. The first stage of calcification may involve the deposition of octacalcium phosphate at nucleation sites containing the complexed metal, with a later conversion to hydroxyapatite.

Fluoride Metabolism

The mechanisms governing the deposition of fluoride in calcifying structures are not understood. The role of fluoride in the inhibition of caries and its possible inhibitory effect on bone loss in resorptive bone disease, require resolution of the mechanisms by which these effects occur. A number of studies, therefore, are being carried out on the effect of fluoride in resorptive bone disease in the human, and in experimentally induced bone loss in the rat. Fluoride inhibited alveolar bone loss induced in the rat by either hydrocortisone or a low protein diet. However, maximum levels of fluoride tolerated by the rat did not reverse the decrease of bone protein and citrate synthesis as induced by hydrocortisone.

In one individual having multiple myeloma, the administration of about 30 mgs of fluoride per day for about two years, reduced the mobilization of fluoride from the bones.

Salivary Biochemistry

Phosphates have been shown to markedly reduce dental caries in the rat and hamster, and an increase of phosphate in the saliva of humans has been reported to be associated with a low caries experience. Since phosphate is involved in the glycolytic cycle, studies have been initiated on the relation of various glycolytic enzymes of saliva to dental caries. Some enzymes involved in the so-called "shunt" pathway have also been studied. Thus, hexokinase, aldolase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase have been found in whole saliva, whereas in parotid saliva, only hexokinase, aldolase and phosphoglucose isomerase were present. Bacteria, cellular debris or perhaps the submaxillary saliva thus may serve as a source of enzymes not found in the pure parotid secretion. The activity of these enzymes in caries-free, caries-normal and caries-rampant individuals will be studied.

In collaboration with the Oral Medicine and Surgery Branch of NIDR, calculus formation in the rat was related to a high protein concentration in whole saliva and with an elevated acid and alkaline phosphatase activity. Ratstrain differences seemed to be of more importance than diet variables, as a factor in calculus formation.

EPIDEMIOLOGY AND BIOMETRY BRANCH

During the year the Epidemiology and Biometry Branch continued its studies of the epidemiological characteristics of oral diseases. Activities included: (1) nutrition surveys in cooperation with the Research Branch of the Office of International Research; (2) investigations of the epidemiology of dental caries, including studies of the fluoride-dental caries relationship, clinical trials of caries inhibitory agents, and the nature of the disease in children fed by intubation; (3) studies of periodontal disease; (4) dental arch dimensions and

occlusal anomalies in populations; and (5) collateral activities.

Nutrition Surveys

In previous years the branch has participated directly in surveys organized by the organization then titled the Interdepartmental Committee on Nutrition for National Defense. Survey sites included locations in Alaska, Asia, Africa, and Central and South America. Data from previous surveys has been collated with findings from each new one as it is developed. During the past year data from the survey in Nigeria were analyzed and assistance in the field was given to survey teams working in Guatemala and El Salvador.

Analysis is continuing with the objective of discovering specific population factors which are associated with relative susceptibility to disease, on the general hypothesis that any causative factor should be found associated with disease in a consistent and predictable manner, population after population. Such associations should have been demonstrable because the prevalence of oral disease was found to vary, area by area, by factors of 30 to 60-fold.

Optimum (or even excessive) intakes of fluoride were invariably associated with an inhibition of dental caries and there was a general relation between caries prevalence and per capita consumption of refined sugar, but other widely-held theories were not supported. Very low caries prevalences were found in populations subsisting principally on such foods as rice, cassava, or yam, which are highly cariogenic in laboratory animals. No support could be adduced for the theory of a "protective" food or food element other than fluoride. Leads for further studies came from such findings as the observation (in Viet Nam, Lebanon, and Nigeria) of children with deciduous teeth attacked by caries alongside permanent teeth seemingly immune. One ominous note emerged: there was clear evidence that caries prevalence is rising, sometimes swiftly, in most populations now relatively free from this disease.

Tooth loss in some of these populations was high from another disease—periodontal disease, which leads to deterioration and loss of the structures which support the teeth in the mouth. In such areas as Lebanon, Trinidad, Viet Nam, Thailand, Burma, and Nigeria the onset of disease was early and advanced destruction was common even in young individuals. Noma, a disease which has virtually disappeared in western civilizations, was common in children in Nigeria. It was usually associated with protein-calorie malnutrition, exposure to malaria, recent history of rubella, and an overwhelming infection with Vincent's organisms.

As a general rule high prevalences of periodontal disease were seen in those populations where children exhibited signs of malnutrition in calories, protein, and vitamin A and carotene. There was a consistent and positive relationship between deposits of oral debris and calculus and the severity of periodontal disease in every population studied. When these factors, and age, were held constant no consistent or strong relation could be demonstrated between disease and nutritional status. Estimates of nutritional status were based on biochemical determinations, dietary studies, and the character of diets followed in the past. Factors brought under study included such items as vitamin A, beta carotene, ascorbic acid, thiamine, riboflavin, niacin, total proteins and protein fractions in serum, calcium, iodine, and iron. On the basis of the findings the value of any of these agents in therapy may be questioned.

Dental Caries

Studies of dental caries within the United States included investigations with tube-fed children, clinical trials with a phosphate-fluoride dentifrice and a fluoride gel carried in a mouth applicator, and further analysis of findings in a long-term study carried out with children during the first ten years of use of a fluoridated community water.

Studies of dental caries in laboratory animals fed by stomach tube have yielded much basic information about the relative roles of substrate and disease in these animals. Hitherto there have been no parallel studies in human beings. During the year an investigathe bacterial nature of dental caries which

tion was begun with tube-fed children in the Sunland Hospital, Orlando, Florida. This study should establish or deny basic inferences about have not heretofore been studied directly in human beings.

Other laboratory investigations have demonstrated that dental caries activity can be controlled at will in hamsters through the application of fluoride gels or antibiotics, topically to the teeth using a fitted vinyl mouthpiece as a carrier. A field trial of a fluoride gel applied in this manner is in its second year with children of Cheektowaga, N.Y. Preliminary findings indicate that (*a*) much higher concentrations of fluoride appear in the outer layers of tooth enamel after this than after conventional methods of application, and (*b*) that a single hygienist can supervise many more children when this technic is followed. An interim examination disclosed caries had been inhibited in the study children but too little time has elapsed for determination of the magnitude of the effect.

Principal findings in the longitudinal fluoridation study have been published in previous years. This study involved the reexamination of individual children annually for a period of ten years. The basic data are being reentered to test such hypothesis as the possible beneficial effect of prenatal ingestion of a fluoride water. The population is being utilized further in studies of occlusion and hygiene.

Periodontal Diseases

Within the past few years periodontal disease has been recognized as a major public health problem in the United States, where it is a major cause of oral pain and tooth loss. Clinical management of the conditions is difficult, expensive and time consuming and the number of available specialists is quite inadequate to meet the need for such treatment. On a world-wide basis, people of the United States are probably the most favored in this regard; elsewhere the periodontal diseases tend to be more prevalent, more severe, and earlier in onset, and professional personnel are even less able to cope with the problems. Studies conducted by the Branch have led to the clear inference that control of this disease entity must depend upon control of deposits of dental calculus, with little benefit to be gained by nutritional therapy.

Occlusal Anomalies

Orthodontic care claims a considerable share of the funds spent to improve dental health, but the natural history of occlusion has received little emphasis in epidemiological study. The present program of study in the Branch is designed to determine the sequence of events, and the causative factors, which lead to undesirable states of occlusion, in the hope that some of these factors may be susceptible to simple methods of control in early stages of development.

Principal focus is on a longitudinal study in which individual children are examined and reexamined over a period which includes critical periods of tooth emergence and jaw development.

Collateral Projects

In addition to direct research activities and biometric services to our professional staff, a considerable amount of time was devoted to consultation on the design and conduct of field studies being undertaken by others. In some instances this involved direct participation in the first stages of the field work. Principal beneficiaries were research grantees of the Dental Institute, the World Health Organization, the Pan American Health Organization, and la Instituto de Nutricion de Centro America y Panama.

CLINICAL STUDIES

As Clinical Investigations have grown and appropriate staff has been recruited, every effort for the utilization of the Dental Services Branch for human research studies has been made. Where at one time, few joint studies were operating, now many are in progress with considerable productivity. It is our philosophy that what cannot be accomplished elsewhere should be attempted here. Emphasis is placed on urging and supporting to our maximum, human research over animal studies. Because human research is very frustrating

in terms of patient problems, delays, and extended experimental periods, we permit our investigators to become involved in animal projects to the extent that it represents at least a back-up to some proposed study or a human project recently initiated.

The summary that follows merely represents a broad panoramic view of all our activities. It should be appreciated that with NIH facilities the depth of study of even one patient represents a research study and permits us to excel over many other institutions with greater census potential.

In the human dental pulp studies, we have now collected over 4,000 human teeth that were normal to begin with but have received a known traumatic episode for microscopic interpretation. This number of teeth represents a collection larger than all the human teeth used for intentional experimental purposes in all the world's literature previously reported. This collection presents us to the field of dentistry as an international leader for such studies. With the use of collaborative funds the program has every opportunity to expand in the future.

The opportunity for an oral surgeon to work with general anesthesiologists in a dental operatory provides us with information that is sadly deficient throughout the dental profession. Again the Clinical Center provides us with a unique opportunity. Since in some areas there are almost as many general anesthetics administered in dental offices as in local hospitals, the basic physiological data from this study should be invaluable to the specialty of oral surgery.

In human dental caries, the importance of snack habits has become most enlightening. With back-up studies in rats and hamsters, it has been shown how destructive in terms of dental caries certain food substances can be when used individually and how protective others can be in other instances. This project is continuing with pertinent combinations to further our knowledge of controlling rampant caries through diet.

The one disease in dentistry that is most neglected is aphthous stomatitis. Here is an area where patients truly suffer over long periods of time and often without let-up. This is a disease that is continually referred to the next dentist down the street. With Clinical Center facilities we have approximately 90 patients suffering with this ailment under continual observation. By calling on the skills and know-how of many disciplines, progress has been made in controlling the disease and aborting recurrent episodes of this painful disorder. With antibiotics and vaccines there is developing a basic understanding of how sensitization of an individual to a very low order of an infecting organism can lead to an acute exacerbation of the disease.

New diseases continue to be recognized. So-called Heck's disease (focal epithelial hyperplasia), although first described in the American Indian by members of our staff, has now been found in Central and South America, Alaska and in a recent migrant from Samoa. Although the viral etiology of this disease has been temporarily ruled out, the genetic aspect of it has yet to be dealt with.

The use of radioactive isotopes has become an essential tool in many of our studies.

Another bogged down area of dentistry has been the field of tooth transplantation. Although attempted for thousands of years, progress to date has not greatly improved. Although many such studies are presently going on throughout the world, the simple knowledge regarding the significance of the regeneration of cementoblasts has been continually neglected. Yet without a complete knowledge of this important cell, any hope to encourage the true reattachment and function of a periodontal membrane is handicapped.

We have been deficient in studying oral cancer in terms of experimentation. Although we can thoroughly evaluate selected patients, here again is an instance where animal back-up is necessary. A hamster study involving tobacco, gambier and calcium hydroxide in causing betal quid oral carcinoma is in progress. Although cellular atypism has been observed, no outright cancer has yet developed.

The Oral Pharyngeal Development Section continues to provide basic information to stabilize this area. Vital staining demonstrations of differential skeletal growth patterns in face

and cranium of four laboratory mammals is in progress. Analogous studies of facial and cranial skeleton continue in human subjects distorted by anomalies and/or accompanying neurological impairments.

Transducer methods of observation of tongue contacts and margin pressures have been developed with multiple-site inputs being recorded in parallel with speech and swallow. The development of oral perception in terms of form (oral stereognosis) continues to be calibrated in normal and abnormal children. Syndromes of disability of perception are being described.

Similar studies related to chemosensation (small and taste) have been adapted to children and to neurologically impaired persons. Related studies of the orofacial area of cats concerned with the interaction of sensory stimuli in association areas of the cerebral cortex are continuing.

Epidemiologic studies related to congenital malformations have been very productive. It has been established where the incidence in American Indians fits in relation to Caucasian and mongoloid groups. Biochemical studies have elicited information regarding genetic background and potential for dental caries. Also neonates metabolize different elements in different manners according to certain ethnic groups. The relationship of the effects of parathyroid hormone in various syndromes is being studied.

Unexpectedly, no increase in dental anomalies occurred in children exposed to atomic fallout although an increased incidence of thyroid nodules did occur.

In salivary studies it has been found that a polymorphism in human amylase isoenzymes exists, that different forms of the enzymes are consistently produced in particular individuals.

In cytogenetics it has been shown that phytohemagglutinin has an important action on the cellular mechanisms which regulate the lymphocytes. This system may provide a test for understanding the regulatory mechanisms which govern transcription of genetic information within the mammalian cell.

Considerable effort has been made to hold this summary within a summary down to a few pages. By being less specific in describing our studies, some brevity has been maintained but at the same time it is obvious that prejudice exists and certain emphasis has been placed where the personal experience has been greatest.

In the future, it might be recommended that every investigator provide a one-sentence summary of the meaningfulness of each of his projects.

DENTAL SERVICES BRANCH

During the past years the research programs of the Dental Institute have become more integrated with the activities of the dental clinic area. A close working relationship exists between investigative staff and the patient care dental officers in many collaborative studies. Concomitantly, our major responsibilities to provide optimum dental care for the research beneficiaries of the various categorical institutes has continued in an effective manner. The Dental Services Branch provides a complete oral examination, evaluation, consultation and dental therapy for the patients of the Clinical Center.

The above objectives are being met by the Branch's contributions to important National Institute of Dental Research programs in the various specialties in dentistry, such as: Oral surgery with general anesthesia methods, human dental pulp studies for the biologic evaluation of restorative dental procedures, alterations in the maxillo-mandibular jaw relation in the transition from natural dentition to dentures, control of rampant caries, post surgical tissue healing of impacted third molars related to periodontal involvement.

Commensurate with our responsibilities in performing optimum dental care for the 500-bed Clinical Center, the Dental Services Branch has actively collaborated with the total National Institute of Health research effort. The following examples may be cited:

National Cancer Institute

Participation in operations about the head and neck, including neck dissections. This has

been of mutual benefit to our staff and the maxillofacial surgeons. Suggestions made by dentists during surgery are of major importance to the ultimate success of the final prosthesis and rehabilitation of the oro-facial region.

During the past year an increasing number of maxillo-facial prostheses have been constructed for patients with cancer. An efficient working relationship continues between the dental clinic and the National Cancer Institute surgery staff in the management of cases surgically treated for neoplasm. Notable are those patients with paranasal sinus disease surgically approached by the combined intracranial facial procedure. A three-stage maxillofacial prosthesis is designed and fabricated for each patient.

The Branch renders further important contributions to the Leukemia Service of the National Cancer Institute in handling a myriad of oral problems associated with this disease.

National Institute of Arthritis and Metabolic Diseases

In a study of Fraction 1 of Cohn, a fibrinogen and AHG (anti-hemophilic globulin concentrate), the Dental Institute's role has been to assess the response of hemophiliac patients to multiple dental extractions. The results of these collaborative studies have been prepared for publication.

National Heart Institute

Patients with congenital defects, who are to have heart surgery, pose problems of dental management in both the pre- and post-surgical periods. Special considerations also are necessary in the performance of dental operations on patients with hypertension where fear and anxiety present particular problems. In this project considerable experience and care is required for proper dental evaluation and treatment of patients being maintained on anti-hypertensive drugs.

All patients with rheumatic heart disease present particular difficulties. As an example, in the absence of proper dental care and preparation, even such a simple procedure as oral prophylaxis can precipitate a fatal, acute bacterial endocarditis. The same holds true for patients who are to undergo or have undergone cardiac surgery, resulting in prosthetic heart valve replacement.

During this fiscal year consolidation and revision of the medical and dental records was completed. This now permits a more efficient flow of records within the clinic as well as to and from the Medical Records Department, Clinical Center. This procedure satisfies the requirements of the Medical Board, Clinical Center; Medical Records Department, Clinical Center and Dental Services Branch, NIDR.

Extensive renovation of the dental clinic was planned and instituted during this fiscal year. The major objective was to modernize the existing modules in order to provide a more efficient physical facility within the limited space available. The installation of easily maintained, bright operatories allowing the dentist and dental assistant to be seated during the four-handed dental procedures results in performing more dentistry for chronically ill patients with less time and energy expended.

The following areas have been completed or underway:

1B–04	Special Prosthetic Operatory
1B–06	Special Prosthetic Laboratory
1B–07	Oral Surgery
1B–09	Oral Surgery
1B–12	Dental Operatory

ORAL MEDICINE AND SURGERY BRANCH

As the Oral Medicine and Surgery Branch has grown, we have attempted to acquire staff that would give emphasis to all the main problem areas of dentistry. Although some areas are still understaffed because of space and recruitment problems we feel that we are finally on the way.

A brief review of the activities in the broad program of the Oral Medicine and Surgery Branch follows:

Studies of the Human Dental Pulp

In this project there is a continuing evaluation of the response of the human dental pulp to changes induced by dental drilling proce-

dures and by various restorative and related materials, such as cavity liners. This study has furnished the dental profession with some very practical information on operative procedures, particularly in regard to optimal cutting speeds, the proper use of coolants, and modifications in technic necessary for the safe placement of experimental restorative materials.

Because of the reduced inflammatory response of the pulp following high speed cutting technics, the incidence of reparative dentin production has been greatly reduced. Thus dentinal tubules remain open and permit the toxic or irritating products of sterilizing agents, cements and silicates to permeate to the pulp tissue and cause further damage. This lack of reparative dentin formation is creating a formidable problem in restorative dentistry, especially in the field of full mouth rehabilitation where often the entire coronal dentin is exposed. Experimental drugs designed to reduce sensitivity of teeth (i.e., corticosteroid compounds) and to more effectively seal the dentinal tubules are being sought, as well as drugs and technics to increase the incidence of reparative dentin formation.

When prepared cavities are washed with a steroid formula containing 1% prednisolone in a vehicle of parachlorophenol, cresatin and gum camphor, before restoration with zinc oxide and eugenol, it was found that the pulpal response to the cavity preparation was minimized about 50%. When the prednisolone was used without the vehicle, the inflammatory response was sustained only 12 days. Also when the same formula was applied to the cavity preparation several days after the full potential of the response had occurred, the resolution period was still shortened.

In an attempt to find more ideal restorative materials, collaborative research is being conducted with the Eastman Dental Center and with an industrial non-dental manufacturer who are providing adhesive filling materials which will mechanically and chemically bond with tooth structure. The biologic effects on the human dental pulp tissues of two such experimental materials are currently under investigation.

Because the Clinical Center can supply only about 300 human teeth per year for such studies, it has been necessary to supplement our needs with contract arrangements with several universities and other government facilities.

Anesthesia Studies

A collaborative study with the Anesthesiology Department of the Clinical Center on general anesthesia in ambulatory dental patients is developing important information concerning the physiological effects of various anesthetic agents and oral surgical procedures. These data provide a continuing record of pulse, blood pressure, arterial O_2 saturation, respiratory phenomena, cortical brain activity and the electrical activity of the heart. The accumulated data of over five years of study have been and will continue to be used as a baseline of comparison for the new anesthetic drugs which are being introduced for use in oral surgery. All of the agents and combinations of agents commonly used in oral surgery have been used and evaluated in this study including Fluothane, which drug is of special interest at the present time because of its alleged hepatoxicity. The incidence of this complication is extremely low, so that our failure to find any evidence of the association may be statistical rather than factual.

Among the more significant findings from a practical standpoint are:

Consistant hypertension in all ambulatory anesthesias, which directly parallels the intensity of the surgical stimulation;

Preoperative and operative tachycardias in almost 100 percent of the anesthetics (the pre-operative changes in rate being apprehensive in nature) whereas the operative changes are due primarily to the pharmacologic action of the intravenous barbiturates and secondarily, to the surgical stimulation in extremely light anesthetic planes; and

That depression of arterial oxygen saturation is a controllable factor related to anesthetic management and drug adminis-

tration (i.e., avoidance of obstructions and drug overdosage).

Since in some areas there are almost as many general anesthetics administered in dental offices as in the local hospitals, and since there are no other such studies being conducted, the basic physiological data from this study should prove important for the specialty of oral surgery.

Dental Caries

Rampant dental caries is a very severe form of disease in which practically all of the teeth are attacked by decay in a relatively short period of time. It is found chiefly in young children, but may develop in adults who previously had little or no caries experience. Under suitable experimental conditions, comparable forms of rampant caries can be developed in laboratory animals such as rats and hamsters. From a research standpoint, rampant caries offers a most favorable opportunity to study the basic factors which activate or control the caries process because the lesions is reduced to a minimum, and the determination of caries activity can be much more certain than in caries of usual severity. The purpose of this study is to evaluate in clinical studies the many factors which may be important in different cases of rampant caries, and to study the more important of these factors in laboratory and animal experiments with the goal of establishing more effective means for solution of the caries problem.

The study of new patients with rampant caries during the past year has further extended the evidence for the basic conditions underlying the development of rampant caries. For example, the social aspects of this problem were illustrated in a child with rampant caries which had developed after the family had moved to a new neighborhood where she was the only young child and the neighbors continually gave her "sweets to eat." A consistant finding in rampant caries patients has been the uncovering of some source of fermentable foodstuff above that in the average diet.

The animal experiments testing these foods shows that some are very cariogenic, many are moderately cariogenic and a few are noncariogenic to rats. During the past years experiments were also conducted to determine the extent to which the very cariogenic foods would be made less cariogenic and the noncariogenic foods such as cookies, adding water, grinding, adding sugar versus adding fluorides, and other modifications of the food.

It was found that certain noncariogenic foods such as "dog biscuit" were not made very cariogenic by even large additions of sugar, and contrariwise, some highly cariogenic foods were not made non-cariogenic by the addition of fluoride. These experiments indicate that cariogenicity is affected by several factors including chemical properties, physical properties, taste of the food, and the time it takes for it to be eaten. Several of these factors seem to be favorable in "dog biscuits," and these are being studied.

Another objective of this project is to identify and classify human oral streptococci and to determine whether or not specific strains of streptococci can be identified as etiologic agents in dental caries. The flourescent antibody technique is employed to identify these strains in histologic sections of carious teeth and in the mixed cultures of dental plaque smears.

Continued serologic screening of plaque smears from patients has indicated that certain serologic groups of streptococci are not involved in the caries process and has therefore intensified the implications of certain other serologic groups of streptococci in the caries process.

Experiments with gnotobiotic rats have indicated that some adaption of the rodent mouth, sometimes involving antigenic shift is necessary in order for these human oral streptococci to establish themselves and produce caries.

Studies of Soft Tissue Lesions

The transitional L-form of an alpha streptococcus has been isolated from oral lesions in patients with recurrent aphthae and periadenitis aphthae. This organism was consistently recoered from lesions in numerous

patients on repeated examinations over a 12-month period. Blood obtained for culture during two exacerbations in one patient was found positive for the same organism. A stable L-form was obtained from scar tissues at the site of previous lesions during a remission.

The injection of a licensed intravenous vaccine (Strep. indifferent, Lilly) into one patient with periadenitis aphthae, over a period of several months reduced the severity of the ulcers but did not prevent their recurrence completely. Therapeutic investigation is also under way on all these stubborn and resistant chronic debilitating diseases with a limited success observed with various forms of acromycin and topical steroids.

Animal studies have indicated that hypersensitivity to the antigens of the alpha streptococcus isolated from recurrent aphthae is an important factor in the development of these lesions. Positive skin tests (delayed type hypersensitivity) to these antigens are obtained in patients with aphthous stomatitis but not control individuals. The degree of the skin test reaction is directly proportionate to the severity of the disease in the patient tested.

The injection of a vaccine intravenously for 5 days prior to skin challenge reduced the size and duration of the ulcers experimentally produced in the hypersensitized and control guinea pigs. The greatest protective effect was noted in the non-sensitized (controls) guinea pigs.

Mucous Membrane Changes Associated with Age and Certain Disease. It is apparent that human buccal mucosa, although appearing clinically normal, may undergo various changes with the increasing age of the patient. Since the buccal site is frequently biopsied, standards need to be established on normal mucosa to eliminate errors in diagnosis due to the age factor.

Further work is being carried out regarding the previously undescribed "focal epithelial hyperplasia" in Indian children. Special studies including viral analyses and electron microscopy failed to reveal evidence of a viral agent. The present knowledge concerning this lesion indicates either an environmental or genetic factor is involved in the development of this lesion.

Preliminary studies using tritiated thymidine on oral mucosa involved in verrucous carcinoma have revealed epithelial turnover rates remarkably similar to those believed to be found in normal oral mucosa. Similar studies have been performed on the oral mucosa of patients with Darier's disease. Preliminary results reveal a need for further utilization of this technique in the study of normal as well as disease states involving the oral mucosa.

Clinical Periodontal Studies

Very recently a protocol for autogenous reimplantation of human teeth was approved. It is hoped that by studying the response of the periodontal tissues to tooth reimplantation that some knowledge relative to the factors permitting the differentiation of fibroblasts into cementoblasts will be obtained. After reimplantation, the teeth became firmly attached with new alveolar bone formation. In the absence of continued cemental production, periodontal fibers lose their attachment to teeth with eventual exfoliation. New functioning cementoblasts have not been found earlier than 29 days.

In the treatment of the osseous defects in periodontosis, preliminary results indicate that the autogenous transplantation of developing third molars into the first molar sites can be an effective way of inducing healing of the alveolar bony lesions and in restoring periodontal health.

Animal Periodontal Studies

Dental abnormalities were produced in Syrian hamsters by infections with H–1, OLV, Krisini and Rat Virus. The abnormalities produced were generally somewhat similar and consisted of root resorptions, misshapen roots, bony ankylosis of roots and microdontia of the third molars. The differences noted between these four viruses were: the absence of mandibular third molars in the animals infected with OLV virus, a severe inhibition of osteoblastic activity and bone formation of the mesial and mesio-lingual aspects of both maxil-

lary and mandibular first molars in animals infected with OLV and H1–virus, enabling Stenson's gland, which is a normal component of the sinus, to come into close apposition with the mesial root of the maxillary first molar.

In studying the effects of various viremias on healing wounds in adult animals, thirty five-day old male Syrian hamsters were injected intravenously with OLV infected tissue culture fluid which was obtained from rat embryo tissue cultures, at time intervals of 0 hours to 6 days following fracture of the left forearm bones (Group I) or extraction of the maxillary right second molar tooth (Group II). The intravenous injections of OLV healing when administered prior to the third postoperative day. The delay in healing was transitory, i.e., by 15 days postoperatively no alteration in healing could be discerned.

In a study comparing the healing of surgically exposed dental pulps in germfree and conventional rats, conventional animals showed an immediate, severe inflammatory response which quickly led to total pulpal necrosis; while the germfree animals, without exception, showed minimal inflammatory response (in spite of food impaction) with subsequent dentinal bridging.

Oral Pathology Investigations

In a study of hamsters' pouches to determine the individual and/or combined roles that calcium hydroxide, tobacco and gambier might play in causing betel quid induced carcinomas, it was found that the affected pouches treated with calcium hydroxide showed one or more of the following lesions: deposits of calcium, inflammation, giant cells, and fibroblastic proliferation in the lamina propria; and inflammation, ulceration, atrophy, hyperplasia, hyperkeratosis, parakeratosis, acanthosis, and cellular atypia in the epithelium.

No squamous cell carcinomas were produced in any of the groups, and no changes were noted in those cheek pouches treated with snuff or starch powder alone. Several hamsters treated with gambier developed minute ulcers with inflammation.

In determining the genetic mode of inheritance of taurodontism by means of radiographic surveys and clinical histories of the involved families, four families in which complete radiographic documentation was obtained, revealed no parent possessing the taurodont tooth form. The trait occurred in ratios of (*a*) one of three children, (*b*) one of two children, and (*c*) two of two children. Taurodontism was found in both deciduous and permanent teeth of both males and females.

The mode of inheritance of taurodontism is apparently compatible with a recessive characteristic, but our evidence is not definitive. It is not a dominant characteristic as supposed in the German literature. Histologic examination revealed normal odontoblastic and periodontal membrane activity, lending further support to the original theory that the developmental site of the taurodont tooth form is Hertwig's epithelial sheath.

In a study to classify and determine the tissue of origin of benign fibro-osseous lesions of the jaws from the vast wealth of case material in the files of the AFIP, the following information has been derived:

Ossifying fibroma, cementifying fibroma, and cementoossifying fibroma appear to arise predominantly from the periodontal membrane but they can also arise from the medullary bone.

Oxytalan fibers may occur in most benign fibro-osseous lesions of the jaws, regardless of their tissue of origin, provided that mature collagen fibers are also present in the lesion.

Inasmuch as oxytalan fibers and pre-elastic fibers cannot be distinguished with present histochemical methods, the demonstration of fibrous elements stained with the oxytalan fiber method does not constitute conclusive evidence of odontogenic origin of the tumor.

The birefringence pattern under polarized light does serve as an excellent differential for diagnosis. Fibrous dysplasia gives a random irregular birefringence, indicative of woven bones, whereas, the other fibro-osseous lesions manifest birefringence as parallel light and dark bands, indicative of the varying degrees of lamellar bone formation.

In a study to define the behavioral patterns of osteosarcoma and chondrosarcoma of the jaws, the following findings have been derived:

There is no apparent racial or sex predilection but females appear far more likely to have mandibular tumors than maxillary ones, whereas males are about equally divided between maxillary and mandibular tumors. Radical resection appears to offer the best hope for cure. Tumors in the mandibular symphysis area are almost amenable to cure; those in the maxillary sinus are least amenable. The average age of occurrence for osteosarcomas of the jaw is about a decade older than for osteosarcomas of the jaws, but there is a quite wide range, and the range is wider for the mandible than for the maxilla. Early findings are quite variable and often non-specific. Early diagnosis depends upon correlation of clinical behavior, histologic appearance, and roentgenographic appearance, any one of which may be deceivingly benign appearing. An important early finding may be roentgenographic evidence of symmetrical widening of the periodontal membrane space, with maintenance of an undisturbed lamina dura radiopacity.

Oral and Pharyngeal Development Section

The Section has continued in basic experimentation and clinical application studies of the structure and the motor performance of the mouth and pharynx. *Basic anatomical studies* include: (1) vital staining demonstration of differential skeletal growth patterns in face and cranium of four laboratory mannals, (2) detailed development patterns, in separate items of skeleton cultured *in vitro*, (3) sequence of histological demonstration of responses of bone to deforming forces.

In the human, changes have been described in spatial relation to nasal septem and base of cranium during postnatal development.

Analogous studies of facial and cranial skeleton have continued in subjects distorted by anomalies and/or neurological impairments. These have included standard methods of radiological cephalometry and also adaptations of laminography which demonstrate the mid-line structures. In clinical application, evaluations of the basic orthodontic methods of tooth displacement have been performed, using precision techniques of portrayal of spatial changes of teeth.

Basic physiological studies include: (1) elicitation of varied patterns of cry and related arousal response by electrodes placed stereotaxically in the brain stem of cat, and the demonstration of action details by correlated pressure recording, sound recording, laryngeal photography and regional cineradiography; (2) neurophysiological studies of patterns of representation of sensation in the trigeminal nucleus, mechanisms of integration of afferent information in the cerebral cortex, and (4) patterns of respiratory motor response to stimulation in the pharyngeal area.

Studies of upper respiratory and feeding functions have continued in an increasing number and variety of subjects impaired by anomaly, such as the Pierre Robin syndrome. The methods of cineradiography, cinephotography, respiratory displacement, and sound recording and spectrographic display have been utilized in study of selected patients having cleft palate and other pharyngeal and oral anomalies. Transducer methods of observation of tongue contacts and margin pressures have been developed; multiple-site inputs are recorded in parallel with speech and swallow indications. By these methods, we have come to re-definition of their disabilities in terms of their deficiencies and compensations of pharyngeal actions, rather than the overt deficiency of their palate and related structures. These demonstration methods have also been extended to subjects having neurological impairments of the oral and pharyngeal area, and initial descriptions have been made of the particular distortions of feeding, or pharyngeal airway maintenance and of vocalization in subjects having spastic dyskinesia, athetosis, lower motor neuron disorders, and regional kinesthetic sensory disorders.

Clinical experimental methods have been developed of evaluation of oral sensation and perception, and the development of oral perception of form (oral stereognosis) has been calibrated in normal children. Syndromes of disability of perception have now been de-

scribed in dysarthric and orally dysphagic subjects.

Analagously, studies of the chemosensations: smell and taste, have been adapted to children and to neurologically impaired persons. First demonstrations have been made of disabilities of these special senses in facially deformed children.

In a study on cats concerned with the interaction of sensory stimuli in association areas of the cerebral cortex, it has been found when peripheral stimuli were employed (light flash, auditory click, and forepaw shock) most cells in anterior lateral gyrus exhibited preferential responsiveness to auditory click stimulation. This preferential responsiveness, however, was dependent upon the types of stimuli employed; when cells were activated by electrical stimulation of optic and auditory nerves and the dorsal column of the spinal cord, equal responses to the stimuli were most prevalent. A comparison of response characteristics in anterior lateral and anterior middle suprasylvian gyri revealed that responses to sensory stimuli were not identical in these two association areas, irrespective of the types of stimuli.

In a study on the functional organization of the trigeminal brainstem in the cat, it was found that the chief sensory nucleus of the tirgeminal nerve contains cells with different response characteristics. Almost all cells studied to this date had small receptive fields in the oro-facial area and exhibited a short latency (1–5 msec) response to electrical stimulation of this field. The modifying influences upon these cells by peripheral and central inputs can be separated into three groups: (1) cells whose activity was not modified by cortical, thalamic, or light flash stimulation; (2) cells whose activity was modified by cortical stimulation and orthodromic thalamic stimulation; and (3) cells whose activity was modified by both peripheral and central inputs. Very few cells were activated antidromically by stimulation of the contralateral arcuate nucleus. Histological analysis of electrode track placements indicates a more medial location of cells whose activity was modified by peripheral or central input, or both.

HUMAN GENETICS BRANCH

Congenital Malformations

One of the major efforts of the Human Genetics Branch has been the establishment of a data retrieval system for research on congenital malformations among all American Indian neonates born in PHS facilities. To date, we have completed approximately 10,000 birth records of the Indians born during the last year and a half. These data have yielded some unexpected results:

Only 5.1 percent of all Indian births are premature compared to 70 percent for white and 9.7 percent for non-whites.

Indian stillbirths were 4/1000 births compared to 13.7/1000 U.S. whites and 26.7 1000 for non-whites. Indian neonatal death rates are 13/1000 births compared to 16.7/1000 for white and 26.1/1000 for non-whites.

Major congenital malformations were 15/1000 births and minor malformations were 34/100 births.

Approximately 32/1000 births or 3.2 percent Indian children either die in the neonatal period or have major malformations. When corrected for underreporting, this is about the same value obtained in Japanese infants (4.3 percent).

The data suggest that the malformation pattern is intermediate between Caucasian and mongoloid patterns.

Study of specific malformations among Brazilian Indians indicates a striking absence of malocculusion in primitive groups, i.e., 5 percent compared to approximately 50 percent for a comparable group derived from the same tribal stock who have been in contact with civilization for only 20–30 years.

A newly described benign oral lesion, focal epithelial hyperplasia, was encountered only among persons of Indian ancestry in North America, Guatemala, El Salvador and Brazil.

Studies of cleft lip and palate children and their families indicate that, among the relatives of children with oral clefts, 27 percent have a hearing loss of 20 db or more at 500, 1000, 2000 or 4000 cps, compared to only 13 percent among control proband relatives. Other parameters such as visual acuity, minor

anomalies (possibly indication of a cleft palate habitus) did not show a significant difference between the two groups. This would indicate that there are familial factors (possibly genetic) operating to cause a local (first arch syndrome) disturbance rather than a general disturbance in development as regards to oral clefts.

A population of 4200 Indians in North Carolina have been exhaustively examined for clefts of lip and palate and 42 cases have been found which makes this frequency the highest known in man. A number of other congenital anomalies appear to be associated with this type of defect in this population which may help define a specific form of the disease.

One of the major undertakings of the branch was the preparation of a chapter on malformations of the head and neck for a Handbook of Congenital Malformations for the National Foundation.

Genetic Factors in Dental Caries

In the past, two major factors (fluoride and refined carbohydrates) have been shown to consistently influence the amount of dental caries in a population. A third consistent relationship has been established between the inherited ability to taste phenylthiocarbamide and dental caries, such that tasters for this substance have from 28 to 40 percent lower def rates than nontasters. This finding was verified in a number of studies. Investigation of the thiourea concentration in saliva indicates this was not the basis for the caries difference observed.

Biochemical Genetic Defects

Investigations of biochemical genetic defects in families and populations indicated that Indian infants do not metabolize bilirubin in the same manner as Caucasian neonates. They have higher and more prolonged neonatal levels of unconjugated bilirubin, which apparently is normal for children in these ethnic groups. Thus indications for exchange transfusion are different in Indian neonates than they are in Caucasian neonates.

Investigation of Gardner's syndrome which consists of multiple odentomas, multiple osteomas of the jaw, multiple polyps of the intestine, and subcutaneous tumors which are inherited revealed that these patients are refractive to the effects of parathyroid hormone. Thus, it is possible that a group of diseases including pseudohypoparathyroidism, basal cell nevus-jaw cysts syndrome, and Gardner's syndrome, share some defect in parathyroid hormone utilization. Other diseases with similar signs and symptoms are being investigated for this biochemical abnormality.

Genetic studies of inherited sensory traits indicate that the inability to smell potassium cyanide is a polymorphism and a recessively inherited trait.

Effects of Radiation Exposure

Children in three Utah communities were investigated for possible effects of atomic fallout. Study of dental hypoplasias, abnormalities of pigmentation, taste and smell ability, did not reveal any detectable difference between the exposed and unexposed communities. Seventy of 2,000 children in Washington County (the exposed community) and 25 of 14,000 children in Graham County (the control community) were thought to have nodular growths by at least one examining physician. Studies of a sample of these children are being conducted by the University of Utah Medical Center.

Biochemistry of Normal Variation and Cellular Biology

Saliva studies have yielded a major finding concerning amylase isoenzymes in humans. A polymorphism in man has been defined such that different forms of the enzyme are consistently produced in a particular individual and that a generic difference exists in parotid amylase between man and rat.

A discrepancy in the classical method for determining secretor status was found and tested on 2,875 individuals, which indicates that approximately 6½ percent more aberrant secretor individuals can be detected by this new method.

SECTION ON CELLULAR BIOLOGY AND CYTOGENETICS

The section on cellular biology and cytogenetics is conducting research into the translation, transcription and replication of genetic information at the cellular level. The translation of genetic information into cellular products is being investigated through studies of salivary enzyme polymorphism. A polymorphism among humans has been defined in the salivary amylase isoenzymes. That the composition of these isoenzymes is under general genetic control has been demonstrated by showing a generic difference in these isoamylases.

Studies relating to the transcription of genetic information and its regulation within the cell are being conducted, using the lymphocyte-phytohemagglutinin system. In this system, non-dividing lymphocytes are stimulated to enlarge and divide in vitro by treatment with phytohemagglutinin extracted from kidney beans.

Studies of RNA metabolism of human lymphocytes stimulated with phytohemagglutinin indicate that the effect of this extract is to produce a synthesis of non-ribosomal RNA whereas stimulation by specific antigens (streptolysin O or tuberculin) results in cell growth with predominant synthesis of ribosomal RNA. The characteristics of non-ribosomal RNA are like those of messenger RNA. If this is messenger RNA, it implies that PHA has an important action on the cellular mechanisms which regulate the synthesis of messenger RNA. This system, therefore, may provide a tool for understanding the regulatory mechanisms which govern transcription of genetic information within the mammalian cell. Malignant lymphoma cells resemble antigen-stimulated lymphocytes more closely as regards RNA metabolism than they do PHA stimulated lymphocytes. This may reflect the fact that the lymphoma cells still have greater control over their messenger RNA synthesis than the PHA stimulated lymphocytes have. This information is important for an understanding not only of the control of cell growth but, also, of cell differentiation.

Future Studies

Present plans are to pursue the study of congenital malformations with particular reference to oral and facial clefts, the genetics of various sensory mechanisms affecting taste, small and hearing, the biochemistry of saliva as it relates to PTC taste ability and caries immunity and the regulatory mechanisms of cell growth and differentiation.

NATIONAL INSTITUTE OF MENTAL HEALTH

INTRODUCTION

During the year just ended the NIMH Intramural program has had another very productive year, as evidenced by the summary reports prepared by the laboratory and branch chiefs in the pages which follow. Despite chronic crowding in every laboratory, the important questions have been vigorously pursued, and there is every reason for pride in the accomplishments of the dedicated members of the Intramural staff. As has been true for several years, this year again saw our scientific staff remain intact. No major investigators left, despite numerous invitations from universities at generally much higher salaries.

It is a pleasure to mention the appointment of two new Laboratory Chiefs during the year. Dr. Gian C. Salmoiraghi was appointed Acting Chief of the CNRC (soon to be made permanent), succeeding Dr. Joel Elkes, and Dr. Ichiji Tasaki was appointed Chief of the Laboratory of Neurobiology, succeeding Dr. Robert Livingston. Both are old friends and long-term members of the staff. We are grateful to them for undertaking the greater responsibilities of chief of a laboratory.

We expect our most serious crowding to be relieved by the new space that should be available in fiscal 1968. Construction is ahead of schedule on Building 36, the basic research building which we are to share with the NINDB, and if the pace continues it may be completed in November, 1967. We anticipate completion of Buildings 110 and 111, the two laboratory buildings at the NIH Farm, also in November, 1967, and have just let a contract (June, 1966) for construction of a temporary building to house Dr. Calhoun's rat population studies at the Farm. It should be completed by late fall of 1966. Further delays about site have dogged the progress of the new building for the Child Research Branch, and the prediction now is for completion in May, 1968. A site has finally been approved at the southwest corner of the NIH campus, with an access road for subject families directly into the community, while access to the rest of the campus is to be by another, non-connecting road. It promises to be a good location in most of the important ways.

The year past saw a tentative conclusion to the process of planning for use of the new space to be made available by completion of Building 36. Existing plans called for moving to that building the Laboratories of Neurophysiology, General and Comparative Biochemistry, Neurochemistry, and Neurobiology, and the Section on Technical Development. To those units we have now added the Sections on Cerebral Metabolism and Biochemistry of the Laboratory of Clinical Science, a new section or laboratory on neuroanatomy, the office of the Associate Director, and an Associate Director's reserve of some 3,000 square feet. The latter is intended for use by assignment to investigators in amounts needed for specific projects over limited periods of time. Sceptics feel that once in occupancy an investigator can never be uprooted, and that such a reserve will soon be dissipated. I trust that this pessimistic prediction will turn out to be in error.

Building 10, after the above moves, will then house all of the research in Bethesda (except that of the Child Research Branch) which involves the study of patients or of other human subjects. Included will be the Adult Psychiatry Branch, the Socio-environmental Studies Laboratory, most of the Psychology Laboratory, the clinical half of the Clinical Science Laboratory, the Office of the Director of Clinical Investigations, and a proposed new section on psychopharmacology. The latter term is used to refer to research which relates pharmacol-

ogy-biochemistry to behavior and mental process.

We had hoped that the accession of 37,000 square feet of new space would make possible a greater variety of new programs than the above represents. That it did not is testimony to the crowding which now exists and to the too long put off provision of adequate facilities for the able senior investigators on our staff. Their needs must get priority over new programs.

During most of the past year, because of the difficulties in scheduling, no member of the staff was on work assignment abroad.

The NIMH Board of Scientific Counselors met twice during the year. On November 4 and 5, 1965, two half-days were spent in a review of several of the major clinical research programs of the adult Psychiatry Branch and the Laboratory of Clinical Science. From the former, Dr. Wynne reviewed the Branch program and described his own work on schizophrenia and family interaction; Dr. Pollin reported on his study of schizophrenic twins and siblings; Dr. Shapiro described his work with the families of disturbed adolescents; and Dr. Bunney summarized his research on depression. From the Laboratory of Clinical Science, Dr. Durell outlined his study of families of psychotic patients in milieu therapy. On March 10 and 11, 1966, the first day was largely devoted to a review by Dr. Dan Bradley and the members of his staff of the research program of the Section on Physical Chemistry of the Laboratory of Neurochemistry. In addition to Dr. Bradley, the others participating were Drs. DeVoe, Merril, Nash, Neville, Small, and Stone. On the second day the Board and Laboratory Chiefs traveled by bus to the NIH Farm at Poolesville to hear a review of plans for the research in brain and behavior scheduled for the Farm, and to see the terrain and present facilities of the Laboratory Aids Branch. Drs. Calhoun, Stanley, and Eberhart took part. Dr. MacLean was scheduled to participate but a death in his family prevented his attending. Dr. Robert Morison presided in the absence of the Chairman, Dr. S. Bernard Wortis.

The Intramural staff were saddened in March to learn of the death of Dr. Heinrich Waelsch, a valued friend and adviser and a member of the Board of Scientific Counselors since 1964. His wisdom, warmth, and unfailing helpfulness will be very much missed.

CLINICAL INVESTIGATIONS

If one were to write a job description for a research director, there are three qualities which might be considered as among those which are essential: he should be able to recognize potential creativity in investigators; he should muster the supports which promote the actualization of this potential; and he should worry—by that I mean that he should have a continuing concern about the program as an organism greater than the sum of its individual parts. This latter area is obscure and ill-defined, yet I am certain that it exerts an important influence on the work carried out by the individual investigators.

One cannot help but be impressed by the range and excellence of the studies described in the following pages. If one measures success by the esteem of professional colleagues, it is worth noting that each year has brought to members of our staff some of the highest awards offered by the various profssional associations to which they belong—distinctions in which each branch and laboratory has shared; for example, of the six Salmon lectures which will have been given between 1961 and 1966, three of the lecturers were invited while they were members of the NIMH staff. It is not an exaggeration to say that our participation is sought in every important national or international conference in those areas in which we are doing research. Staff members are repeatedly offered professorships in outstanding universities. It is doubly gratifying that much of this represents recognition for work conceived and carried out here by investigators who established themselves as important figures in their respective fields after they came to the National Institute of Mental Health. I cannot look back to the early days of the program without some sense of chagrin, thinking of how eagerly and persistently we sought to recruit a larger number of established and widely-recognized research authorities, and of how frustrated we felt at our failure to enlist

their interest. It does those highly-respected men no discredit to say that this failure was the best thing that could have happened. It forced us to give more substantial and long-term support to a group of younger investigators who have proved to be so outstandingly productive that they have made us look very good indeed, and much wiser than we, in fact, were.

In the face of this undoubted success, is it at all reasonable to find that one does have some worry? I find that I do, and yet the fact that the current system works so well makes me diffident and hesitant about expressing any degree of concern. For whatever the observation may be worth, however, it seems to me that our program specifically, and perhaps medical research generally, appears at times to be in a pressure-cooker. Everything is heated up and speeded up, there is a certain air of fierce determination and relentless forward movement which would make a slave-driver feel useless and unnecessary. I cannot say that I identify any bad effects from this; an amazingly large number of the staff want more space and more support so that they may do more work. The work that is done is neither shallow nor incomplete; conclusions are amply documented. When people leave it is often because they are offered even more resources than we can make available to them. I realize that in coming to NIMH in 1952, I had in my mind's eye returning to the cloistered atmosphere of the Department of Physiology at the University of Chicago twenty years earlier—it did not take long to realize that the dead past is indeed dead. It may exist in the individual laboratories, but I find myself somewhat frustrated at the lack of any sense of leisure in the program as a whole, at the relatively infrequent opportunities for contact between members of the various disciplines in which ideas may be casually exchanged, at the fact that it is necessary to make an appointment days in advance to see almost anyone. I shall have to offer it as only an intuitive guess that if we could find a mechanism which would result in some slowing down and in providing occasions for increased casual but interested contact between the several laboratories and branches, our work would be no less thorough and just possibly more rewarding. I realize that many things seem to work against this; the sheer size of the program for one; the factory-like architecture of the Clinical Center with its lack of common rooms and its emphasis on what is utilitarian for another; and the understandable desire to participate in the increasingly large number of national and international meetings for yet another. But I believe that an effort to bring about some such change is worth making.

In my opinion, the growth in scientific stature of our relatively young staff is a bit of history which merits serious thought. The success the program has enjoyed is the result of the fact that these men and women were given career-type support and a level of research resources which they would not have been granted so soon at a university or by us if we had succeeded in recruiting more senior men. They were freed from the burden of developing a series of individual project applications which would have had to earn the approval of a variegated review group. Although there were some critical pressures on them from other investigators (for the most part silent) and although their promotions but not their levels of support depended on some evidence of productivity, by far the major pressures were self-generated and led them to develop increasingly more significant and powerful studies. If our experience can be generalized, granting agencies might seriously consider the advisability of decreasing project-type and increasing program-type support for young men and reversing that procedure for established figures. This, in effect, is what happens in the intramural program; whenever a branch or laboratory chief wishes to change his research without discontinuing his current studies, his request for new resources is by necessity rigorously reviewed since he is always competing with others for very limited reserve funds.

The past year has brought a major change in direction of the program of the Clinical Neuropharmacology Research Center, which had been established in collaboration with Saint Elizabeths Hospital in 1957. As originally conceived by its first Chief, Dr. Joel Elkes, and

as its name implies, its aim was to conduct both clinical and laboratory studies in a large mental hospital setting—studies which, it was hoped, would prove mutually enriching to the clinician concerned primarily with his patient's behavior and to the laboratory scientist concerned with behavior at the segmental or even the cellular level. Several years ago Saint Elizabeths was granted direct research funds by the Congress for the establishment of a Clinical and Behavioral Studies Center which, to a degree, complemented the work of the Clinical Neuropharmacology Research Center. After considerable thought, it was decided that the total collaborative research program could best be developed if Saint Elizabeths Hospital were to assume all responsibility for clinical care, and if the energies of the Clinical Neuropharmacology Research Center staff were to be directed primarily toward the study of fundamental neurophysiological and neurochemical processes involved in behavior. Dr. C. G. Salmoiraghi, Chief of the Clinical Neuropharmacology Research Center, was appointed Director of Research for Saint Elizabeths Hospital by Dr. Cameron; Dr. Francis N. Waldrop, Director of Training and also Director of the Clinical and Behavioral Studies Center of Saint Elizabeths Hospital was appointed Special Assistant for Research and Training to the Clinical Neuropharmacology Research Center, NIMH, by Dr. Yolles. This new plan places responsibility where there is the greatest concentration of competence, and we look forward to an even more productive and cooperative relationship than we have enjoyed in the past.

There has been another development in NIMH indirectly related to the intramural program. This is the establishment of a Field Studies Program Branch under the direction of Dr. James Osberg, formerly Chief of the Mental Health Study Center. The Study Center will be transferred to the new program, which will be concerned with operational research in the increasingly broad areas of responsibility with which the Institute has been charged. This new development is gratifying on two counts: it recognizes once more the importance to the Institute of maintaining a strong program of basic research free from the many practical problems pressing urgently for investigation and solution. At the same time, it provides another research program within the Institute with which some of our investigators may collaborate from time to time. The possibility that staff might wish to move in either direction from one program to the other on occasion increases the richness of our research resources.

ADDICTION RESEARCH CENTER

General

The past year has been an especially productive one at the Addiction Research Center, with real progress being made in several important areas of drug abuse and drug dependence. Important advances have been made in the understanding of some of the basic pathological processes that occur in narcotic addiction, as well as advances in the mode of treatment of these defects. Additional evidence has been acquired indicating that signs of abstinence in the rat can be conditioned. Further, strong supporting evidence has been obtained concerning the existence of protracted abstinence. Protracted abstinence can at least be partially characterized as an appetitive state in which there is an increased consumption of food, water, and narcotics. Preliminary studies have indicated that there may also be biochemical alterations in the brain. The brains of addict rats that have been withdrawn for six months have larger quantities of brain norepinephrine than comparable control animals. During abstinence in man the respiratory center becomes hypersensitive to carbon dioxide, and this observation may provide a precise and sensitive measure of a basic pathophysiological alteration produced by addiction.

Studies showing that the effects of narcotics can be effectively blocked by chronic administration of the narcotic antagonist cyclazocine have been concluded. Cyclazocine is currently undergoing clinical trial in the treatment of drug addiction in New York City, and the preliminary observations seem encouraging. They have confirmed the basic pharmacologic observations made at the Addiction Research Center, namely, that the subjective

and dependence producing effects of narcotics are diminished, and in most instances completely antagonized when cyclazocine is taken daily.

Studies of derivatives from cannabis are continuing. Several isomers of tetrahydrocannabinol isolated and furnished by Prof. Dr. F. Korte of the Organic Chemical Institute, University of Bonn, Germany, have been studied.

Studies on the measurement of the relative strength and duration of sedative-hypnotic agents have also been continued. It has been shown, for example, that both meprobamate and chlordiazepoxide, like pentobarbital, can prolong the duration and increase the frequency of postrotational nystagmus. It is hoped that this method will permit the quantitative study of these types of agents.

Studies on the treatment of addiction to sedative-hypnotics have revealed that chlorpromazine does not effectively antagonize the very serious abstinence sign, convulsions.

A number of drug state and personality scales have been developed, using the Addiction Research Center Inventory, which are proving to be of great value. Since many of these scales were developed using prisoner subjects, an extensive effort has been made to standardize this inventory on other populations. Thus far, studies have been made of over 100 mentally ill subjects (tested by R. Long and P. Philip, St. Peters State Hospital, St. Peters, Minn.), normal subjects (college students tested by S. Grupp, Illinois State University, Normal, Ill., and W. Johnson of Morningside College, Sioux City, Iowa), alcoholics (G. Fuller, Willmar State Hospital, Willmar, Minn.), and criminals (J. Panton, Central Prison, Raleigh, N.C.). In this regard, an alcohol withdrawal scale has been developed. In state dependent studies, the observation of Overton that rats make differential responses on the basis of prior injection of pentobarbital has been confirmed; however, under morphine there is almost complete response fixation.

Fundamental to our understanding of problems of addiction is the mode of action of addicting drugs, for drugs produce their actions by interacting with other molecules of the body. It has recently been demonstrated that narcotics alter phospholipid metabolism. These results may be of great importance since there are indications that neurohumors may have their effects upon lipid membranes of excitable tissues. Further, it has been demonstrated that phenobarbital can alter phospholipid and microsomal membrane metabolism *in vitro*.

Studies of the impact of addictive processes on the social functioning of the individual is one of our most important concerns, and yet one of the areas in which it is most difficult to conduct well-controlled experiments. Important advances in this problem have been made using follow-up studies of addict patients released from the Public Health Service Hospital. One of these, the Kentucky follow-up study, is nearing completion and a most significant and important observation has been made—namely, the first documentation of the fact that addiction is followed by the deterioration in major social roles. Thus employment is less stable, most marriages end in divorce or separation and subjects become more deeply involved in criminal behavior.

Administrative

Surgeon D. R. Jasinski came on duty as medical officer on 1 July 1965. Surgeon C. W. Gorodetzky, medical officer, was assigned to the Department of Pharmacology, University of Kentucky, for out-of-Service training as of 1 September 1965. It is hoped that this will be the beginning of a joint training effort by the University of Kentucky College of Medicine and the Addiction Research Center which will, hopefully, lead to the development of both clinical and basic investigators who have a high level of interest in problems of drug abuse and who will develop expertise in this area.

Surgeon D. C. Kay, a Mental Health Career Development officer, has spent two-fifths of his time during fiscal year 1966 in the Addiction Research Center and will be assigned to the ARC on a full-time basis as of 1 July 1966.

Dr. C. M. Redman, chemist, joined the staff of the ARC as a staff fellow on 1 October 1965. Dr. W. M. Bates transferred from the ARC to the PHS Hospital, Fort Worth, Tex., where he accepted a position as director of research.

For all intents and purposes the new Basic Research Laboratory is complete. It was approved for beneficial occupancy 21 September 1965 and is now almost completely occupied. An occupancy inspection of the building has been made and, although there are a number of minor items to be corrected, it is hoped the contractor will have the building in shape for final inspection by May.

Studies on Addictive Properties of New Analgesics and Patho-Physiological Processes Associated with Physical Dependence

Studies of the ability of chronic administration of the narcotic antagonist cyclazocine to block the euphorogenic, lethal and physical dependence producing properties of narcotics have been completed. It has been clearly demonstrated that cyclazocine can be administered in sufficient doses (4 to 8 mg/day) to protect subjects from narcotics without the production of undesirable side effects. Two clinical trials of cyclazocine for the treatment of narcotic addicts are currently being undertaken in New York City. At the present time the results of these clinical trials are definitely encouraging.

Naloxone (N-allyl-noroxymorphone) has been studied for its abuse potentiality. This interesting antagonist appears to be entirely devoid of agonistic action. Thus, unlike nalorphine and cyclazocine, naloxone does not produce dysphoria, ataxia, uncontrolled thoughts or other subjective effects, nor does it produce pupillary constriction or respiratory depression. On the other hand, studies of this agent's ability to precipitate abstinence indicate that it is probably at least six times more potent than nalorphine as an antagonist.

It has been demonstrated that in patients physically dependent on 240 mg of morphine per day and who are mildly to moderately abstinent, the respiratory center becomes hypersensitive to carbon dioxide. Thus, the slope of the alveolar carbon dioxide-minute volume stimulus response curve is almost twice as steep in the abstinent physically-dependent subjects as it is in the same subjects before they were made dependent. It thus appears that the abstinence sign hyperpnea can be explained on the basis of increased sensitivity of the respiratory center.

The finding that pentobarbital prolongs postrotational nystagmus has been confirmed in studies, using electro-oculography, and that this prolongation is proportional to the dose of pentobarbital. Further, it has been found that the frequency of postrotational nystagmus is also increased by pentobarbital and that this increase is also proportional to the dose administered. Studies concerned with the mechanism of prolongation of post-rotational nystagmus indicate that this is mediated in part by decreasing the ability of subjects to attend.

Studies on the addiction liability of alpha *d*-2-acetoxy-1,2-diphenyl-3-methyl-4-pyrrolidino butane hydrochloride (Lilly 31518, ARC I–C–27) have been completed, and indicate that I–C–27 is a typically opiate-like drug that is between one-half and one-fifth as potent as morphine.

The effects of intramuscularly administered codeine (90, 180 and 360 mg) and morphine (7.5, 15 and 30 mg) have been compared on pupillary diameter as well as subjective effects as assessed by the single dose questionnaires. By all measures, codeine appears to be approximately one-tenth to one-fifteenth as potent as morphine. Of fundamental importance is the observation that the effects of codeine increase up to the dose level of 360 mg. This observation confirms Houde's findings, but is in disagreement with previous observations which have indicated that the effects of codeine reach a plateau at a much lower dose level. Codeine may have a slightly more rapid onset of action and shorter duration of action than morphine, but these data are not clear-cut.

Dihydrocodeinone - O - (carboxymethyl) - oxime dihydrate (codoxime, ARC I–A–41, WSM–7051), an agent that is being considered for use as an antitussive, has been assessed for abuse potentiality and compared with hydrocodone. The results indicate that both codoxime and hydrocodone possess typically morphine-like properties. Codoxime is one-seventh to one-fifteenth as potent as morphine; whereas, hydrocodone is approximately

equipotent. Both drugs have time-action courses that are similar to that of morphine.

The effects of morphine (12 and 24 mg/70 kg) and pentobarbital (50, 150, 250, and 300 mg/70 kg) were compared, using single dose questionnaires and a recently designed questionnaire containing ten items from the morphine-benzedrine group scale and ten items from the pentobarbital-alcohol-chlorpromazine scale. The scores obtained with 150 mg of pentobarbital were equal to those obtained by 24 mg of morphine; however, scores were not increased by larger doses of pentobarbital. Responses on this scale to morphine were, however, dose-related. On the other hand, pentobarbital produced increasing responses on the chlorpromazine - alcohol - pentobarbital scale with increasing doses; whereas, responses under the morphine condition were only slightly greater than placebo responses.

Acute and Chronic Intoxication with Drugs Other than Analgesics, Barbiturates and Alcohol

Studies in the Marihuana Group

We are now collaborating with two groups in the study of effects of compounds isolated from marihuana or of synthetic materials chemically resembling compounds found in marihuana. The two laboratories are the Organic Chemical Institute of the University of Bonn, Germany (Dr. F. Korte), and the Psychopharmacology Service Center. We have previously shown that tetrahydrocannabinol isolated from hashish is an active material which causes subjective symptoms recognized as being similar to those of marihuana by experienced marihuana smokers. The activity of crude extracts of hashish or marihuana correlates only with the tetrahydrocannabinol content. The exact chemical structure of tetrahydrocannabinol has, up until recently, not been known, but it seems likely that the exact structure of natural tetrahydrocannabinol has now been determined and supplies of synthetic materials will soon be available. Studies on two synthetic samples of tetrahydrocannabinol showed that both are relatively inert, as was a sample of synthetic cannabidiol. Natural tetrahydrocannabinol is about 2.5 times as active when smoked as when taken orally. In sufficient dose (200 to 250 mcg/kg smoked), natural tetrahydrocannabinol is a psychotomimetic drug. Smoking can be used as an assay method in man.

It now seems quite likely that the exact chemical structure of natural tetrahydrocannabinol has been determined and that a chemist will soon synthesize the active materials in quantity. If this is achieved a cooperative detailed study of the general pharmacology, neuropharmacology and psychopharmacology of tetrahydrocannabinol will become possible. Since marihuana is one of the most widely used intoxicants of the world, and since the United States has a considerable problem of abuse of marihuana, the importance of such studies is evident. The role of the Addiction Research Center in these investigations might include a study of chronic intoxication with the natural tetrahydrocannabinol, including determinations of the degree of tolerance developed, a more complete delineation of the psychopharmacological effects in man, and studies of possible potentiating agents other than tetrahydrocannabinol *per se*.

Clinical Studies of Intoxication with Alcohol, Barbiturates and Related Drugs

During the current year studies on the subjective changes associated with alcohol withdrawal were completed. Scales for alcohol withdrawal have been developed with the Addiction Research Center Inventory (ARCI). These scales may be useful for following the course of withdrawal, evaluating the efficacy of treatment of withdrawal, and, finally, from a methodological point of view, to determine when personality tests may be administered so that the results will not be contaminated by withdrawal. Severity of withdrawal symptoms as measured by the withdrawal scale is correlated with subjective need for alcohol or barbiturates, course of treatment, occurrence of confusion and hallucinations during the last alcoholic spree, and time since the last alcoholic spree, but not with the use of specific alcoholic beverages such as beer, wine or whiskey during the last

spree, length of alcoholism and amount drunk, or membership in AA.

A psychopathic scale developed from the ARCI highly differentiates criminals and opiate addicts from normal and mentally ill subjects, and is judged to be better than other available tests for this purpose. Alcoholics obtained intermediate scores on this scale. The scale is significantly correlated with several measures of psychopathic deviation on the MMPI and the California Psychological Inventory.

Two editions of the 16 Personality Factor Questionnaire were studied to determine their equivalence. Certain scales on these versions of the questionnaire were found to be nonequivalent. One scale from the 1950 edition indicated that addicts are high in ego strength, whereas the equivalent scale on the 1956 edition indicated that addicts are low in ego strength. This study clearly indicates that caution should be exercised in generalizing about the true validity of scales that have face validity.

Biochemistry of Addiction

The effects of chronic morphine treatment, as well as primary and protracted abstinence from morphine, on urinary epinephrine, norepinephrine and dopamine, food and water intake, urinary output, body weight, and temperature were studied in the rat. In addition, the weight and catecholamine levels of brain and adrenal glands were determined 143 days after the last injection of morphine. During addiction the mean excretion of urinary catecholamines was significantly higher in the addicted animals than in the control animals. Following abrupt withdrawal of morphine, epinephrine excretion increased and reached the maximum within 48 hours, a time at which dopamine values were also at their peak. Norepinephrine excretion became pronounced on the third day of abstinence. By the end of two weeks, urinary catecholamine values were normal or subnormal. Epinephrine and dopamine excretion in the abstinent rats did not vary significantly from control values throughout protracted abstinence; whereas, urinary norepinephrine levels fell below control levels and remained subnormal. During protracted abstinence rats ate and drank more than comparable control groups. These and other physiological abnormalities were clearly evident during the first half of the protracted abstinence; however, all measures were normal at the time of sacrifice. No significant difference was observed between brain and adrenal weights and brain epinephrine levels of the control and addict rats. However, the brain concentrations of norepinephrine were significantly higher and the concentrations of brain dopamine were significantly lower in addict rats than in control rats. Adrenal epinephrine and norepinephrine tended to increase but these changes were not statistically significant.

Neurophysiology and Neuropharmacology of Chronic Intoxication with Barbiturates and Related Drugs

The Effects of Chlorpromazine on Barbiturate Withdrawal Seizures

Dogs chronically intoxicated with and addicted to sodium barbital were given chlorpromazine following abrupt withdrawal of barbiturates. Both the chlorpromazine-treated and control dogs developed abstinence convulsions, as well as other signs of barbiturate withdrawal. It is concluded that chlorpromazine is not a useful therapeutic adjunct in the treatment of the serious sign of barbiturate withdrawal, major seizures.

Barbiturate Withdrawal in Adrenalectomized and Hypophysectomized Rats

Wistar rats were adrenalectomized and maintained on 1 mg daily doses of deoxycorticosterone during progressive intoxication with sodium barbital. Sprague-Dawley hypophysectomized rats that were maintained on special rat chow were also chronically intoxicated with sodium barbital. Following abrupt withdrawal of sodium barbital the control, adrenalectomized, and hypophysectomized rats developed abstinence convulsions. These experiments suggest that the pituitary and adrenal glands probably do not play an important role

in the mechanism underlying barbiturate convulsions in the rat.

Free Choice Between Tap Water and 5% Alcohol in Rats During Barbital Withdrawal

After drinking increasingly concentrated solutions of sodium barbital, a group of rats was given access to both tap water and 5% alcohol during withdrawal. There was no preliminary training or conditioning. During withdrawal there was no indication that a preference for alcohol developed. Perhaps the significant reduction in fluid consumption that developed in the rats during the first several days of barbiturate withdrawal interfered with the development for the preference for alcohol that might have been the consequence of an induced need.

The Effect of Sodium Barbital on Electroconvulsive Threshold Elevation in Cats

Daily induced electroconvulsions in cats resulted in a gradual tolerant-like elevation of the electrical threshold for seizures. The hypothesis that an anticonvulsant drug, such as barbital, might interfere with the development of electroconvulsive tolerance was tested. Fixed daily doses of sodium barbital were administered to a group of cats during a three-week period that were also subjected to daily electroconvulsions. The sodium barbital augmented rather than decreased the elevation of electroconvulsive threshold when compared to control cats. The increase was not entirely due to a direct threshold elevating effect that is a consequence of the anticonvulsant effects of sodium barbital. Further studies of this phenomenon will be undertaken.

Psychological Studies on Addiction

During the year the Addiction Research Center Inventory (ARCI) was administered to newly admitted addicts at the Public Health Service Hospital at Lexington, with the cooperation of Mr. M. J. Meketon, with the object of defining subjective changes associated with opiate withdrawal, and to study the differences between newly admitted addicts and subjects who are accepted for research studies. Preliminary analysis of these data suggests that newly admitted subjects are more like the nonaddict clinical groups than are research addicts on the general drug effects scale, which may suggest that addicts on the research ward answer questions within a more restricted frame of reference, or, alternatively, that addicts in general deny experiences which suggest change when they are not on drugs.

The study comparing physician addicts and general hospital population addicts with a representative group of practicing physicians collected by Drs. W. G. Dahlstrom and R. S. Spain, University of North Carolina, using the MMPI has been completed. Further, a comparison of a new group of addict physicians collected by Mr. Meketon have confirmed the observations made on the initial group of physician addicts. As indicated previously, the representative physicians scored within the normal range on all scales, while, strikingly, the addict physicians produced significant elevations on all clinical scales except for hypomania and social introversion. Except for the masculine-feminine scale the mean scores of the general hospital addicts were much more abnormal than these of addict physicians. In addition to psychopathy, neuroticism, depression, and social maladjustment being shown by the addict physicians, the masculine-feminine scale indicated a considerable degree of sexual instability and deviation without showing homosexuality. Judging by the progressively increasing degree of personality abnormalities from the representative physician to the addict physician, and from the normal physician to the general hospitalized addict, abnormal personality characteristics become increasingly influential in the onset of addiction as legal and quasi-legal availability of narcotics becomes less.

Studies on state dependency behavior of the rat in an escape from shock situation using two safe goal boxes have been initiated. These studies have been used to test the Overton hypothesis of state dependency, which may be stated as follows: A response learned while an animal is under the influence of a specific drug will occur more frequently when the animal is tested under the influence of that particular

drug than under any other condition. Results have indicated that rats trained under pentobarbital make differential response when again pentobarbital, confirming the observations of Overton. However, animals trained under the influence of morphine show almost complete stereotopy or response fixation under both morphine and saline conditions when subsequently tested. If such response fixation occurs in man, adaptability would be reduced by morphine. In addition, since the effects of morphine apparently generalize to the no-drug condition, readjustment would be more difficult in the drug-free state following the use of morphine.

The Mode of Action of Central Nervous System Depressants

The effects of graded doses of nalorphine, cyclazocine, morphine and naloxone were studied on the flexer reflex of the chronic spinal dog. It was first observed that naloxone did not depress the flexor reflex even in subconvulsant doses in the dog; whereas, nalorphine produced partial depression of the flexor reflex. The depression produced by nalorphine was dependent upon the strength of the stimulus used to evoke the flexor reflex (0.8 kg stimulus produced an 84% depression, 1.6 kg stimulus, 75% depression, and 3.2 kg, 61% depression). On the other hand, morphine and cyclazocine produced almost complete depression of the flexor reflex for all strengths of stimulation. These results can at least be interpreted as indicating the narcotic antagonists can differ one from the other with regard to their intrinsic activity. Thus, naloxone has zero intrinsic activity. Nalorphine has less intrinsic activity than either cyclazocine or morphine. Further, it has been shown that the apparent intrinsic activity of the narcotic antagonists depend upon the strength of the nociceptive stimulus that evokes the suppressed reflex.

Studies are being continued in an attempt to understand neurohumoral mechanisms that are responsible for the flexor reflex in the chronic spinal dog, as well as mediating the morphine abstinence syndrome in this preparation.

It has been observed that gamma hydroxybutyric acid will suppress the running movements and hyperexcitability of the flexor and crossed extensor reflexes in the abstinent dependent spinal dog. However, since gamma hydroxybutyric acid will also suppress the flexor and crossed extensor reflexes in the nondependent nonabstinent chronic spinal dog, it is felt that this represents a more general depressant action rather than a selective depression of facilitatory influences caused by abstinence.

Conditioning Factors in Opiate Addiction and Habituation (relapse)

The methods employed in the studies herein reported have been described in detail in previous annual reports for fiscal years 1964 and 1965. However, the designations of the various subgroups of rats in the replicate study (see project report for 1965) were changed to the following:

ADT (morphine-addicted, etonitazene-trained)
CDT (saline-injected, etonitazine-trained)
AUT (morphine-addicted, untrained)
CUT (saline-injected, untrained)

During the last year a total of sevel relapse tests have been completed, extending to 142 days after the termination of all injections (morphine in the cases of ADT and AUT and saline in the case of CDT and CUT). In addition, extensive statistical analyses of the data have been made obtained in relapse tests I (9 days abstinent), II (23 days abstinent), III (44 days abstinent), and IV–VII combined (58–142 days abstinent), with regard to (*a*) "wet dog" shake frequencies in home cage and in linear maze, and (*b*) free choice drinking of water or etonitazene from 8 p.m. to 8 a.m. in the linear maze.

Analysis of variance of "wet dog" shakes indicated that previous addiction, place of abstinence, and an interaction (previous addiction x place of abstinence) all were significant on each of the three relapse tests. These results clearly indicate the abstinence phenomena had become classically conditioned to the environment (linear maze or home cage) in which the appropriate subgroup (ADT or AUT) had been abstinent from morphine each night during the six week morphine injection prior to permanent morphine withdrawal. However, such

evidence of classical conditioning was not obtained from the analysis of data for relapse tests IV–VII, indicating that extinction of this conditioned response had occurred between the 44th and 58th day of abstinence.

Analysis of the free choice data confirmed previous impressions, namely, that postaddict subgroups (ADT and AUT) drank significantly more etonitazene solution than their respective nonaddict controls (CDT and CUT) on every relapse test through 140 days after termination of injection, excepting only the first relapse test. There was, however, no significant difference between the trained and untrained postaddict subgroups for any of the relapse tests.

From the data obtained in both the original and present studies, it is now clear that, in the rat, previous physical dependence on morphine *per se* is an important factor in generating a disposition to relapse long after morphine withdrawal and the subsidence of the primary morphine abstinence syndrome. For reasons mentioned in the individual project reports for 1964, it is considered unlikely that such a disposition is due to residual tolerance to opiates, rather the nonaversiveness of etonitazene for postaddict rats relative to nonaddict rats may be due to persistence of homeostatic imbalance, as manifested by the long-enduring abstinence syndrome of which total wet dog counts (unconditioned plus conditioned) may be an indicator in the present study. These findings and conclusions emphasize the need for intensive research on the possibility that secondary abstinence also occurs in man.

Confirmation of the conditionability (classical) of at least one morphine abstinence phenomenon (wet dog) in the rat supports the hypothesis that in man relapse may be due in part to the recurrence of the abstinence phenomena (as conditioned responses) long after morphine withdrawal. Theoretically, prior reinforcement of opioid-acquisitory behavior by suppression of morphine abstinence phenomena during periods of active addiction to morphine should play a role in generating a disposition to relapse, but the influence of this factor, as well as of conditioning of wet dogs on etonitazene drinking in relapse tests, could not be demonstrated by the techniques employed in both the original and present studies in the rate.

Currently, studies are underway to determine if hustling will increase the probability of relapse. In addition, it is hoped to initiate a study of the effects of lesions in the limbic system (e.g., bilateral cingulumotomy) on both conditioned and unconditioned abstinence phenomena and on relapse tendencies of morphine addicted rats, provided that appropriate means are found for obtaining additional equipment and the services of an additional technician.

Experimental Studies with Human Subjects and Studies on Learning

A series of primary and supplemental experiments have been concluded which dealt with the effects of single doses of morphine upon conditioned and unconditioned electrodermal responses. These experiments have studied the effects of morphine (16 mg/70 kg) upon (*a*) the acquisition of a classically conditioned electrodermal response, (*b*) the retention of a previously established differentially conditioned electrodermal response, and (*c*) evocation of conditioned anticipatory electrodermal responses. One of the major purposes of these experiments was to seek evidence in support of the hypothesis that the morphine produced clinical analgesia is related causally to the reduction by morphine of conditioned autonomic responses. The overall results of the experiments yielded little, if any, support for this hypothesis.

With regard to the acquisition of conditioned electrodermal responses, we observed a moderate, though statistically significant ($P<0.05$), reduction in the level of conditioning compared with placebo. The reduction was not greater than that produced by 200 mg/70 kg of pentobarbital, however. Thus, though there was a reduction in the acquisition of conditioning, the size of the reduction in absolute terms was not remarkable and not likely to be of significance for the mechanism of morphine produced analgesia.

In the case of conditioned anticipatory electrodermal responses, in which subjects were able to anticipate a noxious stimulus, morphine

did not produce any reduction in the frequency or magnitude of responses.

Probably most pertinent for a test of our hypothesis was the study of the effect of morphine upon electrodermal responses which had been established before the morphine effect was tested. For this test a differential, or discriminative, conditioning procedure was used in which one particular tone was reinforced while another tone was not. The test for the effect of morphine was made only after the discriminative response had been established during several weeks of training. In this situation morphine did not reduce the frequency or magnitude of conditioned responses. Morphine did, however, produce an increase in the frequency and magnitude of the partially adapted response to the nonreinforced tone.

A new laboratory has been established in the Basic Research Laboratory to study aspects of the neuropharmacology of learning. The initial aim of this study will be to study conditioned responses using direct cerebral stimulation for both the conditioned and unconditioned stimuli. It is intended by using this model of learning to study the facilitatory and inhibitory roles of stimulating reinforcing systems; the effects of single, multiple and temporally and spatially summed stimuli; and the role of neurohumors in the conditioned response. It is further hoped that this method can be employed for studying the role of nucleic acids and proteins in memory.

Social Science Section

Analysis of data from the Kentucky follow-up study has been completed and a final report prepared. The last annual report mentioned the high death rate and the relatively high rate of abstinence among subjects. Other findings include clear signs of personality problems in subjects prior to the onset of addiction in poor employment records, poor military adjustment, and alcoholism. Although many subjects felt that their addiction was a consequence of medical treatment or treatment of alcoholism, more than half of the men and one-sixth of the women gave pleasure seeking as a reason. After addiction began, most subjects became involved in an addict subculture. The extent of this involvement was determined in part by the reason for becoming addicted, by age on onset, by decade in which addiction began, by sex, and by urban-rural status. The major determinant of involvement was the source of the narcotics. Those subjects who had a stable (medical) source of narcotics showed the least involvement.

Addiction was followed by deterioration in major social roles. Employment became less stable, most marriages ended in divorce or separation, and subjects began to acquire criminal records or committed more crimes than before addiction.

While characteristics of subjects as individuals were associated with later relapse and abstinence to a statistically significant degree, these associations do not seem to be efficient predictors of post-hospital drug status. The abstinence in the sample and the degree of prevalence in addiction in Kentucky seems to be a consequence of increasing difficulty in obtaining narcotics. The unavailability of narcotics in turn seemed to be due to severl factors, including the improvement in medical practice, more vigorous law enforcement efforts, growing public disapproval of drug use, and probably shipping restrictions that occurred during World War II. Subjects varied as to the means to which they would go to obtain narcotics. Thus, some refused to steal narcotics, to forge prescriptions, or to try to "make" physicians. The vast majority of abstinent addicts did not take a step that was available to all, namely, moving to places such as Chicago or New York where drugs were available.

Computer programs have been written and tested for analysis of hospital admission data (Lexington and Fort Worth) for the 1935–1965 admissions. A study of the validity and reliability of field data obtained in the Puerto Rico study has been completed. Interview responses have been checked against prior hospital records, FBI arrest records, and the results of urine testing with the finding that addicts can and will recout their prior criminal hisory and current drug use with considerable accuracy. Several small scale studies based on inter-

views with Lexington patients were initiated during the year.

Biochemical Pharmacology

It was previously reported that morphine and nalorphine stimulated the incorporation of P^{32} into phosphatidylinositol, phosphatidic acid, lysophosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and diphosphoinositide in cerebral cortex slices while inhibiting the incorporation of P^{32} into phosphatidylcholine. These studies have been extended into morphine-tolerant, abstinent, and dependent guinea pigs. No change in phospholipid metabolism was observed in cortical slices of chronically morphinized animals when incubated alone. However, the effects of morphine in stimulating and inhibiting phospholipid metabolism was less than that previously observed in nontolerant animals. Although variable effects have been observed in morphine-dependent guinea pigs during withdrawal, marked stimulation of the increased phosphate into phosphatidic acid and triphosphoinositide have been observed. These findings provide additional support for the hypothesis that the narcotics may produce part of their effects in altering metabolism of phospholipids.

Studies of the metabolism of tritium-labeled cyclazocine in nontolerant and tolerant dogs have been nearly completed. The egress of cyclazocine seems to be more rapid than egress of morphine from the brain of the nontolerant dog.

The effects of sodium phenobarbital and morphine on phospholipid metabolism in microsomal membranes *in vitro* have been studed. Both of these compounds inhibit the metabolism of the major lipid component phosphatidylcholine. The kinetics of the inhibition of these two drugs on this compound differ. The metabolism of other phospholipid components has not been affected. The antibiotic puromycin was used to cause the release of incomplete proteins from their site of synthesis on ribosomes attached to membranes. The released peptides were found to pass through the membranes. The metabolism of components of the membrane during the transfer of newly synthesized proteins is being studied.

LABORATORY OF SOCIO-ENVIRONMENTAL STUDIES

The work of this Laboratory is focused on the study of social influences upon personality and behavior. It is convenient to group our current investigations into four content areas: the social context of personality development, the social psychological correlates of occupation, social factors in mental disorder, and the methodology of social research.

Studies of the Social Context of Personality Development

This is the domain in which the largest portion of the Laboratory's work is concentrated.

Social Class, Occupation, and Parental Values

The first completed analysis to come from Dr. Leonard Pearlin's study of parent-child relationships in Turin, Italy, demonstrates the value of cross-cultural, comparative research. Pearlin's investigation was designed as a replication and extension of a study of social class and parent-child relationships we had previously conducted in Washington, D.C. The earlier study had shown a distinct difference in emphasis in middle- and working-glass parents' values for their children: middle-class parents value self-direction more highly than do working-class parents; working-class parents emphasize, instead, conformity to external proscription. The present analysis is addressed to two questions: (1) Is social class related to parental values in Italy in the same way as in the United States? (2) If so, to what extent is this due to the characteristically different occupational experiences of middle- and working-class parents? Self-direction seems more possible and more necessary in middle-class occupations; working-class occupations allow much less room for, in fact might penalize, anything other than obedience to rules and directives set down by others.

The cross-national comparison shows that Italian parental values are more adult-centered, American more child-centered. Despite this cultural difference, the relationship of social class to parental values is much the same in Italy as in the United States. There is some-

thing intrinsic to social stratification that has strikingly similar effects in the two countries.

To determine the degree to which differences in the occupational circumstances of the middle and the working class might underlie class differences in parental values, fathers were questioned about three aspects of occupational life which together define the limits of and demands for the exercise of self-direction at work. These are the closeness of supervision to which a man is subjected, the substance of the work he does—that is, whether he works primarily with things, with ideas, or with people—and the degree to which his work requires self-reliance. Each of the three is independently related to fathers' values for their children: fathers who are closely supervised, who work primarily with things, whose jobs do not require much self-reliance, are more likely to value the child's conforming to adult direction, fathers who are not closely supervised, who work primarily with ideas or with people, whose jobs do require a large measure of self-reliance are more likely to value the child's self-direction. These three aspects of the fathers' occupational experience, which of course are highly correlated with social class, account for a very large part of the difference between middle- and working-class fathers' values and a smaller, but still substantial, part of the social class difference in their wives' values.

The next step will be to analyze more fully the functioning of the family in Italy.

Values and Behavior

When Pearlin completed his survey in Turin, Dr. Marian Yarrow proceeded to study a subsample of the families experimentally. This provides an opportunity to study the relationship of expressed values to actual behavior. The analysis of these data (being carried out by Drs. Pearlin, Yarrow, and Harry Scarr) is less advanced, but some intriguing results have emerged.

In the experimental situation, the child was given simple tasks, presented so as to arouse an effort to achieve the best possible performance. During some of the experiments, the child's mother was present, during others his father. The parent was instructed not to do the child's tasks for him, but was otherwise free to act as he thought appropriate. Some parents actively intervened,, to direct and control the child's actions. Those who did were disproportionately the ones whose aspirations for their children surpass their own achievements. More than that, they were disproporionately the ones who value the child's conforming to adult authority—not those who value the child's self-direction. The twin conditions of having high aspirations for one's child and disvaluing his self-direction are highly predictive of whether or not a parent will intervene to direct his child's actions rather than allow him opportunity for self-direction.

Maternal Care and Child Behavior in Japan

Dr. William Caudill has been carrying out a systematic observational study of mother-child relationships in Japan, and comparing the results with those that he and Mrs. Helen Weinstein have obtained, using the same methods, in the United States. The analysis is in process, but far enough along for some conclusions to be clearly established. (These are based on a study of three-to-four month old infants. Data on these same children at age 2½ have been collected but are still to be analyzed.)

There is, of course, a basic similarity between Japanese and American practices in that maternal attention in both cultures is centered on the infant's needs for sleep, food, and clothing. Beyond this, however, the contrast between the two cultures is great. The American mother talks to her baby more, and seems more apt to encourage him to respond and be active. The American baby is more often alone, is more active in the manipulation of his body and in the use of objects, and vocalizes more. The Japanese mother rocks her baby more, and talks to him less. Her actions seem directed to soothing and quieting the baby rather than to encouraging response and activity. The Japanese baby is less often alone, and is both physically and vocally quieter. These differences between the cultures do not occur as isolated characteristics of behavior, but rather are in-

terwoven into culturally dissimilar patterns. Even by the age of three or four months, a great deal of cultural "learning" has occurred.

Among middle-class families in Japan, the husband's occupational locus is related to the behavior of his wife and of their infant. The baby in families of small independent businessmen is less often alone, and more often awake and protesting. The mother in such a family talksmore to her baby, holds and rocks him more. In contrast, the infant in families of salaried employees seems more quiet and passive. The wives of salaried employees, in their move toward modernity, seem to have subtracted from traditional ways of caretaking rather than to have added anything new.

Children's Orientations to Health and Illness

Dr. John Campbell's study of how children develop orientations to health and illness is in the data-collection phase, but there are some intriguing preliminary findings from the data already in hand. One is that nine to twelve-year old children are more resistent to "giving in" to illness—to immobilization and dependency—than are six to eight-year olds. Another is that children who, by their own and by their mothers' testimony, are more inclined to take risks, are less inclined to give in to illness. With more such information, Campbell hopes to ascertain the processes that go into children's developing conceptions of health and illness.

The Experimental Modification of Children's Behavior

Drs. Phyllis Scott, Roger Burton, and Marian Yarrow conducted an experiment (reported last year) in the modification of a child's undesirable, aggressive behavior; the experiment was notable in its successful transplantation of the principles of social reinforcement from the laboratory to the natural situation of the nursery school classroom, with its many uncontrolled variables. Further analyses of these data have clarified some of the issues involved, and have encouraged Drs. Scott and Yarrow to go one step further: to attempt to use modification procedures such as social reinforcement and modeling, again in the natural situation, to inculcate desirable, "non-egocentric" behavior in nursery school children. This study is presently being planned.

A Review of Research on Social Development

During this past year, Dr. Roger Burton had the opportunity to review and assess past studies of social development for the *International Encyclopedia of the Social Sciences*. His principal conclusions are: (1) many of the findings derived from interviews about child-rearing practices can be organized along the same three semantic dimensions found in studies of the meaning of words generally; it may be that the underlying dimensions of language, rather than dimensions of the behavior being spoken about, account for the relationships found; (2) the theoretical model underlying most research on socialization, that of a passive child acted upon by the adult world, needs revision to take into account new evidence on variations in the constitutional characteristics and predispositional tendencies of the infant; (3) studies of the effects of stress on infants indicate that too little stimulation is deleterious, that an extra amount of regular or "normal" stimulation under appropriate conditions can promote desirable behavior, and it is even possible that stressful stimulation may cause some desired consequences; and (4) unambiguous labeling by parents of what behavior they approve and what they disapprove increases the likelihood of the child's behaving appropriately in a variety of relevant situations.

Three major trends are foreseen. First is the application of learning principles from the experimental laboratory to more complex kinds of human behavior as they occur in natural settings. Second is an increase in self-consciously critical assessment of the method and data used in studies of social development; few of our theories can actually be tested with data now available. Third is increasing attention to studies of the newborn, and how his constitutional characteristics and predispositions interact with his social environment.

Population Genetics

Dr. Gordon Allen, the sole geneticist in this

Laboratory, is now planning a study to be done in collaboration with Dr. Calvin Redekop of Earlham College, of Old Colony Mennonites in Mexico. The intent is to detect and describe variations in survival and reproduction in a long-isolated rural population. This population is unique in North America for its large size, combined with social isolation, cultural uniformity, "natural" reproduction and preservation of family structure. Under these conditions it seems likely that survival and reproduction may depend to a significant extent on emasurable behavior differences. This would constitute natural selection of psychological variables as hitherto postulated but never documented.

The Social Psychological Correlates of Occupation

We are processing the data from interviews with three thousand men, representative of all men employed in civilian occupations in the United States. The interviews were conducted for us by the National Opinion Research Center, using a schedule developed by Drs. Melvin Kohn, Carmi Schooler, and Morris Rosenberg. The intent here is to trace some of the major social psychological correlates of a number of presumably important dimensions of occupational position and experience. (The analysis, from the Turin data, of the relationship of three aspects of occupational experience to parental values is a good example of what is intended, and in a sense is a happy pre-test of some of the ideas that motivate this study.)

The year and a half since the interviews were conducted has been devoted to coding the data, checking reliability, carrying out elaborate checks on possible sources of error and inconsistency, and planning the analyses to come.

Studies of Mental Disorder

Work in this area, this year, was largely devoted to reviewing and assessing past research on schizophrenia.

Quantitative Studies of the Psychology of Schizophrenia

Dr. Carmi Schooler (in collaboration with Dr. Solomon Feldman of Northern Illinois University) undertook the massive job of abstracting and organizing all of the quantitative studies on the psychology of schizophrenia that had appeared in standard journals (or were mentioned in major review papers) since 1950. In reviewing these studies, Schooler found that a major portion of the research has centered on five hypothesized decrements in the functioning of some or all schizophrenics: an avoidance of intense or novel stimuli, a decrement in set and attention to stimulus input, a decrement in appropriateness of generalization and categorization from input, a decment in motivation to undertake action, and an avoidance of interaction with and psychological closeness to other people. Some studies of these hypothesized decrements have compared schizophrenics to normals, others have compared different types of schizophrenic patients, for example, those with good *versus* those with bad premorbid histories, paranoids *versus* nonparanoids, acutely ill *versus* chronically ill patients. At the present time, all the decrement hypotheses are incompletely proved but viable. Even if all were accepted as proved, however, grave theoretical problems would remain. If any one, or some combination, is taken as basic, it is possible to predict the remaining decrements as likely outcomes. The number of alternative theories imaginable is staggering.

The Social Epidemiology of Schizophrenia

Dr. Melvin Kohn reviewed studies of the social epidemiology of schizophrenia, with major emphasis on assessing the evidence for the hypothesis that social class is related to the incidence of schizophrenia, and on considering the implications of this possibility for our understanding of the dynamics of the disorders. A large number of complementary studies all seem to point to the same conclusion: that rates of mental disorder, particularly of schizophrenia, are correlated with various measures of socio-economic status, at least in large cities, and this probably is not just a matter of drift or duration of illness or who gets hospitalized or some other artifact of the methods we use. In all probability, more schizophrenia is actually produced at lower socio-economic levels. At minimum, this seems to be a potentially useful working hypothesis.

There is some evidence that the greater amounts of stress suffered by people at lower class levels enters into the apparently greater incidence of schizophrenia, but that this is not all that is involved. Class differences in patterns of family relationships may matter, too. To date, however, there has been no evidence of any difference between the family relationships of schizophrenics and those of normal families of the lower and working classes. It may be that the family patterns of the lower classes are in some way broadly conducive to schizophrenic personality development. Clearly, though, these patterns do not provide a sufficient explanation of schizophrenia.

A review of this field necessarily forces one to be aware of the serious methodological deficiencies of past studies. It would be erroneous, however, to conclude that improvement in method is what is principally needed to advance this field. Important as that is, it is not nearly so important as a new stance toward ideas. Rarely have investigators designed their studies to pursue some definite idea or to choose definitively between two alternative interpretations of past results. There has, in fact, been a fear of theory in this field of investigation, with the predictable result that the most preposterous *ad hoc* theories have been dragged in to explain or explain away ambiguous findings. Perhaps the most important thing to be learned from an examination of past research is the desperate need for bringing theory in, in time, when designing our investigations.

Studies of the Methodology of the Social Sciences

One major empirical investigation and one important analysis of the logic of our methods of data analysis have been pursued this year. The first, by Drs. Marian Yarrow, John Campbell, and Roger Burton, is specifically concerned with the field of developmental psychology. The second, by Dr. Morris Rosenberg, is specifically concerned with the methodology of survey research. Both have ramifications that affect all social science research.

Assessment of the Methods of Developmental Psychology

Several coordinated studies are being pursued simultaneously. Essentially, they involve the systematic comparison of most of our major methods of doing research—comparisons of interviews to observations, of retrospective interviews to contemporaneously conducted interviews, of various methods of observing and of recording and classifying observations —as well as the replication of significant past research, all to determine just how much credence can be placed on the methods on which virtually all our research has been based. The results thus far are dramatic and disquieting. Here are some of the recent findings:

A replication of one highly respected study, the Sears, Maccoby, Levin study of parental antecedents of children's aggression, dependency and conscience, produced many inconsistent findings even though very similar techniques of measurement and analysis were used. A re-examination of research by many investigators extends this picture of little systematic consistency of findings about antecedent-consequent relationships. A close assessment of more favorable reviews of this field indicates that they are based in large measure on the selective inclusion of studies and occasionally even of specific findings.

Response reliability of mothers' reports has been assessed by a comparison of mothers' answers to a self-administered questionnaire and to an interview, conducted about nine months apart. The two sets of data, on similar dimensions of childrearing and children's behavior, show considerable inconsistency. For example, correlations between pairs of questions regarding the child's dependent behaviors ranged from +.17 to +.32; the correlations on mothers' levels of demands on the child range from −.03 to +.32. Such low correlations suggest that a considerable proportion of the variance in such measures has not been adequately accounted for. The utility of such items for systematic hypothesis testing is considerably limited.

Comparisons of three sets of data on identical dimensions of childrearing (derived from contemporaneous records, mothers' later recall, and children's later recall) show correlations that range from +.04 on the use of particular disciplinary techniques to +.37 on ratings of

maternal warmth. Children's reports of their earlier development and experiences correspond less well to contemporaneous records than do mothers' reports. Dimensions crucial to developmental theory (such as early environmental traumata, early relationship with mother, early signs of the child's inability to relate to others) yield correlations ranging from +.20 to +.38.

A score was obtained for each mother on the degree of correspondence between the earlier contemporaneously obtained data and her later recall. Degree of correspondence was not related to the sex or to the ordinal position of the child. It was slightly related to how much time had passed (the shorter the interval, the greater the correspondence). Mothers whose recall is similar to the baseline data tend to have had good relationships with their children (at the baseline period), and their children tend to have scored well on positively valued personality attributes. Where mothers' recollections differ from the contemporaneous accounts, either the mother-child relationship of the child's personality was more problematic.

Examination of observational techniques shows that certain procedures for assessing reliability have built-in inadequacies. For example, correspondence between profiles of a given person's behavior (i.e., the rank-ordering of categories of behavior) as appraised by two different observers has two major drawbacks as a measure of reliability: (*a*) since a profile is a summary of the frequency of interaction over a period of time, the extent of agreement on classification of *specific* acts within that sequence cannot be assessed. (*b*) The degree of correspondence between the behavior profiles of two randomly chosen children is nearly as high as that between two independent sets of observations of the same child. The correspondence presumedly indicative of reliability of observation may merely reflect a more general common patterning in the observed behavior.

Methodological Principles of Survey Analysis

Dr. Morris Rosenberg has been systematically re-examining just what it is we do in analyzing survey data. In a series of papers, he has specified the various analytic purposes served by the introduction of "third variables," reconsidered our assumptions about the relationship of indices to concepts, further developed his method of test factor standardization, and clarified the distinction between symmetrical and asymmetrical relationships. No more than a skeleton picture of this work is possible here.

One major area of his work is in the specification of symmetrical, reciprocal, and asymmetrical relationships, and on the elaboration of three-variable relationships. Symmetrical relationships are those which are alternative indicaators of the same concept, consequences of a common cause, functionally interdependn or elements of a common complex. Asymmetrical relationships are those involving an association between a stimulus and a response, disposition and a response, a property and a disposition, a necessary condition and an effect, an ends and a means, and a structure and its imminent outcome.

When a third variable is introduced into a two-variable relationship, it may serve as a test factor, a specifying condition, or as another independent variable. Six types of test factors are distinguished and their theoretical implications elaborated: extraneous variables, component variables, intervening variables, suppressor variables, distorter variables, and antecedent variables. When the third variable represents a specifying condition, it may challenge, confirm, refine, or radically revise a theory; it may enable one to select between alternative hypotheses, reveal positive results in non-correlations, reveal trends or processes, and clarify the nature of the relationship, the independent variable, and the specifying condition itself. When the third variable serves as another independent variable, it may reveal independent effects, relative effects, cumulative effects, and typological effects.

The value of this is in the added power it affords us to know precisely what we are doing, and to be certain that we have not overlooked theoretically fruitful possibilities for analysis and interpretation.

LABORATORY OF NEUROPHYSIOLOGY

Section on General Neurophysiology is proceeding with analysis of shifts of electrical potential of the brain which appear to be primarily due to complex metabolic transactions across the blood-brain barrier. It has been shown that there is great species difference between rabbit, rat and dog on the one hand and the cat and rhesus monkey (and presumably man) on the other hand. The brains of the first group shift positive with respiratory acidosis, and the brains of the second group shift negative. It has been found that some injury factor reverses this response in the latter animals. The injury factor has not been specified, but general deterioration of the preparation and hypoxia seem to be involved. Concussed monkey brains also show reversed reactions. It appears that the capillaryglia blood-brain transport systems are involved in this reaction and that vasomotor disturbances are important. It is now probable that the voltage shifts occurring in the three stages of sleep, in arousal reactions and possibly the voltage shifts observed in certain kinds of conditioning and learning experiments are all of the same general nature. That is, not primarily neuronal but a complex metabolic phenomenon.

These observations have considerable interest for all kinds of brain disease and injury including circulatory disturbances and cerebral vascular accidents.

Another project deals with the problem of data handling to retrieve not exactly synchronized evoked reactions which occur at the various stations of a physiological reflex response. The glabella reflex is the example under study. The technical procedures have been proven and the method will be applied to other systems.

As part of general program on CO_2 and pH effects on neurons, a study of the nerve cells of the *Aplysia* is under way. Currently temperature functions of the pacemaker cells are under study.

The instrumentation section is proceeding with numerous improvements in existing technics and development of new ones.

The Section on Limbic Integration and Behavior has continued its investigations on two broad aspects of the functions of the limbic system, a basic part of the forebrain inherited from lower mammals and now recognized as playing an important role in emotional behavior and visceral functions. The first problem pertains to the relationship of the visual and limbic systems in the evolution of primate behavior, and the second concerns the central representation of pain and the role of the limbic system in modifying neural mechanisms responding to noxous stimuli.

The importance of the first problem is emphasized by a number of anatomical and clinical considerations. In the evolution of higher primates and man, the limbic cortical areas adjacent to the primary visual areas undergo a remarkable expansion. The fusiform gyrus lying parallel with the hippocampal gyrus develops as new structure in the brains of higher primates. The clinical observations of Penfield have revealed that irritative lesions in or near the medial temporal limbic cortex may result in a variety of visual illusions and emotional manifestations. Patients may experience subjective feelings of deja vu, macropsia, micropsia, feelings of being in a dream, feelings of familiarity or strangeness, paranoid feelings, feelings of fear, loneliness, sorrow, disgust, and sometimes ecstasy. Recently Slater and Beard have analyzed 69 cases of temporal lobe epilepsy in which the clinical picture was indistinguishable from that of chronic schizophrenia. Finally, the problem is relevant to the unanswered question of how the visual system establishes a working relationship with the hypothalamus.

In neuroanatomical and microelectrode studies on this problem the Section has gained basically new information indicating how the medial temporal convolutions are implicated in visual symptomatology and hypothalamic regulation. Heretofore it has been classically believed that no fibers from the optic radiations terminate in the medial temporal convolutions. In a neuroanatomical study, however, in which lesions have been placed in 17 squirrel monkeys, it has been found with a

modification of the Nauta stain that following lesions placed in the lateral part of the lateral geniculate body, a continuous band of degeneration is traced not only into the cortex of this gyrus, but also into the adjacent parts of the fusiform and lingual gyri. The band of degenerating fibers would appear to correspond to that part of the optic radiation in man known as Meyer's temporal loop. Some degeneration can also be traced further caudally into the prestriate limbic cortex of the retrosplenial region. Another new and highly significant finding is that following a lesion placed in the inferior pulvinar (a nucleus generally regarded as a visual association nucleus) degeneration develops in an outermost band of fibers leading to the posterior hippocampal gyrus.

Electrophysiological support for these findings has been obtained in microelectrode studies in which the representation of visually responding units has been mapped in the hippocampal gyrus and neighboring convolutions in waking squirrel monkeys. Recordings have been made from more than a 1000 limbic units. These experiments have confirmed findings of the initial exploratory study in anesthetized animals that visual stimulation activates units in the posterior hippocampal gyrus and adjacent parts of the fusiform and lingual gyri. Of particular interest has been the identification of a new class of cells in the posterior hippocampal gyrus that responds similarly to slowly adapting "on" units of the retina. In the retrosplenial region some cells have been found that respond only to stimulation of the contralateral eye, suggesting that they are activated by the primitive temporal monocular crescent. As both the posterior hippocampal gyrus and retrosplenial cortex are a major source of connections to the hippocampus, which in turn projects to the hypothalamus, it is apparent how the present findings have significant implications in regard to the influence of the limbic system on circadian rhythms, neurovegetative and emotional mechanisms, and the dreaming and autonomic aspects of the rapid eye movement (REM) phase of sleep.

The Section's current investigation of the interaction of the fornix and fifth nerve on units at the thalamic level was suggested by previous experiments in which it was found that hippocampal seizures propagating in the limbic system result in an elevation of the threshold of the behavioral response to noxious stimulation of the face and other parts of the body. The present microelectrode studies in awake squirrel monkeys take advantage of the finding that a stimulus to the fifth nerve of sufficient intensity to induce signs of pain if given repetitively does not appear to disturb the animal when administered singly. This provides an experimentally innocuous means of mapping the distribution of thalamic units responding to a potentially noxious stimulus. In addition to units in the classical relay nucleus (n. ventralis posteromedialis), fifth nerve stimulation has been found to activate units in the caudal intralaminar nuclei and in parts of the tegmental area. A prior shock to the fornix may inhibit the fifth nerve response of units in the caudal interlaminar nuclei for a period lasting up to 200 msec, but has no effect on cells of the classical relay nucleus. Following a hippocampal after-discharge induced by tetanic stimulation of the fornix the inhibitory effect on intralaminar units may last as long as two minutes. This latter finding is of particular interest in regard to limbic seizures seen clinically in which patients may burn or otherwise injure themselves because of apparent unawareness of painful stimuli.

One of the most interesting findings, to neurophysiologists, of the electro-microscopic studies of nerve and muscle cells has been the redefinition of the boundaries of the cells. Previously it was simply a matter of a cell having an inside and an outside, but now we find that there are channels which connect the extracellular space to the more internal portions of the cell. The channels are separated from the cytoplasm by membranes. Examples of these systems are the transverse tubular of striated muscle fibers and the invagination in the giant neurons of the snail, *Aplysia*. The Section on Membrane Physiology has been investigating the properties of both of these systems in order to define the mode

of transfer of substances along them. The first step is to determine the electrical properties of these pathways as a means of describing the relative ease of transfer along them. In the *Aplysia* neurons, it appears that the invaginations are the major pathway for transfer in and out of the cell and may be considered to be extension of the surface membrane. In muscle, the transfer across the walls of the transverse tubular system along the tubules has greater restrictions. These restrictions must be incorporated in the development of models which describe the mechanism by which the electrical activity of a muscle fiber induces activity in the contractile apparatus of the muscle fiber.

LABORATORY OF PSYCHOLOGY

Since I shall organize this report around major areas of investigation which cuts across Section boundaries, I am introducing the report with a statement of the general aims of the various Sections.

The *Section on Early Learning and Development* is concerned primarily with learning and development in both human and animal subjects. Investigation in the human area is directed toward learning in social contexts (specifically the mechanisms of adaptation and social learning) in the early phase of life. The methods used are controlled observation and experimental control. The overall research aim of the animal unit is to contribute to our understanding of learning by analyzing the action and correlates of reinforcer presentation and omission. To determine which facts and principles discovered are more general and which are specific to a particular class of reinforcers, to a particular class of behavior, or to a particular age of the animal subject, studies are being carried out on early ingestive behavior, early instrumental behavior, and juvenile social behavior, as well as traditional adult instrumental and operant behavior. The effect of both homeostatic reinforcers, such as food, and nonhomeostatic reinforcers, such as a passive person for a juvenile puppy, are being investigated.

In the *Section on Personality*, two major research programs have developed within the overall goal of investigating factors which facilitate or interfere with the effective functioning of the individual: the identification of factors which influence personality development and change; and the study of factors affecting cognitive processes and development.

In the *Office of the Chief* two major areas of research are represented, a study of the nature and etiology of schizophrenia, and a study of the process of communication.

The *Section on Perception* is primarily interested in two major and separate areas: the nature of the perceptual process, its development, character and aberrations; and the environmental and genetic variables affecting biological systems.

The *Section on Higher Thought Processes* studies the process of problem-solving. Its investigators are interested in the fundamental nature of these processes as well as their operation in various normal and disturbed groups.

The *Section on Neuropsychology* is concerned with the relationships of the brain to behavior in human and animal subjects. These relationships are studied through both ablation and stimulation techniques.

In carrying out these programs some of the work is carried on entirely within the Section structure, some in cooperation with other Sections, some in cooperation with other Laboratories and Institutes, and some with outside agencies both here and abroad.

Against this background I have organized the report into five major substantive areas, dealing with topics without regard for Section lines. I have also added some general material concerned with advances and problems in the Laboratory. The five substantive areas are: (1) Development of behavioral functions; (2) Analysis of developed behavioral functions; (3) Biological substrate of behavioral functions; (4) Forces destructive of behavioral functions; (5) Forces that enhance behavioral functions. The two additional areas are: (6) Theoretical, methodological and technical advances; (7) Administrative aspects.

Development of Behavioral Function

In recent years a substantial amount of the investigative effort in the Laboratory has concentrated on one aspect or another of early development—both in animal and man.

Aside from some planned work of Dr. Rosvold's group with monkeys and Dr. Calhoun with rats, the major attack in animals has been made by Drs. Stanley and Bacon at Poolesville. They have worked with social behavior in young dogs and are presently experimenting with early learning in neonatal puppies.

Neonatal Learning

Sucking behavior of neonatal mammals is a classic example of an innate action pattern present at birth in a remarkably organized form. When attached to a teat, the sucking puppy is likely to obtain milk correlated with waves of negative pressure generated in its mouth. From the point of view of reinforcer action, the components of sucking make up the only possibly operant responses that a newborn mammal can perform fast enough to provide a sufficiently large behavioral sample for analysis.

Accordingly, puppies were trained to suck on an artificial nipple, followed by repeated sessions with a fixed time interval of milk reinforcement, the milk being delivered contingent upon the first suck 10 seconds after the preceding milk delivery. Two of six 2-week-old puppies displayed a pattern of sucking in accord with the temporal spacing of milk deliveries, indicating discriminative operant learning.

The discriminative learning prowess of the infant puppy has been directly confirmed by Dr. Bacon who joined the staff this year and is investigating instrumental approach and avoidance behavior as distinguished from ingestive behavior. He established that 1-week-old puppies can discriminate between the hardware cloth and the terry cloth sides of a discrimination box because they gravitate toward the side where they had received milk. These findings add to the growing body of data showing that the newborn puppy, which was thought to be devoid of any learning ability less than 10 years ago, can learn, and apparently does so on the same basis as adult organisms.

Social Behavior

The young dog readily forms a strong emotional attachment to people. This fact suggested that such attachments could be measured by the speed with which a dog runs repeatedly to a person. If the speed of running improved with such opportunities, viewed as training trials analogous to food-reinforced training trials in alleyways for rats, it would follow that a person or some aspect of handling the puppy was a reinforcer for the dog. Data, collected at another institution by Dr. Stanley and Dr. Bacon, showed not only that a person was a reinforcer for the dog, but that the most consistent reinforcer was simply a passive, unreactive person. Additional data on passive person reinforcement was analyzed and reported this year. Basenji puppies given intermittent passive person reinforcement learned and extinguished approach behavior in the same fashion as puppies given continuous or 100% reinforcement. This finding is comparable to that found with small amounts of food reward in the rat. It suggests that the passive person functions as a weak reinforcer in the basenji. This work will be continued using other breeds.

The other studies on early functioning are focused on similar problems in human development in different contexts and with different degrees of analytic detail. They range from studies of perceptual and cognitive process, through a detailed study of the process of social learning, to a study of intellectual development in molar settings.

The first is a study of object-perception in children conducted by Drs. Carlson and Rapoport. Generally accepted hypotheses concerning the development of object-perception often assume a progression from relatively greater perceptual dependence upon the visual angle subtended by an object to a more complete perceptual integration of angular size with various sensory cues to object-distance. Recent work done here and elsewhere on size-

and distance-perception in adults has seriously questioned the theoretical basis for this assumption. In perceptual experiments adults can produce size-judgments which approach geometrically correct angular values and which deviate correspondingly from an accurate judgment of object-size. These deviations now appear to be due to a *cognitive attitude* about size-distance relationships rather than any basic capacity to perceive angular size as such. This possibility has important implications for hypotheses of neural mechanisms underlying perception and for the interpretation of apparent differences in object-perception between normal adults and other populations such as children and psychologically deviant groups. Comparable procedures for testing size-judgment in both children and adults have been worked out in preparation for a study of the age range from approximately 4 to 10 years.

Dr. Caron, in his studies of *conceptual development,* has extended his investigation of the processes underlying the phenomenon of conceptual abstraction in the preverbal child. A widely held view of concept formation and concept transfer may be characterized as the "verbal hypothesis." This view states that the ability to treat similar instances of a concept equivalently is dependent on the acquisition and application of common *verbal* labels for each instance of the concept. Dr. Caron has undertaken to test an alternate hypothesis, i.e. the "discrimination hypothesis," which holds that conceptual abstraction and transfer are not dependent on the application of common verbal labels but rather on the individual's ability to differentiate the common *sensory* feature or property found in each instance of the concept.

Dr. Caron and his associates have conducted a series of four studies testing the adequacy of the discrimination hypothesis to explain the development and transfer of concepts in preschool children. One study directly compared the relative effectiveness of verbal and discrimination training on the ability of children to transfer learned responses to conceptually more complex but related stimuli. The evidence thus far obtained appears to support the discrimination hypothesis. The other three studies involve examination of the factors which appear to facilitate or impede discriminative abstraction. The results of this series of studies demonstrate that children may be assisted to develop concepts at a very early age without reference to verbal training. The fact that man's ability to think abstractly precedes the development of an appropriate verbal response system permits the inference that humans possess an *elemental representational system* to which language and other coding systems are later coordinated. A practical implication of these findings is that the current programs which are concerned with the enrichment of the culturally marginal, handicapped or retarded child should place greater emphasis on developing techniques to highlight the sensory features which define concepts rather than continuing to stress verbalization of concepts as such.

Dr. Gewirtz' program of *Research on the Impact of Environmental Stimulation on Children's Learning and Development* has Five Overlapping Foci

The *first* general focus has been on *behavior,* and has included many topics only a few of which we have the space to mention: the determination of infant behavior repertoires in early life, with the attempt to separate unlearned from learned components of those behaviors; the acquisition of responsiveness to the environment; the determination of the ages at which different learnings can first occur; the acquisition of control by infant and child behaviors of conditioned discriminative and reinforcing stimuli (non-social and social) provided by the environment; the determination of the control by the infant over behaviors of parents and caretakers; and the determination of the consequences in key infant behaviors of the stimuli provided through caretaking.

The *second* general focus of this research program has been on *environment,* and has involved attempts to specify the nature of functional stimulus units for learning, in everyday social contexts. Attention has been

directed to identifying critically and rigorously useful dimensions and units of the environment, implied by terms such as "richness," "privation," "deprivation," and "separation."

During the past year several reports have been published from the project under these two research foci. Two of these reports, of monograph length, have dealt with the age course of infant smiling in the first 18 months of life in four Israeli child-rearing environments. The forms of the age curves for smiling differed among the enironmental groups. Attempts were made to understand these differences in terms of what we knew about the different conditions they provided infants in them. The possibility that the smiling habituation curves (to a constant stimlus across 12 minutes of time) for individual infants differ in the several Israeli environments is currently being explored. The form of decline of the habituation curves was found to be dramatically like the form of similar response curves in which responses were made to constant stimuli by lower animal species.

Slow but steady progress has been made during the past year on the large companion study (being conducted in collaboration with Dr. H. B. Gewirtz) in which 108 infants in four child-rearing environments were studied for one entire day at four age points during the first year. Stimuli provided by the environment to the infant, the infant's behaviors and the contingencies between stimuli and behaviors have been catalogued. After considerable preparatory work several computer programs were recently completed which have permitted the start of analyses of two major segments of data.

A *third* focus of this program has been on conceptualizing the different processes that could be considered to represent learning and, more generally, adaptive behavior change; and, when required, on extending basic learning conceptions to the analysis of some of the complexities and aberrations of life in the early years (e.g., "privation," "deprivation," "separation"). In this attempt, a variety of possible processes of change are scrutinized, including the separate effects of stimulation, conditioning, performance, and setting-drive conditions. Further, these processes have provided the bases for attempts to *design* environments that would enhance early dimensional and contextual learnings, as well as the possibilities for efficient adaptive and social learning and development. Dr. Gewirtz has prepared a major theoretical analysis of these issues for the Committee for Re-examining Early Child Care. It is directed to various basic and applied fields interested in early child growth and development and is to be published this year.

A *fourth* focus of the program has been on the strategies and tactics of caretaking of infants and older children in intact families, in day-care installations, and in residential institutions. This research has involved analyses of concepts like "tender loving care," which reflect aims of child rearing valued by society. Further, in terms of what is known of early learning, the approach has been involving the design of effective patterns of caretaking to (1) maximize the possibilities of attaining socially desirable child-rearing outcomes in the behaviors of children, (2) facilitate bringing out the full capabilities of the developing child, and (3) make unambivalent the caretaker's relationship to the child (which could result from the caretaker's behaviors coming under the child's control).

For the above ends, a study was carried out on the instrumental crying (for attention) of infants as young as six weeks of life in a well-baby ward of a children's hospital. It was found that the crying of the infants was maintained, indeed strengthened, by the attention readily provided by the well-intentioned caretakers to the infants when they would cry. A research carried out illustrated clearly that, by not responding to apparently unwarranted crying together with responding to socially approved behavior like smiling, the infants stopped crying unnecessarily and became "charming" companions for the caretakers.

A *fifth* focus of the Section's research program has been on the *setting* conditions that determine stimulus efficacy for behavior on a particular occasion. These conditions may operate at one time point (e.g., "ground" for

"stimulus figure") or across time points (e.g., deprivation or satiation contexts for a stimulus) to heighten or lower the efficacy of stimuli for behaviors. Such setting conditions are particularly important for an understanding of child behavior systems in natural settings, given that the child's capacities are developing and limited. In a series of studies, Dr. Gewirtz and his associates have found that the more frequently social stimuli are presented to children ("satiation"), the less effective those stimuli are as reinforcing stimuli in a subsequent learning situation. Further, the more recovery time between the receipt of stimuli and the learning task, the greater the recovery was found to be. This type of relationship has been identified heretofore primarily with organismically relevant appetitive stimuli with lower organisms. Two reports of this research have been submitted for publication this past year, and have been well received.

As seen in some aspects of the work done by Dr. Gewirtz' Section such as Dr. Etzel's study on the crying child, and in Dr. Schaefer's work with culturally deprived children, the implications of basic studies naturally lead to the testing out of the hypotheses developed in the real situation.

Intellectual Development

Dr. Schaefer, on the basis of Dr. Bayley's findings in her normative study, is currently conducting a research program aimed at determining whether it is possible to raise the level of intellectual performance of Negro infants of lower socioeconomic status, through a program of intellectual stimulation. Part of the effort is to determine whether this training program, which does not involve removing the child from the home, can provide significant influence on the child's motivation, language development and functioning intelligence. If this program is successful, that is, if it turns out that lower functioning intellectual performance is not a result of maturational factors, a new method of assisting the child of lower socioeconomic status will become available. The research, which has been undertaken in collaboration with the Bureau of Social Research, Catholic University of America, includes an experimental group of 30 Negro infants of lower socioeconomic status and a control sample of 30 comparable infants. The experimental group is being tutored in the home beginning at 15 months. Tutoring sessions are offered five days a week, one hour a day. Both the experimental and the control groups will be tested at 21, 27, and 36 months to assess what specific abilities have been affected and what differences may be found between the tutored and untutored groups.

A related study is being carried out with another group of Negro infants from a more stable suburban Negro community, in association with the Home Study Program of Kensington, Md. Some of the infants will be tutored beginning at one year and others beginning at two years of age in order to determine whether significant differences may be attributable to earlier intervention. Both of these groups as well as a "no-tutoring" control group will also be tested at three years of age to determine the effects of the tutoring program.

Analysis of Developed Functions

There are three areas of investigation of developed, rather than developing, functions going on in the Laboratory, namely, communication, thought, and environmental and genetic variables affecting biological systems.

Communication

The study of human communication involves two sections of the Laboratory, the Office of the Chief, represented by Dr. Allen T. Dittmann and the Section on Personality, represented by Dr. Donald S. Boomer. The principal research focus remains the study of the phonemic clause which takes into account the rhythmic characteristics of speech. A theoretical analysis of the processes involved in the communication of emotions or affects is also under way.

Dr. Boomer has continued his research aimed at formulating a psycholinguistic theory of speech encoding and decoding. He has systematically tested the hypothesis that the pho-

nemic clause is the unit of speech which bears on speech formulation and comprehension. During the past year he published the first major statement regarding the phonemic clause and its role in the interrelationship between speech and thought. His current research has provided further evidence that the phonemic clause rather than the isolated word is the functional unit of speech and encoding. He has initiated a study aimed at testing the hypothesis that the phonemic clause is also related to the decoding process as represented by the comprehension of orally presented material.

Interest in the phonemic clause has increased because of evidence gathered in past years that this is the fundamental unit in which spontaneous speech is formulated and uttered by the speaker, and by which it is understood by the listener. On the encoding side the psychological process is seen as successively assembling groups of words into syntactic patterns. On the decoding side the hypothesis states that temporal strings of phonetic signals are accumulated and held in abeyance until the end of the phonemic clause, at which time the entire assembly is decoded as a pattern and understood as a single meaning unit. Most of the evidence already at hand concerns the encoding side. Examples of this type of research include the study of hesitations and filled pauses. Since these pauses are found to occur toward the beginning of the clause, it appears that decisions about the whole unit are made at the outset, after which the remainder can be spoken relatively fluently.

Data for evidence like that from hesitations are also being collected from the study of retraces and false starts in spontaneous speech. The hypothesis under test here is that (1) these phenomena are confined within phonemic clauses; (2) they do not cross clause boundaries; and (3) they occur at the outset of phonemic clauses. A parallel hypothesis about the phonemic clause from the decoding side has also been formulated and data are being gathered for its test. The hypothesis states that in conversational speech, the listener indicates his understanding by certain behaviors which occur only at the end of phonemic clauses, behaviors like head nods and vocal insertions like "Mm-hmm," "Yes," and "I see." Since vocal behaviors lend themselves readily to study, a recording situation for brief conversations between pairs of normal control subjects has been set up so that the coding of the speaker's clauses and identification of the listener's vocalizations can be done completely independently. Preliminary examination of other interview material, collected for the body movement study to be reported below, indicates that the "Mm-hmm" is inserted almost exclusively at junctures between clauses. The response is sufficiently selective, however, that it may be possible to identify some larger unit, based on differentiating among the three types of juncture.

Instrumental work is being done on one of the distinctive features of the phonemic clause —primary stress—which, by definition, can occur once and only once per clause. Primary stress is being studied because it is more amenable to physical measurement than is the other distinctive feature—the boundary between clauses, or juncture. The identification of such boundaries depends on linguistic judgment and is not communicable in more objective terms. Measurements are being made of three acoustic aspects of a series of primary stresses, peak amplitude, integrated amplitude, and duration, with a view to specifying its characteristics well enough for test in speech synmethod of data collection, i.e., output from ac-

One of the basic contributions of Dr. Boomer's research extends beyond the field of psycholinguistics in that it provides a more precise tool for a broad range of studies involving speech and comprehension. If future work continues to support the hypothesis that the phonemic clause is the natural unit of syntactic processing, both in speech transmission and in reception, then a meaningful unit for speech analysis will have been provided mental health investigators concerned with such problems as psychotherapy, psychopathology, cognitive development, etc.

In order to provide a direct test of Dr. Boomer's hypothesis that the phonemic clause is the basic unit in speech formulation and comprehension, it is necessary to vary intonational features in otherwise identical utterances in

order to assess the effects of these features on recognition and comprehension. A human speaker, however, cannot simultaneously control his voice on all of the relevant dimensions; such stimuli can be produced only by an electronic speech synthesizer. In order to gain experience with such an instrument it is planned that Dr. Boomer will spend a year, beginning June 1966, working at the Department of Phonetics, Edinburg University, Scotland.

Study of the relationship between body movements and speech rhythm patterns continues this year with a replication of the study completed last year in which hand and head movements, like hesitation forms in speech, were found to occur toward the beginnings of phonemic clauses, while foot movements were found to occur independent of these speech rhythms. The replication uses a different method of data collection, i.e., output from accelerometers which are attached to head, hands, and shoes of the subjects. The signals are written out on an operations recorder along with an oscillogram of speech for location of pauses and collation of the phonemic clause coding, which is done from audio tape and typescript. Subjects engage in 15-minute conversational sessions on two successive days during which the interviewer makes every effort to reduce the stress which might result from being in this unusual situation. A great deal of development work was necessary, in collaboration with the Technical Development Section, to perfect the apparatus, and 10 normal control subjects have been run in the experiment. The results are only partially analyzed.

The theoretical work on emotional communication is an outgrowth of a literature search begun by Dr. Dittmann during his assignment in Milan, Italy. Originally intended to cover only a small aspect of the topic, the work has broadened and is seen as an application of the mathematical theory of communication proposed by Shannon. In some areas the theory must be added to, because of the special problems of human communication: first, emotional information is more complex than that encountered in telecommunication where the theory was developed; second, in social interaction there is confusion of the functions of the communication system, such as encoding, choice of channel, and the like, which can be performed by independent components in the original applications of the theory; and third, the types of interference or noise in human communication are more varied and more complex than the random noise encountered in telecommunication. These differences do not seem, however, to mean that communication theory is irrelevant to problems of emotional communication. Rather, the theory is a general one, and details must be filled in wherever it is applied. Dr. Dittmann has been working up notes for a monograph which will present the details of this theoretical analysis.

Thought Process

In the area of thought process, which has emphasized problem-solving, extensive studies have been carried out. The ability to avoid or correct heuristically crude or detrimental methods of inquiry has been studied with more than 150 subjects using an elementary set of problem-solving tasks monitored by HEP, the Heuristic Evaluation Programmer I, which was designed and developed in our Laboratory. The following are, in quite general terms, the principal heuristic deficits identified thus far: (*a*) Sequential inquiries were poorly organized so that the cognitive strain associated with a solution effort was much greater than it needed to be. (*b*) The questions reflected by the subjects' operations were so specific that the information elicited was near minimal. (*c*) Poorly adapted behaviors were allowed to perseverate without exploring obvious alternatives. (*d*) Quite direct implications of information elicited were not used. (*e*) Failure to use negative information prolonged the use of inefficient procedures.

In order to secure an opportunity to observe the interpersonal relations between individuals engaged in a cooperative solution effort, HEP has been redesigned to operate alternately from two display panels located in separate cubicles. The cooperating problem-solvers can communicate over a speaker system and are free to question, advise, or criticize one another

during the solution effort. Pilot work has been carried out on 10 pairs of subjects to standardize procedures and instructions preliminary to a study of interaction and cooperation between schizophrenic patients and their mothers. The amount and type of verbal interaction which occurs in a structured problem-solving situation and the effects of heuristic performance of the subjects will be studied.

A group of college students has been tested on a set of very difficult, three-element HEPP problems. Using a procedure whereby displays are automatically read into the apparatus, efforts were made to teach strategies for solving these complex problems. Although the subjects were told that a number of solution strategies would be shown, only a few changed their previously developed solution procedures. There was evidence of a surprising inability to invent techniques for reducing cognitive strain or to learn from the displays rather obvious methods for doing so.

Enviromental and Genetic Variables Affecting Biological Systems

Dr. Calhoun has been responsible for this area. His analysis has concentrated on two major areas: drinking and the development of indices for the assessment of behavior. His emphasis on the first, drinking behavior, represents a strategy of gradually presenting, in published form, aspects of a new and comprehensive theory of behavior. He is presently limiting himself to the lever-pressing-drinking behavior which is so familiar to many experimental psychologists. In fact, drinking behavior does mirror most of the processes typical of other behavioral states with regard to the control of their duration. Then, once this baseline publication has been completed, he plans to proceed with more concise treatments of the similar processes exhibited by sleep, locomotion, eating, and grooming as major behavioral states of the rat. Only after each of these is treated separately will he engage in the examination of the more complex processes of initiation and sequence of behavioral states, since these two topics can only be treated in the context of the entire repertoire of hehavioral states. Then, after the presentation of all of this separate material, he will present the broader theoretical formulation which he believes to form the heuristic basis for a more precise understanding of emotion, mood, motivation, drive, learning, conformity, and creativity.

With regard to the second major area, the development of indices for the assessment of various factors involved in behavioral states, Dr. Calhoun is now approaching a stage of computer analyis where he has available indices on: (1) growth, (2) vitamin A storage, (3) reproduction, (4) arthritis, (5) serum lipids, (6) blood clotting, (7) mortality, (8) social withdrawal, (9) social relations, and (10) spatial attachment. These indices will be utilized initially to explore the degree to which individuals with similar histories (in part determined by these indices themselves) do in fact resemble each other with regard to these indices. Although several strategies will have to be followed in this assessment, the final objective will be to expand the conceptual framework of the stochastics of social systems with primary emphasis on the index of social withdrawal.

Biological Substrate of Behavioral Functions

In this major category of research being carried out in the Laboratory the emphasis is on the organic structural factors which underlie behavioral functions, an area of investigation belonging almost exclusively to the Section on Neuropsychology. Important advances have been made in each of three major fields in which the Section has been involved. Since it would extend the report inordinately to separate out from the discussions of the biological substrates determined by experimental intervention from those interventions occurring naturally, such as epilepsy and Parkinson's disease, the consideration of the latter has been included with the present discussion of the work of the Section. It is clear, however, that the methods conventionally used by the Section have relevance as well for the subsequent section "Destroyers of Behavioral Function." The three main areas of work of the Section may be considered under: cortical mechanisms in sensation and perception, cortical mechan-

isms in problem solving, and cortical-subcortical relations and the regulation of behavior.

Cortical Mechanisms in Sensation and Perception

Vision.—The experiments on visual functions have been concerned largely with the way in which the cortical visual areas interact. Specifically, the aim is to test the proposal that the prestriate cortex is an essential relay in a pathway linking the striate area with the inferotemporal "visual" area. The implication of a series of studies is that the inferotemporal area is important for pattern perception not only in macular vision (i.e., the field of greatest visual acuity), but also in peripheral vision. This tentative conclusion has an important bearing on the visual studies being carried out on neurosurgical patients, described next.

The first experiment on the effects in man of unilateral temporal lobe excisions (performed by NINDB surgeons for the relief of epilepsy) indicated that letter recognition was impaired in the visual field contralateral to the removal. The finding is consistent with the view, first suggested by some work on monkeys, that each temporal lobe is more intimately related to the striate cortex of the same hemisphere (and hence to the contralateral visual field) than it is to the striate cortex of the opposite hemisphere. An alternative explanation of the contralateral impairment is possible, however. A temporal lobe removal in man is nearly always accompanied by damage to the genicule-striate radiations, resulting in a contralateral upper quadrantic field defect. Although care is taken to expose visual material in an intact part of the field (e.g., along the horizontal meridian or even in the ventral quadrant) the possibility exists that the so-called intact field is in fact defective in subtle ways as a direct result of the radiation damage. To investigate this question, a second experiment was performed in which, in addition to pattern discrimination, basic sensory capacity in the "spared" fields was determined in 15 cases with left and 15 with right temporal lobe removals together with 15 normal control subjects matched for age, sex and education. As before, discrimination of letters and patterns was found to be impaired in the field contralateral to the excisions. Thus, the pattern discrimination loss in man would seem to be explicable in the same way as the pattern discrimination loss in monkeys, i.e., as a result of damage to the temporal (and perhaps, specifically, the inferotemporal cortex).

Audition.—The work this year in audition has failed to confirm an earlier suggestion that the superior temporal cortex serves functions in audition analogous to those served by the inferotemporal cortex in vision. Specifically, lesions oof the superior temporal convolution which spare the primary auditory projection area in the supratemporal plane do not seem to interfere with auditory discrimination performance. Since negative results were also obtained following lesions of the primary auditory area alone, an experiment has been undertaken which involves total lesions of the superior temporal gyrus, combining the two areas removed separately in the previous studies. Preliminary results indicate that the total removal does produce losses in the retnetion of an auditory frequency discrimination and in auditory frequency thresholds. The defect does not appear to be confined to audition, however, but extends to visual and spatial problems as well, thereby raising a puzzling problem for interpretation.

Somesthesis.—The over-all aim of this program is to identify the cortical mechanisms serving somesthetic sensation-perception, learning, and memory. In the past year, the work has concentrated on contributions to discrimination learning and performance made by regions *outside* the classical sensorimotor area, located in the hemisphere *contralateral* to each hand. One such contribution, made by the sensorimotor area *ipsilateral* to the hand tested, was described in last year's report: monkeys with *ipsilateral sensorimotor lesions* showed deficits in learning difficult form discrimination, whereas they were unimpaired in learning preliminary, easier discrimination habits. The opposite pattern of deficit has now been found in monkeys with *contralateral nonsensorimotor lesions*: They have enormous difficulty in learning the pre-

liminary habits, whereas once this basic strategy has been acquired, they show the normal rate of learning on the difficult form discriminations. With respect to thresholds for detection of size or roughness differences, the two lesions had similar effects: size thresholds were unaffected, whereas roughness thresholds were elevated. The experiments show that all the factors necessary for normal somesthetic learning are *not* contained within the contralateral sensorimotor area. Instead, it seems likely that the deficit depends on afferents to or efferents from the ipsilateral area and in this sense should be regarded as a true sensory or sensorimotor impairment.

By contrast, the unilateral nonsensorimotor lesion (which included section of the forebrain commissures) has effects which are strictly contralateral and which appear to depend crucially upon the loss of cortico-cortical connections.

The effects of cerebral lesions on somesthetic functions are also being studied in man. Patients admitted to the Clinical Center for surgical therapy of Parkinson's disease (lesions of nucleus ventralis lateralis) are being studied pre- and post-operatively. This nucleus is the origin of the major afferent system to the motor cortex, a region which may serve somatosensory functions as well. Quantitative tests of punctate pressure sensitivity, two-point discrimination, and point localization are applied to the face, hands, trunk. and feet in order to determine the nature and distribution of the possible impairment. Only a few suitable patients are obtainable each year, and more are needed before results can be reported.

Visuomotor Coordination.—Observations made on the monkeys with the unilateral nonsensorimotor lesion including section of the forebrain commissures suggested that, in addition to the somesthetic loss described earlier, there is a striking deficit in visual guidance of the limbs contralateral to the damaged hemisphere. Tentatively, it can be concluded that there is a loss of crossed integration between the visual input to one hemisphere and the motor output from the other. Crossed visuomotor reactions in animals with section of the commissures alone, as described in the literature, have not yielded as striking or as consistent effects as those described here. The role of a unilateral cortical removal in producing this "disconnection syndrome" appears to be unexpectedly important and calls into question the adequacy of the current conception of the mechanism involved in such syndromes.

Cortical Mechanisms in Problem Solving

Further attempts have been made this year to specify the nature of the behavioral loss produced by lesions of the principalis area in the monkey. It was established long ago that this was the focal lesion in the frontal lobes for yielding the classical delayed-response and delayed-alternation deficits. More recently, evidence was obtained suggesting that the major source of the difficulty in these tests for animals with pricipalis lesions was the spatial feature. To pursue this possibility further, animals have been trained on an object alternation problem which contains the same delay and reversal features as those of the classical tests but differs from them in eliminating spatiality as a relevant cue. It should be noted that eliminating the spatial cue renders the problem inordinately more difficult for normal animals. Despite this, preliminary results indicate that a principalis lesion which is confined to the banks and depths of the principalis sulcus, while seriously interfering with performance on spatial alternation, leaves performance on the more difficult, nonspatial version completely unimpaired. If this preliminary finding is confirmed it will not only provide a dramatic demonstration of the specificity of the defect produced by principalis lesions, but it will also pave the way for an intensive investigation into the nature of the spatial impairment.

Cortical-subcortical Relations and the Regulation of Behavior

The classical view that behavioral responses are produced by cortico-cortical influences playing upon the precentral motor cortex has received little support in recent years. On the other hand there is increasing evidence that there is considerable regulation of behavior through cortico-subcortical mechanisms. Be-

havioral studies implicating the caudate nucleus in behavior known to be subserved by frontal cortex, together with the recent discovery of a topographically organized system of direct connections between cortex and caudate nucleus has focused our attention on this subcortical structure.

Our earlier work had demonstrated that the head of the caudate nucleus subserves many of the functions of the frontal cortex with which it is anatomically related. However, recent anatomical findings suggested that a more precise relationship existed; namely, that on the one hand, the anterodorsal sector of the head of the caudate and dorsolateral frontal cortex would have similar functions, and that, on the other hand, the ventrolateral sector of the head of the caudate and orbitofrontal cortex would have similar functions. In a behavioral study investigating this possibility it was clearly evident that the anterodorsal caudate and dorsolateral frontal cortex did have similar functions, but the evidence for the ventrolateral and orbitofrontal cortical relationship was, at best, equivocal. Further analysis of the data this year, however, indicates that the equivocal result was an artefact of the scoring procedure and that the ventrolateral caudate and orbitofrontal cortex do indeed have similar functions. This same study had demonstrated that the tail of the caudate and inferotemporal cortex also have similar functions, both being concerned with visually-guided behavior.

One implication of this close similarity in function between caudate and cortex is that in the absence of cortex the caudate may be able to subserve cortical functions. If this is proven to be the case, a solution will have been found to one of the major problems in neuropsychology; a mechanism of vicarious functioning will have been discovered. Two strategies are being followed to explore this problem. Both involve placing lesions in infant monkeys since it is known that cortical lesions sustained in infancy are likely to have less severe effects than the identical lesions sustained later in life. The first strategy is to examine the effects of partial and total frontal lobe ablations in infancy (60 days of age) and at a later age (2 to 3 years of age) on a wide variety of tests in which frontal lobe function is implicated. The second strategy is more specifically concerned with the major question as to whether or not the caudate nucleaus subsumes functions of the cortex in its absence. For this purpose both infant and adult monkeys are being prepared with caudate nucleus lesions and with combined caudate and cortex lesions .They will be tested later on the battery of tests used for the frontal animals. The Section has some preliminary data which suggest that a pattern of deficits and sparing will be found as a function of prefrontal lesions which will provide a sound basis for demonstrating whether or not the caudate is a locus of vicarious functioning.

Two other studies have been concerned with an analysis of the function of the caudate nucleus. The one followed the same design that was so successful in dissociating the spatial factor from the reversal factor in a frontal animal's impairment. The other study, analyzing the function of the caudate, utilized the methods of prism-adaptation studies. This work has now been completed. Of the many cortical and subcortical lesions sampled, those in frontal cortex and in the caudate nucleus impaired the process of prism adaptation. These findings lend further support to the notion that the caudate and prefrontal cortex have similar functions. It suggests further that both may be concerned with processes involved in visuomotor coordination.

Destroyers of Behavioral Function

The Laboratory of Psychology is presently engaged in studies of two major areas which concern themselves with forces having a negative impact on the quality of behavioral functioning: the effects of crowding and the effects of schizophrenia. Another area of this kind of effect—that of brain damage—has already been considered in the part dealing with neuropsychology.

Crowding

Dr. Calhoun has conducted a literature survey of population in its relation to mental health, as background for an extensive empirical investigation of the effects of crowding

in rats which he expects to commence in the next fiscal year. A 1000-page anthology based upon 400 recent articles and books has been assembled. This is serving as a basis for assessing the influence of changes in the size of the human population, its geographic distribution, and its composition on mental health, and to identify major problem areas justifying more intensive investigation. This anthology is also serving as the starting point from which to explore a theory of concept creativity arising from his research on the duration and sequence of behaviors in the rat. This theory provides a method for computer-aided identification of ideas that may be most profitably associated in developing a projection from consensus opinion. Furthermore, it provides a basis for associations between novel ideas and consensus ideas which have the greatest probability of generating useful new conceptual relationships.

Dr. Calhoun, in his survey of literature on population and mental health, has concentrated on papers in which the author has attempted to develop a general conceptual framework, or ones which try to define major issues which are now apparent or will be in the future. He is also concerned with methods for dealing with those identified problems, and, while he has by-passed strictly experimental or clinical studies, the major gaps in communication between the several disciplines which study the interrelationship of population and mental health are apparent in these papers. Each discipline seems to have particular concepts of which other disciplines are unaware or unappreciative. Dr. Calhoun hopes to provide a tool which researchers could bring to bear on the problem of population and mental health.

Schizophrenia

The work on *schizophrenia* which are originally concentrated in the Officie of the Chief has enlisted the efforts of several persons outside this office. This work has encompassed studies in perception, behavioral deficits of various kinds, emphasizing particularly deficits of attention and problem-solving, and the relations between performance deficits and psychological arousal. The heredity problem has been vigorously pursued, particularly the heredity response in the "susceptible" person, as has the further extension and development of the theory of "segmental set."

Over the past several yers, Dr. Carlson together with Dr. Feinberg have studied the extraordinary lengthening of response-time which schizophrenic patients manifest in judging the velocity of a moving visual stimulus. This effect is not attributable to inattention, lack of motivation. or misunderstanding of the task. It appears to be a distortion in the functional use of relatively short time-intervals, but from the literature on the subject and from our own results it is clear that the matter cannot be explicated adequately without investigation of some basic methodological and conceptual issues involved in the study of time-judgment. One major problem is that logically equivalent methods of assessing time-perception do not always yield logically consistent results, even with normal individuals. As long as the reasons for such discrepancies remain obscure, it is not possible to determine the validity of any given substantive effect with sufficient generality to be useful.

These investigators have, therefore, undertaken a study directed toward a number of problems involved in understanding time-perception itself, as well as toward determining the nature of the particular deviation which seems to occur with schizophrenics. This study requires the collection of a rather large amount of time-judgment data from normal groups and schizophrenic and neurological patients. Most of the preliminary work involved in determining suitable experimental procedures and methods of analysis have been completed this year.

Dr. Zahn's investigations continue to focus on behavioral deficits in schizophrenia, with emphasis on deficits of attention and on the relations between performance deficits and physiological arousal.

During the past year emphasis has been given to studies involving the concurrent recording of the responses of autonomic variables such as skin resistance, heart rate, finger pulse volume, skin temperature and respiration

to stimuli which may be considered as varying in meaning or in demandingness. Previous work had shown that, while acute and chronic schizophrenic subjects are respectively equal to or greater than normals in physiological responsivity to "meaningless" auditory stimuli, they are significantly lower in physiological responsivity to the more demanding stimuli of a simple reaction time experiment.

Similar investigations are in progress with identical twins and their parents and with parents whose biological or adopted children are schizophrenic. Preliminary analyses of the data of 12 twin pairs who are discordant for schizophrenia show that the relationships of physiological responsivity to the demandingness of the stimuli for the non-schizophrenic and schizophrenic twins are much like those previously obtained for unrelated normal and schizophrenic subjects, respectively. Reaction time differences show a similar relationship. This tentatively suggests that these phenomena are related to the symptomatic presence of schizophrenic rather than to a genetic "substrate." Further and more detailed analyses of the physiological characteristics of these subjects are in progress.

Another study, done with Dr. Schooler of LSES, NIMH, investigated the influence of having a patient cooperate in a block design task with another "patient" (a confederate) as opposed to doing the task with just the experimenter present on performance and on the level of arousal. Contrary to expectation, the performance level was improved by cooperation with the other "patient" but in accord with expectation, the level of physiological arousal was significantly increased by working with the other "patient."

The huge amount of data collected in these studies has necessitated the development of a system for automatically reducing it by computer directly from analog tape records. Such a system for the GSR has been in operation during the past year and data from over 300 experimental sessions have been processed. A similar system for reducing heart rate data is currently under development.

The "set index," a reaction time method of proven effectiveness for discriminating between chronic and acute schizophrenics, brain injured subjects, and normal controls, is being used to test subjects with psychomotor epilepsy before and after surgical (usually temporal lobectomy) treatment of the disorder. Our interest in this group stems from the frequent reports of their schizophrenic-like behavior. Dr. Zahn hopes to be able to determine whether a temporal lobe disturbance might be involved in the attentional deficits in schizophrenia.

Another reaction time study with chronic schizophrenics concerns the effects on reaction time of shock reinforcement for slow responses. Previously we had found that this technique increased the speed of response markedly in chronic schizophrenic patients. The purpose of the current study is to replicate this and to determine the possible roles of information and arousal level in the improvement.

Dr. Jerome and Dr. Young have been studying the problem-solving ability of schizophrenics on HEPP. When conditions are provided to enable schizophrenics to make a maximal effort to achieve an understanding of the instructions, they are usually capable of solving the simpler HEPP problems by some means. They do, however, manifest various deficiencies which are of the kind that have been characterized elsewhere in this report under the discussion of higher thought processes. Schizophrenic performance has been compared with that of normal subjects, normal twin siblings and parents. Normal control subjects (of college sophomore level), whose mental test scores are far superior to those of schizophrenics, solve all problems more efficiently and use more sophisticated heuristics than schizophrenics. This is, of course, expected. The performance of the normal twin sibling is superior to that of his schizophrenic sibling only on the average; the instances in which the schizophrenic is superior to the normal being less frequent than the converse. It is very common for at least one of the parents of a schizophrenic to exhibit obvious heuristic deficits such as disorganization of inquiry and poor comprehension of implications which are characteristic of the schizophrenic himself. Such parents often fail to generalize their solution strategy when the problem size increases and evidence more dif-

ficulty in changing set when problem type necessitates such a change.

The focus of Dr. Rosenthal's research has become more and more sharply outlined around two points: (1) The contribution of heredity to schizophrenia in the context of different early rearing environments. (2) The nature of the personality-behavioral configurations reflecting this heredity component in the non-schizophrenic but "susceptible" person.

In carrying out research on these issues, he is now actively involved in six research projects, but to a different extent in each.

A continuing study of the Genain Quadruplets. His collaborator in this study is Dr. Olive W. Quinn, Professor of Sociology at Goucher College, They are following not only the clinical course of the girls, but also their life experiences and their reactions to them. However, they are not limiting their concern to a narrative description of events and behaviors in the usual kind of follow-up study. They plan to capitalize on the genetic identity of the girls and the mass of detailed history available of their developmental years. They hope to elucidate more precisely the role of various personality functions in the development and expression of mental illness, emphasizing the kinds of experiences that determine the response pattern developed by each girl with respect to the particular function, the reaction of involved persons to that response, and the consequent developmental sequences (especially their adaptational character) that lead toward schizophrenic behavior. We will include such functions as sexuality, aggression, dependency, interpersonal attachments, and perhaps some others.

Studies of discordant MZ twins and their parents. Dr. Rosenthal has reduced his participation in this study for several reasons: (*a*) It does not help to elucidate the contribution of heredity to schizophrenia, as he thought it might when he first proposed the study. This earlier belief was based on differences that were found between discordant and concordant MZ twins in Slater's twin series, but subsequent studies by Kringlen and Tienari did not show the same differences. (*b*) He disapproves of the method of sampling used in the study, and consequently he is not certain that the families brought in for study are representative of discordant-twin families in general. (*c*) The value of studying psychological differences in the twins themselves is limited since the differences by and large reflect the ravages of the illness, which can be studied incomparisons of schizophrenics and normal controls. This is not a final judgment but he does not expect it to change.

A study in Israel which is designed specifically to get at the two main interests mentioned above. His principal collaborator is Dr. Shmuel Nelga rof Kiryat Tivon, Israel. The study involves two presumed genotypes (schizophrenic vs. non-schizophrenic parents) and two different rearing environments: the usual nuclear family setting vs. the kibbutz way of life. The design permits the partitioning of variance into proportions associated with type of parent, type of rearing, and interaction between parentage and rearing with respect to a variety of personality characteristics and test measures in children aged 9 to 12. The study did not get under way until September, 1965 because Dr. Nagler had to fulfill earlier commitments.

The preliminary phase has involved discussions with several heads of hospitals, educational and mental health officials to obtain permission to use hospital and school records for locating index and control families. This phase has not been easy or quick, but Dr. Nagler has managed to obtain cooperation at every point so far. Most cases have been selected and it looks as though we can meet our hoped for n of 20 Ss in each of the 4 cells in our design. A full staff of very able people has been assembled including, besides Dr. Nagler, a psychiatrist, a psychologist, two social workers, a secretary and a consultant who is Professor of Psychology at Hebrew University. The staff has been holding meetings regularly to deal with many problems: the tests and procedures to be used, the approach to the kibbutzim, schools and families, the length of time set aside for the examination of the children, the location of the examination site—which will probably be Hebrew University, the timing of the testing (two half days), observational procedures in

natural settings, the type of interviews to be used with the child and key informants, the selection of controls, etc.

During this phase, Dr. Rosenthal has been in close contact with Dr. Nagler. A dry run evaluation of tests and procedures using non-subjects will be started in mid-April. Dr. Rosenthal will visit Israel in mid-May to review the study and help the staff settle on a final selection of procedures. In all probability, the examinations of the actual subjects will begin in June or July.

A study in Denmark to evaluate the incidence of schizophrenia in the biological and adoptive families of children who were adopted by non-family members and who subsequently became schizophrenic. Dr. Rosenthal's collaborators are Dr. S. S. Kety, Dr. P. Wender, and Dr. F. Schulsinger. At this stage, all index cases (schizophrenic and manic-depressive adoptees) have been selected as well as a number of their controls. In the meantime, the group have begun the study of the families in those instances where selected controls have been obtained. This last phase of the study should take another year or more.

A study in Denmark of children who had a schizophrenic parent but who were given up for adoption to unrelated persons at an early age. Dr. Rosenthal is carrying out this project with Dr. S. S. Kety, Dr. P. Wender, and Dr.F. Schulsinger. This study began in August, 1965 with actual examination of subjects begun in November, 1965. To date, about 25 subjects of each index and control, have been examined, and 20 index cases and 20 controls selected. At this point, it cannot be determined how many index cases the search will yield, but this phase of the study is progressing well and we may not be far off our original estimate of 100 index cases. If the yield of cases reaches that high, it will probably be impossible to test the original group of 100 in the two years for which they originally budgeted. The study will have to be extended or a number of subjects will have to be omitted from the study. Thus far, very few cases have been lost. A very preliminary glance at some data gives the group reason to believe that they will find theoretically relevant differences between the index subjects and their controls on a number of aspects of personality functioning.

A study of adoptive parents and natural parents of schizophrenic subjects, being done at the NIH. To date 10 families have been examined in which the schizophrenic child was adopted and 3 families in which the schizophrenic subject was the biological child. The latter families are matched to the former with respect to SES, sex and age of the child, age of the parents, et al. The two groups of parents will be compared with respect to various aspects of personality functioning. An additional 7 control families have been selected and it is expected that all will be examined by the end of the summer. If the results of the study are promising, the investigators will plan to extend it to include a more systematic sampling and a larger battery of tests.

During the year Dr. Shakow has continued his work on previous empirical studies of schizophrenia and further analysis of the extensive data for its theoretical implications. His three major productions have been the preparation of papers on schizophrenic perceptual processes, a consideration of the implications of the findings from the experimental psychological studies of schizophrenia for the understanding of normal function, and a detailed paper on the problems of nosology in psychopathology and their relationship to research in the field.

The empirical study reported deals with auditory apperceptive reactions to the tautophone by schizophrenic and normal subjects. The vowel patterns which comprise the stimuli of the tautophone, the auditory apperceptive device used, were administered to 25 schizophrenic male patients (11 hebephrenic, 6 paranoid, and 8 of other types) and to 20 normal male subjects. Quantitative, as well as qualitative, analyses differentiated the groups. These analyses indicated that the normal subjects appeared to accept the tautophone as an apperceptive task, the hebephrenic subjects personalized the situation, and the paranoid subjects seemed to impersonalize the task. The profiles found in these groups are consistent with findings from other perceptual, apperceptive and projective studies. The personality

structure of schizophrenics and of schizophrenic subtypes are considered in relation to two aspects of attention—scanning and articulation. The major finding is that schizophrenics show extreme forms of behavior—either under- or over-scanning, or under-or over-articulation.

In the consideration of the implications of schizoprenia research for the understanding of normal psychological function, Dr. Shakow first considered the general relationships of psychopathology to normality, then reviewed several decades of research in schizophrenia, giving the major findings and the inferences for psychological functioning in schizophrenia and normality. These generalizations were then seen in the context of various theories with regard to psychological functioning particularly the different between the establishment of generalized and segmental set.

In the discussion of the problem of nosology, Dr. Shakow considered first the general attitude the investigator should take in confronting the problem of classification of mental disorder. He viewed this in the context of his personal associations with the problem as pointed up by his own studies of schizophrenia. Various underlying issues of the classification problem were considered. Dr. Shakow concludes with a summary of what he learned during his own efforts in the categoraization of mental disorders, both the problems involved and the cautions to be kept in mind when approaching these problems. These included the FOLLOWING POINTS: the use of multiple simple approaches, care in the use of controls with either direct or inferential studies, a proper balance between the holistic and the segmental approaches, stress on the nomethetic based on extensive idographic studies, an increased emphasis on metaphoric as opposed to literal approaches both in the use of generalizations and in the use of structural/dynamic descriptions when they are based on earlier careful use of the empiric, and a concentration on the prior acquisition of context experience as background for the employment of method.

Enhancers of Behavioral Function

Some preliminary work in this broad area undertaken by Dr. Bergman in the context of psychotherapy was discontinued with his premature death. The only investigations being currently conducted in this important area are those by Drs. Parloff and Datta of the Section on Personality on Westinghouse winners.

In view of the fact that the more and less creative samples investigated by Drs. Parloff and Datta are clearly differentiated on personality variables, a study has been undertaken to determine whether characteristic parent-child relationships are associated with demonstrated differences in subsequent creative performance. Subject reports on their relationships with each parent during each of three age periods are currently being analyzed but no conclusions are available as yet.

Further evidence revealed that the personality patterns which are associated with high creative performance in a group of adolescents studied by Dr. Datta and Dr. Parloff are consistent across vocations. This finding is of particular interest since the data analyses reveal many highly significant personality differences which are attributable to vocational interest. A similar analysis of variance was performed on data obtained from four highly creative adult samples and their appropriate controls. As in the adolescent sample, personality factors are clearly associated with creativity level independent of the particular vocation. A comparison of the personality variables which differentiate both the adolescent and the adult creative samples from their control groups revealed the following similarities: high levels of ambition, activity, forcefulness, self-acceptance, and spontaneity; ability to recognize and admit views which are unconventional; a high level of independence and self-reliance; and a broad range of interests. The personality features which characterize and differentiate the adult creative sample are very similar to those of the adolescent creative sample; however, the members of the adult creative group appear to be even less concerned than the adolescent group with making a good impression on others, are more dominant, have a greater sense of well-being, and are more flexible. A two-year follow-up of the adolescent sample reveals that although the personality

characteristics remain fairly stable, the changes which occur in the "high" creative sample appear to be in the direction of moving closer to the personality pattern which has been identified as characterizing the adult creative man. Most impressive, perhaps, is the fact that the adolescent creative sample became significantly more flexible than the control group during the two years since high school graduation. The two-year follow-up retesting of the total sample revealed that the "high" creative group continued to be differentiated from the "less" creative group on the same personality scales which had earlier differentiated them.

Since the data analyses based on the adolescent sample indicate that a longitudinal study is warranted, periodic follow-up studies of this group and the replication sample will be undertaken. Another aspect of the creativity research program will be concerned with experimentally investigating variables that seem to affect creative performance. In the view of the investigators, one of the factors which has been neglected in these studies is the role of the standards and values of the "creative man." It may not be sufficient simply to train individuals to report a number of "original" ideas, for the creative man must also be able to select from among his ideas those which are worth pursuing. On the basis of some preliminary work undertaken by Dr. Datta, a research project will be conducted to assess the relationship between the subject's standards and his judged creativity level. The research will also investigate the subject's capacity to produce "creative" works in accordance with (*a*) the consensual standards of judges, and (*b*) the subject's own standards for creative performance.

Theoretical, Methodological and Technical Advances

During the course of the year several advances have been made along theoretical, methodological and technical lines.

An important advance in the logic of measurement was proposed by Dr. Stanley. Increasing evidence that neonatal dogs can learn, when correlated with similar evidence obtained from human infants by other investigators, has suggested a new approach to behavioral quantification. Rather than taking behavioral theory as the basis for measurement, the approach starts with a *general* theory of measurement. The general theory is then applied to behavior as recorded in learning situations. Considerable work on specific equations remains to be done, but it is already clear that the mathematical manipulations of idealized "data" curves will generate specific research. For example, mathematical analysis implies that continuous (100%) operant or instrumental reinforcer action can be viewed as functioning indirectly. That is, the idealized curves alone do not imply that the reinforced response is being strengthened. Rather, they imply only that nonreinforced responses habituate or extinguish. This view of instrumental and operant reinforcer action is similar to, but not identical with, aspects of contiguity theory, statistical learning theory, and an evolutionary analogy of reinforcer action suggested by others.

Methodological and technical advances have been made in various parts of the Laboratory.

Dr. Schaefer has developed some new measurement techniques. One of the problems facing the investigator who is concerned with the antecedents of personality development is the fact that he must frequently depend upon retrospective accounts from the subject or patient regarding significant life experiences. Dr. Schaefer, in cooperation with Dr. Nancy Bayley, has undertaken a much needed study to test the validity of the adult subject's reports of his early relationships with his parents. By utilizing material obtained from a longitudinal study, the Berkeley Growth Study, it has been possible to compare the subject's ratings of his mother's behavior toward him during adolescence and infancy with the ratings made by psychologists who actually observed the parent-child interaction at the time of the subject's infancy and adolescence. It was found that subjects at age 36 were able to report the behavior of their parents which had occurred 25 years earlier in a

manner which agreed closely with the description obtained at the time from the professional observer. The correlations between the subject's etrospective reports and the observer's reports regarding parent-child relationships during infancy were, however, much lower. This research supports the use of subject's recall of parent behaviors at least as early as age 11. Dr. Schaefer has also investigated a simple technique which could be devised to provide systematic information concerning the individual child's behavior and adjustment from early childhood through late adolescence. He is attempting to determine whether school teachers, if properly trained and if provided with good methods, can be a source of such data. One of the advantages of such systematic data collection would be the early detection of maladjusted behavior.

Dr. Stanley, because of the nature of his research, has worked hard to achieve automation of his apparatus, which would result in the improvement of the measurement of early sucking behavior, make better control over liquid stimuli possible, and reduce the need for arduous work schedules of personnel in the unit. Considerable time has been spent by this unit together with the Technical Development Section in designing and testing automated research equipment. The equipment has proved adequate for maintenance of healthy infant animals, but inadequate for research. It is hoped that the equipment will appreciatively reduce the need for around-the-clock work by personnel.

Dr. Jerome and his group have also made technical advances in the development of a Sequential Relations Apparatus. The SRA, adapted from the Logical Analysis Device, presents a series of problems involving the combination of a number of elements into various causal relations. The subject's task is to analyze the relations that exist between a set of input buttons and a goal signal. When he has discovered how to produce the goal signal under the constraints of the problem, he has solved it. The whole procedure is controlled and recorded by an IBM 1620 computer; this allows a maximum flexibility for developing procedures and experimental controls which would be impossible with special purpose equipment. Five subjects have been run in the exploratory phase of this study;procedures and problems are still in the developmental stage.

The 1311 Disk Drive which has been added to the NIMH computing system operated by the Laboratory has greatly expedited several phases of the data processing work. However, since more than half of the logged time on the computer is consumed by the preparation of copy, the efficiency of the facility could probably be doubled by the addition of a moderately fast line printer.

Mr. John James, the programmer associated with this facility, has written a machine-free macro-language implemented by the IBM 1620. It is anticipated that it will greatly reduce the confusion of tongues created by having access to several machines, each with its own vernacular. The system permits the programmer to write in a single computer language when using an assembler for which Mr. James has developed macro instructions. He is reporting on this language to the Fall Joint Computer Conference of AFIPS (American Federation of Information Processing Societies).

Dr. Rosvold's group has made progress with several new devices. Preliminary testing of a remote stimulation equipment has been completed, and all units are satisfactory. A different switching mechanism will have to be developed, however, for reliable switching from electrode to electrode. General Electric is currently working on this problem and appears to be ready to supply the more reliable switch. The device for locating structures within the brain by using changes in impedance from an electrode passing through tissue has been improved to the point where it is now a highly reliable instrument. It will be used in experiments requiring accurate locating of subcortical structures. An ultrasonic device for making subcortical lesions deep in the brain without damaging intervening tissue should be delivered soon.

Administrative Aspects of Laboratory

I should like finally to review some special aspects of the Laboratory's functioning during

the last year. First let me consider several persistent problems still seeking solution before discussing the general condition of the Laboratory.

Despite a generally favorable atmosphere, four major problems still trouble the Laboratory: animal procurement, isolated units, equipment, and salary level. Difficulty in procuring appropriate animals for research in the behavioral sciences has long been a problem because NIH's system favors users of large numbers of animals whose only requirement is good physical care. Our investigators, however, require animals whose behavioral histories are known. Because of the inadequacies of the NIH system we have been forced, on the one hand, to sacrifice precious experimental space for the mere holding of animals and, on the other, to spend valuable time of experimenters on the mere procurement of appropriate animals. I trust that the work of Dr. Stanley's Committee, together with the efforts being made with the Laboratory Aides Branch, will improve this handicapping situation.

The second problem is that of equipment. A great deal of effort is being devoted to the construction of special apparatus systems. The systems are carefully designed, but their construction has been held up by numerous delays in obtaining funds, in contract negotiations, in delivery by a contractor, or in fabrication by NIH facilities. Professionals within a Section, who should be devoting their time to research activities, would have to do a major portion of this work to avoid delay. Even if this is sometimes possible, as it is generally not, without exception it is undesirable. The result is frequently that experiments which should have been completed last year are not undertaken at least until the next year. NIH's facilities for supplying suitable apparatus for experimental psychological research needs careful examination.

The efficient handling of isolated facilities such as the Poolesville Farm is a third major problem. The administrative problems attendant upon the establishment of a new research unit in rather primitive housing have, during the past year, channelled time and effort away from productive research. The development of such facilities would be facilitated by the assignment of special administrative assistance during the formative stages to help with such particularly difficult problems as the procurement of personnel. Perhaps an administrative officer should give a specified amount of time (say a day, a week, or whatever time seems appropriate) to such new operations until they are well established units with their own administrative force. Despite the problems at Poolesville, the refinement of unique research apparatus, the conduct of current research, and the planning of future research emphasis is gratifying. The morale of the staff remains high and is boosted still higher by the anticipation of organizing a first class productive research laboratory when the prospect of the new Animal Behavior Laboratory building becomes a reality in FY 1967.

The fourth major problem is the super-grade restriction of behavioral scientists. Unless the present unfair discrimination in such promotions is ameliorated within the next few years, the continued employment, as well as recruitment, of promising and productive scientists in our area will be jeopardized.

New developments hold much promise for the Laboratory. The development of the Poolesville Farm Operation and the new Child Research Building should enable us to extend our work in the respective areas involved. I have already said something about the Poolesville operation in this respect. Let me add a few words about the Child Research operation. The research of the Section on Early Learning and Development outlined earlier represents, essentially, the research themes which are to be emphasized in the reorganized Section when it moves in about a year to its new facility in the Child Research Center. Plans are being made to add additional personnel to participate in experimental researches under the themes outlined. Until now, the lack of facilities has limited us considerably in the experimental work we could do. It is hoped that most of the present researches listed under the five foci will be completed and reports from them published before the Section moves to the new research facility where it will continue with new studies in these areas.

The possibility of establishing a Section on Behavioral Genetics should be considered in the context of other possible administrative changes. A separate section in the Laboratory of Psychology concerned with these and related research problems seems administratively desirable because studies in this area are expensive and because they are important to the scientific development of abnormal psychology and psychiatry. The proposed section should remain small for the first few years, dedicated to the successful completion of the studies already begun and additional ones addressed to the same basic issues.

In conclusion let me say that both the morale and productivity of the Laboratory are good as evidenced by the body of this report and the publications which have come out of the Laboratory during this year. We have also had a group of visiting scientists and fellows of high quality, a condition we expect will continue in the coming years. It has also been gratifying to witness the recognition that many of the members of the Laboratory have received in the positions they have been offered by other institutions, as well as the consultations they have been called upon to provide in areas directly connected with their fields of competence.

CHILD RESEARCH BRANCH

Clinical Investigations

Introduction—Now completing its seventh year of longitudinal research on three interconnected problems—the exploration of psychological patterns of marriage, of early parent-infant relationships and of stable individual differences between infants from birth to age three years—the Child Research Branch program is at a major transition point. The data from the early studies at three developmental stages (initial marriage relationship, initial parent-infant relationship and the presocialization period when the first infant is 2½ years old) has not been nearly completely analyzed and reported. Plans for the next longitudinal study are complete. By means of this new program to be initiated in September 1966, we will investigate whether the patterns discovered on pilot samples ranging from 30 to 100 subjects can be replicated on samples ranging from 100 to over 1000 subjects. As always the research planning for the core longitudinal study by three Sections of the Branch is carried out in a coordinated fashion, thus facilitating comparison within the same family of marital variables, parent-infant behavior variables, and infant behavior variables. At the same time, each Section remains free to pursue special interests of primary concern within the particular area.

Closely tied in with the new and expanded size of the program is its steadily growing requirements for personnel and for space. A number of new positions, not now available, are urgently needed. It was indeed disappointing, though not totally unexpected on the basis of past experience, to have another six to nine months delay appear before the implementation of our new Developmental Research Building. This delay was occasioned by a dispute over the location of a new highway on the southern boundary of the National Institutes of Health reservation. Unfortunately the resiting of the building will also necessitate an expensive re-traversing of the tentative stage in the Building planning process; hopefully this will not cut so deeply into available money for construction that either the space within the building itself will suffer or that new funds will have to be found. The plan is for an imaginative new building which will meet the best prognostications not only as to the needs of this program, but also to meet the general requirements of conceivable future basic research programs on early family development and on infant development. The unique and spacious "outdoor" play yard enclosed within the walls and ceiling of the building will provide an unparalleled opportunity for controlling the effects of weather on the behavior of preschool children the year around. Much improved facilities for observing and recording the behavior of adults and children will be provided throughout the building. Limited space will also be made available to investigators within the Laboratory of Psychology and the Adult Psychiatry Branch, National Institute of Mental Health, within the build-

ing. There will also be, at long last, some space for Visiting Scientists and for other collaborative studies which we have not been able to accommodate in the past. As now scheduled, and unless further unforeseen delays occur, the building will be open in time for us to utilize the new nursery school laboratory for the next longitudinal study. That is to say, our present facilities in Wilson House will be used to gather early data on our 1000 or more recent marriages, but by the time their infants have reached age 2½ and are ready for study in the nursery school, we should be moved into our new Building.

One difficult problem in planning the new program is to estimate the readiness of the Branch to engage in collaborative studies with other intramural research groups. It has become obvious that the more diverse the information included within an expensive longitudinal effort of this kind, the more opportunity would result for exploring a wider variety of hypotheses. On the other hand, we have discovered in some past studies that we have begun to pass the point of no return in demanding a heavy workload of time and energy from our families, so that fatigue effects or shifts in willingness to cooperate begin to appear. The program is somewhat different from much research going on both at NIH and in other developmental laboratories in Universities because of its concentration on the effects of relatively concrete and simple variables, like sex of the child, birth order, whether bottle or breast fed, how much involvement the parents have with relatives, and the like. Other investigators have urged greater investment in variables like cognitive or perceptual style—and other personality variables—but as a strategic matter, it does not seem unreasonable to try to define the effects of subculture, birth order, sex of the child, and so forth before trying to incorporate variables which while perhaps more appealing theoretically in an abstract sense, may well have less predictive power when trying to create developmental typologies.

A somewhat similar problem has arisen this past year also having to do with the exploratory and basic-naturalistic orientation of the program, somewhat at variance with the current ethos of mental health investigators. We tried and failed to use the foreign grant mechanism to gain support for a cross-cultural collaborative study with the Department of Neuropsychiatry in Taipei, Taiwan. While many factors contributed to this failure, a significant one was the attitude of Study Section members, an attitude which I have had occasion to observe in relation to other naturalistic and basic exploratory studies of behavior. This is an attitude which places higher value upon preformed and limited hypotheses and upon narrow experimental design than upon an open matrix of alternative hypotheses and a design based primarily upon naturalistic observations, supplemented by more precise experiments only as the problem becomes clearer. Essentially, as for a number of years, I continue to feel that a major obstacle to the progress of mental health research is the underestimation of the complexity of the phenomena with which we are confronted and—much too often under the pressure for quick "clean" results—a too-great need for premature definition of the nature of the problem under study. Particularly with the new mathematical tools and the increased computer resources now available, a more honest and complete confrontation of the problems of developmental research on children and on families is now increasingly possible.

There is every expectation that this program may make a substantive contribution to better understanding of the interaction of congenital (possibly genetic) factors with family relationship factors in shaping the behavior and experience of the infant as he grows to age three years. At this time we have clearcut variables in both these domains of interest which are gathered on all our longitudinal subjects. With this in prospect, it seems appropriate during the next year to lay more firm plans than in the past for cross-cultural collaborative studies. For there is always a considerable danger, no matter how well it appears that we have controlled for intervening variables, that our theory of the interaction of mother-father-and-infant across the early developmental stages of the infant's life

has hidden biases deriving from the fact that all the data was gathered within one single culture. The identical behavior can have completely different experiential meaning not only from family to family within the United States, but in a much more radical sense between different national or subcultural environments. Until these studies are carried out, we will not have tested the limits of our theoretical conclusions about what is truly significant in the interaction of parental behavior with infant behavior. If similar results *can* be obtained crossculturally, and while controlling for the relevant congenital factors in the infant, we will have gone a long way toward demonstrating the validity of specific patterns of parent effects on children and specific child effects on parents.

Patterns of Middle Class Early Marriage

The intensive pilot study of 50 recently married couples strongly suggests that, within the middle class at least, four dimensions of the psychological relationship between husband and wife may in a rather critical fashion serve to shape a great variety of behavioral and attitudinal elements in the marriage. Perhaps the most prominent of these is the question of how involved and how attached the husband and wife are to their families of procreation. We are concerned here with a variety of kinds of involvement, including the frequency of telephone contact, of meal times and of other social occasions together; included also is the participation of the older generation in daily decisions made by the couple, the involvement of the husband in a family business, and a variety of other meaningful week-by-week personal involvements. There is an apparent linkage between high involvement with the older generation during the newlywed phase of marriage and professing the Jewish faith; this linkage may be stronger with the less intellectual couples and with families where there is an investment on the part of the husband in a family business.

Another dimension with considerable statistical power to separate patterns within the pilot sample is the wife's or husband's report of a variety of family difficulties during childhood or adolescence. These include complaints about frequent arguments in the family, overly strict discipline and other forms of chronic disturbance, illness and unhappiness. The interesting finding emerged, which is consistent with the concept that a developmental stage during the adult period of life may have differential impact on the two sexes, that husbands who make these complaints become overinvested in career ambition and less intimately involved with their wives, tending to select rather child-centered (as opposed to husband-centered) women to marry. With the wives who enter marriage with these complaints about their past, one finds a more open expression of conflicts directly with the husband in the newlywed phase, interpersonal marital conflicts which are enacted in many social situations as well as in private with the husband. From these preliminary findings we have derived hypotheses to be tested in the next longitudinal study; should these hypotheses be later confirmed they will be clearly relevant to preventive psychiatric planning for intervening with young families in the middle class community.

In addition the question of whether the young wife is, in early marriage, involved primarily with her marital relationship, or in using the marriage as an immediate jumping off place for producing a brood of children, also has turned out to differentiate many other behaviors and attitudes among our sample of couples. Finally, the direct observation of husband-wife communication during experimental situations has provided the dimension of rationality vs. affectivity while solving a new problem. There is a puzzling but intuitively not surprising connection between logical communication style without laughter, without self-derogation and without hostility, and a close attachment by a couple with their relatives and parents. This too will be investigated in the next longitudinal study. It is an illustration of the fulfilment of one of the early aims of the Child Research Branch program, namely to develop new brief tests of family communication and problem-solving which would bear a dependable relationship to larger social role and family structure patterns. Here

the color matching test and the form matching test have considerable value, although as time goes on very likely there will be further technical improvements. At any rate these techniques—as well as some developed by other investigators elsewhere—do represent a breakthrough methodologically toward the day when brief methodology for studying the psychology of families can be built into large scale epidemiological and population research. In addition, considerable work is ongoing toward the improvement of paper and pencil instruments in our marriage research; indeed the large sample of 1000 couples will receive only paper and pencil instruments with the color matching test.

A variety of new problems are upon us this coming year as a result of processing samples of subjects about ten times as large as we have heretofore dealt with. A new computer remote station is required to prevent immediate backup of large amounts of unprocessed data and also to permit immediate data searching and analysis during the course of the project. In order to deal with this new sample three new staff fellows (psychiatrists) will join us July 1st. We will be selecting subjects from the entire Washington area, with the considerable aid of the Capital Beltway; whereas in previous work we limited ourselves more to the Northwest Washington and Montgomery County areas.

Early Parent-Infant Patterns

Research on the early phases of development of the mother-infant relationship, and to a much less extent the father-infant relationship, has progressed now to the point where useful techniques and variables are in ongoing use during the first month of life, during the third month of life, and at a year and a half of age. During the earlier stages of development the data analyzed and reported so far in the literature from our program is in agreement with other investigators, in pointing to the salience of the sex of the infant and of the state of arousal of the infant as determinants of parent-infant behavior. Our group has been able to link these primary biological variables with pre-pregnancy attitudes of the mother-to-be as regards her desires for maternal contact with an infant. It also appears that when directly observing the mother and infant together in their home, the style of the mother's behavior when attempting to quiet an irritable baby or when attempting to provide the infant with pleasurable arousing stimulation, do systematically interact with the above infant variables. The past findings from this area of research are now being replicated on an independent sample carefully controlled for parity and with a wider range of subjects than has been true before with respect to maternal age, ethnicity and education. This does not represent a serious excursion as yet by the Branch into studies of sub-cultural contrast beyond the white middle class within the urban community but rather represents an attempt to get as great a range of psychological differences in our exploration of early parent-infant behaviors.

Studies of Individual Differences in Infants

Work in this Section has continued to pursue the earlier discoveries of an infant lethargy pattern, which appears to predict a dependency and contact-need pattern in the nursery school, as well as to pursue the relationship between respiratory and feeding behaviors in the newborn period to later nursery school persistent task-orientation behavior, and avoidance of socialization. As a result of these interests, and in order to prepare for the next longitudinal study, there has been a concentrated effort to extend and improve our assessment of the neonate in the areas of sleep behavior and other psychophysiological parameters. As mentioned above, there are strong suggestions of a linkage between arousal and sleep variables in the neonate with mediation of maternal contact-comfort.

The program has also discovered that an index of mild physical anomalies, an index obtained by the nursery school teacher, seems to have considerable value. To be specific, we are investigating the hypothesis that in the third year of life anomalous finger and toe construction, together with a high palate, relates to high scores on impulsivity, emotional lability, low tractibility and high levels of ag-

gression. The interesting thing about this is that these findings are all within a non-brain-damaged group of children from which—by all available criteria—children having even minimal likelihood of brain damage had apparently been eliminated. Thus, as has been increasingly evident from recent literature, the factor of very mild hidden morphological immaturity or very mild brain damage, is identifiably significant in determining psychological development.

In the work being planned there will be a new focus on first born children to fit in with the total longitudinal study as well as on expanded studies of visual attention behavior, feeding behavior, birth weight, respiration and their connection with early style of socialization. Three new staff fellows will join the Section this year (a pediatrician and two psychologists) to assist in carrying forward these new directions.

Toward A Theory of Marital Experience

Until the last few years the focus of most developmental research has been on "hard variables" within the tradition of the natural sciences and of experimentation. This has been a recent historical advance as, with better control over variables and with more resourceful data reduction techniques, many new concrete facts are emerging. Nevertheless there are restrictions upon our understanding of the child's development if research is confined to the study of overt behavior without reference to inner experience. Ultimately the experience of the family constitutes the matrix which nurtures the child's personality; the behavior of family members constitutes only direct or indirect and partial expressions of actual experience.

From a theoretical point of view what is most needed now is the development of new models for understanding process variables within family relationships, models which are tied to operational criteria but which at the same time are concerned primarily with experience. During the past year two investigators on our staff have begun work on such a model, which is concerned with the experience of intentionality at any given moment during an actual ongoing relationship between a husband and a wife. Our interest here is to investigate systematic relationships between several ways of experiencing intentionality and how these may be related to certain forms of conversation between the couple. A variety of techniques are being used: interviews, home visits, and movies. Preliminary theoretical papers are in various stages of preparation, having to do with the concepts of play vs. game as forms of interaction and having to do with imaginative vs. unimaginative rigid modes of expressing intention.

New Developments

The Branch is beginning to put more effort into intensive clinical case evaluations of couples who represent salient patterns with respect to theory or to frequency of occurrence. Completely adequate staff resources for carrying out such clinical investigations are not available but some studies of this kind are getting under way. Carrying out clinical investigations, often involving a fairly intensive relationship between one or more of our staff and one or more members of the subject-family, does not precisely conform to previous administrative models for research on normal volunteers. Although these investigative studies may provide certain learning or insight to the subjects in the course of the study, their purpose is in an actual sense not aimed psychotherapy. In those small numbers of cases where the families do suffer from anxiety, treatment-like issues may, however, become involved in the process of investigation. But this is of course true of transference issues which intrude into any intense continuing human relationship. Without regard to whether the study is being carried on by a physician, psychologist or social scientist on our staff, these clinical investigations are not primarily being carried out in a medical treatment contest but rather are being carried out in the context of social science research.

With a large number of young couples being studied as to matters personal, and with the very brief contacts anticipated with many subjects with whom we do not have the opportunity (unlike in our past work) to develop a

meaningful relationship, it is inevitable that the future will bring an increase in episodes of community misunderstanding. These arise from disturbed families as well as from individuals who do not comprehend the connection between scientific investigation of family life and improving public health practice.

As noted above, the extension of certain studies in a very tentative fashion, to include a few lower socioeconomic subjects and to include those who did not graduate from high school, or who have attained an advanced professional degree, and to include older couples beyond the early stages of family development, will give us a broader spectrum of pretest data to use in planning possible comparative studies with our core group of middle class young family subjects.

The opportunity to be relieved during the coming year by my colleague, Richard Q. Bell, Ph.D., of the administrative work in order to reflect upon the scientific and health implications of our findings, and to engage in needed theoretical work, will provide further perspective on the problems referred to in this Annual Report.

ADULT PSYCHIATRY BRANCH

The activities of Adult Psychiatry Branch staff fall into four main categories: (1) planning and execution of specific research efforts, as spelled out in the Individual Project Reports and summarized here; (2) psychiatric clinical care and clinical training; (3) psychiatric research training; and (4) consultative and collaborative work with colleagues and professional groups both at NIH and elsewhere.

Research activities are divided into five sections, as follows: (1) Family Studies, (2) Studies of Personality Development, (3) Twin and Sibling Studies, (4) Studies in Psychosomatic Medicine, (5) Studies of the Psychophysiology of Sleep. Thus, a broad range of interests are represented, from psychosocial to psychobiologic, in the different parts of the program.

The in-patient clinical work is conducted within two 12-bed wards in the Clinical Center; in addition, the Family Studies and the Personality Development Sections work with a number of out-patient families. Also, when appropriate, the cooperation of hospitals and research organizations elsewhere is obtained in order to gain access to larger samples of research data. For example, we have been able to analyze psychological test data collected elsewhere in order to verify leads which have developed from data obtained in our own program. Although most of the research in the program is with human beings, it has become increasingly important for certain psychobiologic hypotheses to be explored first in animal studies.

Family Studies

Three sections in the Adult Psychiatry Branch—the Section on Family Studies, under Dr. Wynne; the Section on Personality Development, under Dr. Shapiro; and the Section on Twin and Sibling Studies, under Dr. Pollin—all regard the family as an area of concern, although, as will be indicated, they work with somewhat different kinds of families and use different concepts and methods in their work.

The Section on Family Studies is concerned with understanding and treating psychopathology, especially schizophrenia, in relation to the family context. In order to do justice to this complex subject, it is necessary to go both within and beyond the family as such: to study, on the one hand, certain dimensions of individual and psychophysiologic functioning, both of schizophrenics and, for comparison purposes, of nonschizophrenics; and to study, on the other hand, factors of the community and broader social structure and culture which help determine the meaning and impact of familial influences in individual development. At present, these research and treatment interests can conveniently be viewed as clustering into six distinct, although closely linked, categories:

Intrafamilial Relationships and Transactions

As reported in previous years, this research program has established that it is possible to differentiate or predict blindly what kind of psychiatric disorder the off-spring of a family

have from the forms in which the other members of the family communicate and interpersonally relate. This work makes use of the sociologic concept of the family as a partially self-contained social organization or social subsystem and of a psychodynamic theory of normal and schizophrenic ego development. In their conceptualization of "normal" development, which goes astray in particular ways with schizophrenics, Wynne and Singer have reasoned that there needs to be a reasonably appropriate "fit" between the innate characteristics of each child at successive stages of maturation and the characteristics of the environment. The latter includes the patterns with which the parents focus attention and meaning and communicate with one another and with their children. This means that certain transactional conditions are necessary as framework for the child to learn, for example, culturally appropriate skills, such as "reality testing," a sense of self and self-boundaries, an interest and ability in establishing interpersonal relationships, and an interest and ability in establishing directionality and goal-directedness in his life.

The form in which the individual child's development proceeds is a resultant not only of the infant's emerging capacity to engage with and become oriented to significant caretakers from whom he can then learn, but of the caretakers' ability to engage the infant's and later the child's attention and to orient him to those aspects of speech, behavior, and other events which will be important in his expectable life experiences. Thus, it is at the stage of engagement, orientation, and what, broadly speaking, can be called attentional processes that the first difficulties can arise which may lead into schizophrenic forms of ego impairment.

Wynne, Singer and colleagues have found that a fruitful concept for linking the difficulties of individual offspring is to deviances in family communicational patterns is to use a concept which labeled the "sharing of foci of attention." Such sharing of attention is regarded as a pre-requisite for learning to relate to another person, to communicate with consensually understood meanings, and to learn the numerous and essential ways of interpreting physical and cultural reality.

Thus, it is hypothesized that communication difficulties in parents which could affect the development of core ego functions that are in fact impaired in schizophrenics, include, most importantly, defects and deviances in the manner in which foci of attention are shared. These problems lead directly into a variety of other difficulties in such areas as use of language, task-orientation and interpersonal relations.

During the past year scoring manuals have been derived from these principles which can be used reliably and quite simply by other investigators. A paper has been completed by Singer and Wynne describing scoring manuals for use with individual Rorschach and TAT tests obtained from parents. Also, during this year two papers have been published in which the Singer Scoring Manual has been applied by Wild et al. at Yale to Object Sorting Test data obtained from parents of schizophrenics and "normals".

Using a similar emphasis upon the transactional aspects of test behavior, rather than the intrapsychic, projective or symbolic aspects, Dr. Nathene Loveland has been working on scoring manuals for use with various forms of the Relation Rorschach technique. This technique consists of asking two or more people with various relationships to each other, such as husband and wife, families, patient and therapist, etc., to see how many things they can find in a Rorschach inkblot on which they can agree. No tester is present during their discussion, which is tape recorded. This approach lends itself to a comparison of each person's behavior in the different interpersonal settings of, for example, individual Rorschach, Spouse Rorschach, Family Rorschach, and Doctor-patient Rorschach. Material from some 80 Spouse Rorschachs is currently being scored, with comparisons of the couples along a variety of dimensions, including the diagnosis of their offspring.

As still another means of studying marital and family transactions, Wynne, Morris and colleagues have been extending their earlier studies. Dr. Morris had blindly predicted diag-

nostic features of offspring from communication styles of parents as found in excerpts of family therapy from which diagnostically relevant facts about the offspring were excluded. Morris has replicated this study on a new sample of excerpts from six families with three other raters also making predictions. Correct predictions of schizophrenic versus nonschizophrenic offspring were made in 21 out of 24 instances. By a conservative test of significance, $p = 0.01$. This indicates that the methods of the Morris-Wynne study are communicable and replicable.

During this year a new interview format was devised for eliciting communication samples from couples with an interviewer but no offspring present. Methods for evaluating and scoring these research interview protocols follow lines similar to those used with the other ways of sampling communication of family members. Each procedure provides a setting in which certain kinds of deviances are somewhat more easily and frequently elicited. These differences between the procedures are a matter of emphasis and degree, rather than that any of the procedures elicits a qualitatively unique set of behaviors. It is indeed striking that the patterns of these communication deviances can be identified in a great variety of transactions. The patterns appear to be pervasive, structured or "stylistic" features of the ways the family members engage in transactions and, it is hypothesized, have appeared in parental relationships with their growing offspring.

In addition to these detailed studies of communication patterns, somewhat different conceptual vantage points are being used for looking at patterns of role relationships in families and at patterns of emotional closeness and distance between family members. Research and therapeutic interviews, as well as selected psychological tests, are being used in the development of a series of rating scales applicable to these features.

The Study of Psychotherapy, Especially Family Therapy

Treatment indications, techniques, processes, and outcome. Family and marriage therapy have rapidly become recognized as important additions to the traditional psychiatric treatment repertroy. An approach especially emphasized in the Family Studies Section and in the Section on Personality Development (Dr. Shapiro) is the technique of having one or two therapists, most commonly a psychiatrist and a psychiatric social worker, meet together with all of the members of a family. This approach has been variously termed conjoint family therapy, family unit therapy, or family group therapy. The immediate, ongoing transactions of family members with one another and with the therapist are regarded as the most significant starting-point data to be explored, understood and treated in this approach. It has become increasingly apparent, once that therapists have broadened their vision to consider more than an individual patient, that many psychiatric problems cannot appropriately be located within individual family members but are problems of communication and relationships that often involve all members of a family or household.

A special and growing interest in family therapy and family psychiatry arises because it can be regarded as a foothold or starting point for the participation of clinical psychiatrists in broader comprehensive treatment programs and preventive psychiatry. Not only is the family the most immediate and most emotionally significant interpersonal influence in the psychological development and functioning of most individuals, but it also constitutes the most accessible naturally occurring group in society with whom psychiatrists can use the skills developed in ordinary clinical training.

During the past few years the number of psychiatrists, social workers, and clinical psychologists who regard themselves as "family therapists" in all or part of their work has increased at a startling pace. As a member of the Group for the Advancement of Psychiatry Committee on the Family, Dr. Wynne is helping plan a national survey of current practices and objectives of family therapists. Dr. C. C. Beels of this section is making a critical review of the literature on family therapy. Such reviews are especially necessary at this time

before dogmas and doctrines take over the thinking of persons in this still fresh and promising field.

For the same reason, it seems imperative that research on family therapy as a treatment technique be set up. During this year Drs. McCormack and Smith of this section have embarked upon an interesting pilot study in which two techniques of family therapy are being compared in a well-thought out research design that includes a series of new methods for evaluating the quality of intrafamilial communication and family-therapist communication. For example in the Relation-Rorschach technique applied to this study by Dr. Loveland, the family therapist and the family meet together and try to reach a consensus about what they see in the Rorschach cards; the form in which they do so appears to provide valuable data about the quality of the therapeutic relationship.

In addition to the study of family therapy as a treatment technique in its own right, the use of family therapy has served two other research purposes: it has provided a relatively unstructured, free-wheeling source of research hypotheses and unanticipated ideas, and tape-recordings of the psychotherapeutic interviews with families have been extracted for special research purposes in work both of the Family Studies Section and the Section on Personality Development.

Cross-cultural Family Studies

One of the most important research means for advancing our knowledge both of individual psychopathology and of the social context in which it occurs is the cross-cultural approach. This provides a way of distinguishing the conditions under which, for example, a particular symptomatic picture is and is not associated with a family or other social variable. This year Dr. Wynne has been involved in the planning of a cross-cultural International Pilot Study sponsored by the World Health Organization. In this study diagnostic methods and criteria for the evaluation of schizophrenic patients will be worked out for the first time in such a way that diagnostic comparability from one country to another may become possible.

In another study Drs. Singer and Wynne are evaluating whether psychological test protocols obtained from families of schizophrenics and nonschizophrenics in Japan and Lebanon can be scored and assessed with the same criteria which were used in the American family samples. Thus far, it does seem possible to predict diagnosis of offspring blindly from parental tests obtained in these culturally diverse settings. Because the patient-family links studied in this research are concerned with the form or structure of thinking and communicating, rather than the content of thinking, it was reasoned that the methods of scoring should be applicable regardless of cultural background.

Dr. Wynne is also continuing with the study of other kinds of cross-cultural data obtained previous field work in Lebanon, especially data on the value-orientations of five Lebanese subcultures obtained in collaboration with Dr. Herant Katchadourian.

Studies of Family Development

Collection of retrospective data about the sequences of family development through time have continued, particularly by Dr. Juliana Franz and Miss Carol Hoover.

A pilot study was initiated by Dr. Loveland in which the Relation Rorschach technique was applied to the families with very young children. This may provide a useful means of studying family communication and relationship patterns on a longitudinal, prospective basis. Such work is important for the planning of future studies in this Section.

Experimental Ego Psychology

Studies of sensory, perceptual, and cognitive processes, especially in schizophrenics; and

Clinical and Conceptual Studies of Schizophrenia

In these areas some very intriguing and promising methodologic progress has been made during the past year. With the joining of the staff of Dr. Julian Silverman, experimental methods for the study of cognitive and related dimensions in schizophrenics and non-

schizophrenics are being used. The dimensions studied experimentally are being conceptualized in terms that can be approached with clinical ratings as well. This rapprochement between careful clinical research and experimental studies of schizophrenics has long been an important need.

As an example of these experimental methods being introduced, the Mackworth eye-movement camera has provided a direct means of studying scanning behavior. Previous indirect measures of scanning, as applied by Silverman to the study of schizophrenics, have suggested that acute reactive paranoid patients scan more extensively than normal, whereas nonparanoid and chronic paranoid patients scan minimally. Such work has implications for reconceptualizing the heterogeneity and variability of schizophrenic functioning along dimensions that probably will have an improved rationale over those previously used.

At the same time that this experimental work work is proceeding, Dr. Wynne and colleagues have been engaged in defining a series of rating scales for use with individual research interviews and projective tests which will facilitate comparisons of these approaches and, in turn, is related to the concepts derived from the study of family members. The family research has led to a consideration of continua along which clinically symptomatic family and nonsymptomalic but otherwise similar family members vary. Thus, the study of the dimensions in schizophrenic functioning emerging from the experimental psychological studies and from the family studies has come to have significnat overlap.

During this year the contributions to the understanding of schizophrenia and family relations stemming from this program have been recognized in the award to Dr. Wynne of the 1966 Frieda Fromm-Reichmann Prize for research on Schizophrenia by the American Academy of Psychoanalysis and the award to Drs. Wynne and Singer of the 1966 Hofheimer Prize for psychiatric research by the American Psychiatric Association.

Personality Development

The work in the Section on Personality Development has been concerned with classification of psychological issues pertinent to adolescence and has been carired on in two research groups, one under Dr. Roger Shapiro and the other, terminated during this year, under Dr. Earle Silber.

The therapeutic and research activities under Dr. Shapiro are intimately related to some of those taking place in the Family Studies Section. Both sections are greatly concerned with studying the usefulness of conjoint family therapy; the staff of the two sections share the same clinical in-patient facilities, participate in clinical conferences together, and evaluate some of the same families, from somewhat different vantage points, in their systematic research activities.

In Dr. Shapiro's study, families are observed in which there is overt personality disturbance in an adolescent member who has been unable to adapt to living away from his family in a university setting, has developed psychiatric symptomatology, and has withdrawn from school with the suggestion that he get psychiatric treatment. A relation has been hypothesized between parental definition of the adolescent and the adolescent's personality disorder. This hypothesis has led to a research focus upon interactions between parents and adolescents in conjoint family sessions in which the parents' view or image of the adolescent can be directly inferred from their behavior with him. This inferred image of the parents is called "delineation of the adolescent." The determinants of delineation in the individual psychology of the parent are analyzed; and the relation of parental delineation to the identity problem of the adolescent is scrutinized both through study of psychotherapeutic interviews and the use of research procedures, including projective tests, and self-concept interviews. In these ways an attempt is made to define the determinants of adolescent disturbance which are related to his family experience. The program of therapy includes conjoint family therapy, individual psychotherapy for the adolescent, and marital counseling of the parents.

The work under Dr. Earle Silber, terminated in October, 1965, with his departure from the

research staff, was mainly concerned with developing and validating methods for the systematic study of identity problems in late adolescence. Factors affecting the self-esteem of adolescents were studied in considerable detail. The concluding phase of this study involved an experimental procedure in which the self-esteem of a group of adolescents was assessed before and after they were given lower self-esteem ratings by adult authority figures. This experimental group showed significantly greater change toward lowered self-esteem than was found in a control group. Within the experimental group, those subjects who had higher self-esteem, less psychopathology and more autonomy in their relation with their parents responded more selectively with change on those dimensions on which they had obtained information contradictory to their self-image, but also preserved a more generalized high level of self-esteem.

Twin and Sibling Studies

During the past year the Section on Twin and Sibling Studies under Dr. William Pollin has continued to locate and admit for intensive multidisciplinary study additional families with twins discordant for schizophrenia. It has also made a beginning in similarly evaluating families with twins concordant for schizophrenia and with twins without notable psychopathology. A total of 20 families wth twins have now been admitted and studied. the additional families seen during this past year have for the most part demonstrated the same consistent patterns of interrelated biological and psychological phenomena previously defined. In addition, several new aspects of the psychobiological interactions which consistently tend to differentiate the index from the control twins in the discordant pairs have become apparent.

The pattern of consistent life history differences that has been found to differentiate the schizophrenic from the nonschizophrenic twin in the series thus far studied here at NIMH includes the following: (*a*) the schizophrenic twin-to-be has in 12 of 13 cases weighed less at birth and now tends to demonstrate more "soft" neurological signs than the cotwin control; (*b*) tended in childhood and adolescence to be less competent, organized, effective and show less sustained goal directed activity than his cotwin; and (*c*) tended to be the more sensitive, anxious and unhappy of the twins from an early age on. The data, though consistent thus far, do not as yet permit extensive conclusions to be drawn; it is still not clear whether they relate to schizophrenia *per se*, to a susceptibility to various forms of psychopathology in a more general sense, or derive in part or in whole from some as yet unrecognized sampling bias. Our current tentative formulation of these data is that in the group of families studied there existed initial, non-genetic, constitutional differences between the twins. These initial constitutional differences reflected in the different birth weights, involved most importantly a different level of biological maturity or competence, which resulted in less smooth and effective operation of various adaptational and internal environment regulators in the smaller twin. These sometimes slight biologic differences contributed to or determined the very early establishment of role difference within the family relationship patterns. The smaller twin, as a result of these relationship and role differences, experienced a sequence of reinforcing events in childhood years which *in toto* accentuated rather than mitigated the initial minimal disparity in coping potential. The increasing intertwin differences in personality, particularly in ego structure, led to an increasingly unfavorable stresscoping ratio, in the index twin, with passing years. That is to say, there was an increasing tendency to *generate* stress in dealing with ongoing developmental events and transitions, and a relatively decreased ability to cope with them. Drs. Pollin and Jades Stabenau are currently attempting to expand this formulation and to synthesize a multifactorial theory of the etiology of schizophrenia.

Constitutional and biological differences between the index and control twins thus far delineated included in summary the following: the index twins show a higher lactate/pyruvate ration (Frohman; 9/10); have a lower PBI (11/12); more "soft" neurologic signs

(9/11); weighed less at birth (12/13). These biological variables, and other where the data is not yet clearcut, have been clarified in particular by Drs. Stabenau and Loren Mosher, working collaboratively with a number of other investigators, here at NIH, and in other research centers. Dr. Fred Guggenheim has focused on organic factors in the twins' parents and has found a surprisingly high incidence of significant medical illness in them. He found that 7 of the first 13 mothers studied had well-documented thyroid disease, a prevalence rate (58%) which is elevated to a statistically significant degree when compared to other prevalence and case finding studies. Additionally, there appeared to be an elevated incidence of malignant neoplastic disease and an overall pattern suggestive of excessive physical illness in these parents. These findings raise questions about the possible relationship of thyroid function to the phenomenon of twinning, though it is not yet possible to exclude some type of as yet unrecognized sample bias in accounting for the results. It appears most likely, however, that they reflect the heightened degree of intra-familial tension in the prepsychotic years within these families in a manner consistent with the demonstration by Hinkle and others, in large scale studies, of the relationship between emotional crisis and episodes of physical illness.

In the continuing analysis of data obtained from three groups of families with young adult siblings discordant for schizophrenia, or juvenile delinquency, or where both siblings were well-adjusted, Dr. Stabenau has devised a new, additional objective indicator of a greater degree of familial stress during early critical developmental periods in the life of the index as compared to the control. In the S families 7 of 11 indexes as compared to only 2 of 11 controls had younger siblings born within 2 years of their birth; in the delinquent families the corresponding ratios were 7 of 8 indexes and 2 of 8 controls; and in the well-adjusted control families the ratios were only 1 of 5 for both index and controls.

Working with TAT material from these same three groups of S, D, and N families, Mrs. Martha Werner and Dr. Stabenau have noted an additional significant distinction between the normal and psychopathology families. In keeping with their hypothesis concerning the role of repressed rage in the formation of schizophrenic symptoms and delinquent behavior, it was found that on card 13 MF 28 of 39 stories told by the S families had themes of violence, murder or death; that 26 of 32 stories in the D families and only 5 of 20 responses by the N families had similar themes.

In addition to the phenomenon of a differential degree of stress in the family as a whole occurring during the first two years in the life of the schizophrenic or delinquent-to-be as compared to the life of the control a number of other factors have been found which help explain the presence within a given family of psychopathology in one child and not the other. These include (1) a clearcut pattern of differential identification with the index-to-be much more closely identified and allied with the psychologically sicker of the two parents; (2) a psychological Gestalt which places one of the siblings in the role of being much more caught up in or used as a surrogate for some intense rivalry between the parents; (3) certain accidental historical or biological differences determine that the index-to-be to a much greater extent than the control restimulates some unresolved though previously dormant major conflict within one or the other of the parents; (4) the early establishment of role differences cause one of the siblings or twins to receive considerably less by way of psychological growth and ego-syntonic life experiences.

Dr. Loren Mosher has studied the determinants of psychopathology in a twin population by exploring the relationship of such psychopathology to differences in identification and cognitive style. His findings thus far include the following: the index twins, when compared with their cotwin, regardless of sex, are most often identified with their mothers, and are rated and test out as being more "global" or amorphous (field dependent) in their cognitive style (or type of schizophrenic thought disorder); the identification variable appears to have less effect on cognitive style

than does psychopathology) female twin pairs and mothers are more "global" (field dependent) than male pairs and fathers. The fact that females more often have "global" cognitive styles (or amorphous thought disorders) may be relevant to the higher concordance rate for schizophrenia in female twins and the larger number of "chronic" schizophrenic females in mental institutions.

Psychosomatic Studies

The Section on Psychosomatic Medicine is a part of the Adult Psychiatry Branch which is more heavily concerned with relationships between biology and behavior. Although specific projects range from laboratory research at a molecular level to clinical investigations at a molar level, the overriding and unifying theme of the Section's work is a furtherance of a meaningful understanding of the behavioral-biochemical paradigm.

There is a good deal of inferential evidence from both clinical and animal studies that norepinephrine (NE) metabolism in human brain may markedly influence a variety of behaviors. However, due to several methodological problems it has not been possible to obtain direct estimates of rates of metabolism of norepinephrine by brain in clinical investigations. Because of the importance of such knowledge for research into the relationships between brain NE and behavior, much of the work under Dr. James Maas over the past three years has been devoted to the development of a technique whereby reliable, individual measures of norepinephrine metabolism in brain may be obtained. In the initial phases of this work it was found that (*a*) norepinephrine given into the cisterna magna of dogs rapidly enters brain and by five hours is in a pool which is not freely accessible to the cerebrospinal fluid (CSF); (*b*) 50–70% of isotopically labelled NE (H^3NE) enters blood from CSF, but (*c*) when rather large amounts of H^3NE are injected into blood essentially no radioactivity is found in brain or the DSF. These findings suggested that by giving an intravenous infusion of C^{14}NE at the same time that H^3NE was injected into the disterna magna one could derive, from assays of metabolic products of H^3 and C^{14} in urine, direct estimates of the amount of NE which enters brain and the rate at which NE in brain is changed to various degradation products. Experimental data obtained from work with dogs indicate that such a double isotope technique does yield quantitative information as to rates of degradation of isotopically labelled NE by brain. In addition, further animal experimentation has been done over the past year to simplify the mathematics involved in this approach and to find methods whereby the fraction of H^3NE entering brain might be increased. The results obtained thus far suggest that the reliability of the method is such that the application of this technique to clinical investigations is now feasible. During the coming year it is planned to use this double isotope approach in studies of severely depressed patients.

Other studies of norepinephrine metabolism at a more molecular level are also being done by the group under Dr. Maas. The enzymatic steps involved in synthesis and breakdown of NE are fairly well understood, but the mechanisms by which the catecholamines may be bound or stored intracellularly in brain are less clear. Because of the structure of the NE molecule, i.e. a catechol moiety with an ethannolamine side chain, a reasonable hypothesis is that the catecholamines form complexes with metals with a consequent change in physical properties which may explain the phenomenon of binding. Three lines of evidence support this hypothesis; (*a*) it has been found that synaptic vesicles isolated from rat brain have more than enough metal (Mg, Cu, Fe, Zn) to complex with the quantity of catecholamines present in this subcellular fraction of brain; (*b*) ethylenediamine, a chelating agent, prevents uptake of exogenous NE by synaptic vesicles; and (*c*) *in vitro* evidence has been found for the formation of ternary complexes between metal, NE and ATP. These findings as well as data reported by a variety of investigators are consistent with, and supportive of, the hypothesis that coordination between metals and NE are importantly related to the intracellular binding of NE. Present work has been directed towards obtain-

ing very pure preparations of synaptic vesicles as demonstrated by electron microscopy, and then following a variety of treatments, measuring rates of uptake and release of metals and NE.

In the process of the above investigations it became apparent that metals might be also quite important for membrane function. Data obtained indicate that (*a*) metals coordinate with phospholipids and alter their solubility characteristics; (*b*) ternary complex formation between metal, phospholipids, and ATP occurs; and (*c*) the solubility characteristics of the ternary complex is similar to that of the phospholipid alone. From these data a molecular model of neural membranes which can explain reversible transitions between two phases having different physical properties was constructed.

To understand further the role that these transition metals might have in neural functioning, electron spin resonance spectroscopy of subcellular fractions of brain (myelin, mitochondria, and synaptosomes) is now being done in collaboration with Dr. Hideo Kon of NIAMD. Although there was some initial technical problems with this approach, these now seem to be corrected and some interesting and potentially important data are emerging which may aid in our understanding of the molecular mechanisms involved in synaptic functioning.

In addition to these molecular approaches, Dr. Maas and co-workers have been studying the relationships between biogenic amines and critical periods of development. There is a good deal of experimental evidence from a variety of animal studies which indicates that during development there are critical periods in which environmental influences are most important in determining the ability of the adult animal to respond to stress, to learn, and, in the cases of dogs, to form social bonds. In an earlier investigation of some of the biological processes which might underlie this latter phenomenon it was found that during the developmental period critical for primary social formation in the dog there were marked changes in norepinephrine and serotonin levels as well as catechol-O-methyl transferase activity in most areas of the brain examined. More specifically, around the beginning of the period critical for primary social bond formation biogenic amine levels and enzyme activity were high but markedly decreased over the following one to two weeks. As a beginning in the understanding of the possible significance of this finding in relation to behavioral correlates collaborative work with investigators at the Jackson Memorial Laboratories at Bar Harbor, Maine was undertaken in which one group of pups at 5 weeks of age and another at 7 weeks were given Reserpine for a period of five to ten weeks, taken off the drug and then tested in a variety of ways at 26, 36, and 52 weeks of age. Control animals were littermates and were given Nembutal. Experimental and control animals were housed together. From this first study the data indicate that those animals which received Reserpine (a drug which lowers body stores of norepinephrine and serotonin) from 5 to 15 weeks of age are, when tested as adults, somewhat more fearful and do not learn maze problems as well as the control subjects. The dogs which were begun on Reserpine at 7 weeks (and continued until 15 weeks of age) seen as adults to show test behavior somewhat intermediate between these two groups. Due to the small sample size, firm statements must await the outcome of studies now in progress which have as their goal the replication and extension of these earlier findings.

In a somewhat different approach to the relationship of central nervous system amines and behavior, work is being continued in an attempt to obtain neurochemocal-behavioral correlations in strains of mice and rats which have been inbred for differences in *emotionality* or fearfulness as measured by the open-field test. Initially, in working with mice which differed in terms of this behavior, it was found that the more "emotional" strain of mouse (BALM/cJ) had a higher level of serotonin when compared with another less emotional strain. (C57BL10cJ) In addition, the work seemed to indicate that the serotonin differential was due to brain stem and limbic structures rather than other areas. Since this initial finding, work has proceeded along lines which attempt to relate this serotonin difference to

behavior in other strains and species. It has been found that: (*a*) in three other strains of mice, one of whom was "emotional" and the other two not, serotonin differences in the predicted direction were found, and (*b*) reactive and nonreactive strains of rats which were bred by Broadhurst for differences in open-field behavior also show brain serotonin differences in the predicted direction. Correlations between ambulation scores in the open-field and serotonin levels in limbic structures in brain were done and there was found to be a significant negative correlation. Present work in progress deals with obtaining F_1 and F_2 animals from the reactive and nonreactive strains for behavioral testing and serotonin assays.

The clinical portion of the section's work has been concerned chiefly with studies of depressive reactions and has been carried out under the direction of Dr. William E. Bunney, Jr. This program involves the investigation of biochemical and behavioral aspects of depressive reactions. In the last year six reports have been published which deal with a variety of psychological and biological factors in depressive illness. Issues and questions raised in these publications continue to be explored, and in addition four new areas of investigation have been initiated.

The basic research tools used in this research are continuous, longitudinal methods of obtaining behavioral data through the use of rating scales, coupled with an ongoing collection of urine and plasma specimens. Research findings built on this data have been published during the past year and include the following:

Urinary excretion of 17-hydroxycorticosteroids (17-OHCS), an index of anteriar pituitary-adrenacortical activity, is elevated in certain subgroups of depressed patients.

High positive correlations exist between longitudinal behavioral ratings of depression and fluctuations of 17-OHCS levels for a given patients.

Changes in 17–OHCS levels accompany behavioral changes rather than preceding or following them.

Changes in 17–OHCS levels can be used to study and locate specific days for analysis of precipitating events and to characterize the categories of precipitating events that seem relevant to depressive crisis.

It was hypothesized that 17–OHCS levels reflect what has here been termed psychological distress or pain and that this is particularly intense in acutely suicidal patients. The data suggest that elevated urinary 17-OHCS levels may offer a possible biochemical test for suicidal potential.

A review of the current literature plus ongoing investigations indicate that changes in norepinephrine metabolism may have etiological significance in depressive reactions. These findings have been described in detail in one of the papers published during the past year. Analysis of an additional body of data bearing on the relationship of norepinephrine metabolism to depression has been recently completed. Findings show high urinary norepinephrine levels and different excretion patterns of breakdown products of norepinephrine in patients with acute psychotic depressions as contrasted with those having neurotic depressions. This investigation represents a three-year study of urinary norepinephrine and breakdown products in depressive reactions. Based on comprehensive clinical observations, two members of the research group placed the sixteen patients in this study into one of two diagnostic groups, acute psychotic depression and neurotic depression. The accuracy of these judgements were validated by two independent raters. The acute psychotic depressive group showed marked elevations of norepinephrine levels in contrast with the neurotic depressive group. The difference between the norepinephrine levels in the two groups was significant at the 0.002 level. All the individual means of acute psychotic depressive groups were higher than those in the neurotic depressive group. Norepinephrine levels in the neurotic depressive group are more stable than those in thepsychotic depressive group as shown by the ranges and the standard deviations. One possible abnormality in norepinephrine metabolism in depression may involve and inhibition of one of the enzymes involved in the breakdown of norepinephrine.

The findings of this study offer evidence compatible with this hypothesis.

The analysis of additional data confirming the hypothesis that 17-hydroxycorticosteroids may offer a test for suicidal potential has been completed. Through research additional data on a number of suicide attempts have been collected. Steroid data on three additional suicides occurring in the other research centers has also been reported here, which tends to support the original hypothesis. As our body of data concerning this problem increases, more sophisticated analysis and qualification of findings becomes possible.

Two additional studies have been completed and are in the process of data analysis. The first involves a further attempt to investigate the role of norepinephrine in depression and involves infusions of precursors of norepinephrine and serotonin in an attempt to change brain amine levels. Specific and interesting behavioral changes have been observed. The significance of these are currently being analyzed; however, no long lasting improvement of the depressive mood was noted.

The second area involves analysis of precipitating factors occurring prior to the onset of depression. It is hypothesized that investigation of these factors should offer some clues concerning the core problems of depression plus giving us basic information about response patterns of the individual patients to stress. Forty patients and their relatives have been intensively studied to date. The factors have been broken down into external factors, controlled and uncontrolled by the patients, and internal factors. The temporal sequence of these events has been analyzed. Approximately 20 patients who fall according to all diagnostic criteria (except the absence of a precipitating event) into the category of endogenous depression have been studied. In almost all of these individuals clear cut precipitating factors have been present. This finding clearly challenges the validity of the concept of the endogenous depression as an entity and has specific implications for future research. From these data it is hypothesized that *all* cases of depression whether they fit the "endogenous or reactive" category are *stress precipitated.* For this reason studies of the biochemical stress reactions of the depressed patient are being actively continued.

In addition to the work thus far described, which is at or near completion, data for other projects have been collected but are at present unanalyzed. The first of these studies involved an attempt to study the mechanisms by which depressed patients maintain extremely high levels of 17–OHCS. This is a particularly interesting phenomenon in that patients may maintain levels five times normal for six months and yet do not develop symptoms of Cushing's disease. The drugs dexamethasone and metopirine have been used as research tools. Tentative evidence from a number of severely depressed patients suggest that abnormalities may exist in steroid metabolism. Attempts are being made to develop a hypothesis which accounts for the observed changes in steroid, catecholamine and electrolyte metabolism in depressed patients. It seems likely at this time that these factors are interrelated.

A second area of work in progress has involved the use of lithium in the treatment of manic-depressive patients and in the treatment of depressed patients. For as yet unknown reasons, lithium seems to be a highly specific treatment for manic-depressive illness and can be used as a research tool for the study of the mechanism of action of lithium. A number of theoretical concepts concerning the mechanism of action of lithium have been developed and are currently being investigated. If successful, such studies could lead to a more basic understanding of psychopathology than previously anticipated. A few patients whose psychopathology is extremely sensitive to changes in lithium levels have been studied and from this work and from a review of the literature it would appear that the use of lithium in this country is unrealistically conservative and limited.

A third area of work involves interest in studying the mechanism of action of electric shock therapy. During the past year a small study in dogs was initiated to investigate the effect of electric shock therapy on norepinephrine metabolism. A large potential exists in

this area and currently awaits space and personnel.

A fourth area of work in progress involves a study of the thought patterns of depressive patients. During the past year a good deal of progress has been made in this area. Currently, 20 depressd patients and 20 schizophrenic patients are being studied. These are being matched for depressive content so that it is impossible to differentiate them on this factor. Independent raters are now attempting to predict blindly which patient is psychotically depressed and which is schizophrenic on the basis of categories developed from the analysis of their thinking disorders. A number of previously undescribed characteristics of psychotic depressive thinking have been defined.

Future plans involve a continuation of the above four projects and the initiation of three new areas of work. These involve, first, a collaborative project with a number of hospitals to evaluate the usefulness of finding that 17–OHCS levels may offer an index of suicidal potential.

The second area is concerned with a continued study of lithium and a continued investigation into the mechanism of action of lithium. One of the most relevant hypothesis concerning the mechanism of action of lithium and its effect on nerve transmission in the brain involves its possible action on the sodium this it is critical to study intracellular sodium. A method for this has been worked out in collaboration with the Bethesda Naval Research Medical Center and involves the use of neutron activation.

The third area of future research involves a direct testing of the norepinephrine hypothesis through a study of the metabolism and turnover rates of norepinephrine in depressed patients.

Psychophysiology of Sleep

Longitudinal studies of electrographic sleep patterns in hospitalized depressive patients continue to be the principal focus of interest for the Section on Psychophysiology of Sleep. An innovation accomplished during the past year which promises to be widely adopted in clinical sleep studies was the installation of permanent recording cables from the sleep laboratory to the nearby psychiatric ward. This permits electroencephalographic and other polygraphic recording from patients while they remain in the accustomed setting of the ward, rather than requiring that they sleep in the unfamiliar setting of the laboratory.

In retrospect it now appears as though this is the only way in which meaningful studies of sleep patterns in psychiatric patients could be accomplished, and, indeed, current sleep studies of depressive patients have been greatly facilitated. In addition to extending the series of patients for whom three night samples of sleep recordings have been studied at various stages of illness, nightly sleep recordings from selected patients are now being obtained over very extended periods, in one case amounting to 111 consecutive nights. For the first time this type of study provides detailed and precise knowledge about variations in sleep patterns of psychiatric patients in relation to day-to-day changes in clinical status or various therapeutic interventions. In the patient referred to, for example, this data reveals marked variations in total sleep from night to night, yet an unrelenting and cumulative sleep deprivation over the months of the study. The degree of REMS (rapid eye movement state) deprivation is still more severe. Therapeutic trials of an experimental antidepressant drug were associated with total abolition of REMS, and its withdrawal was followed by marked increases of REMS above normal levels, demonstrating that the experimental phenomenon of REMS deprivation can be a naturally occurring one under clinical circumstances.

Although these studies have been handicapped during the past year by the relative unavailability of suitable patients, it is intended that they will be extended to additional depressed patients and eventually to other diagnostic categories as well. Many technical problems remain to be solved, but it is foreseeable that nightly monitoring of sleep patterns may soon become a routine procedure in clinical evaluation and management of psychiatric patients.

The earlier findings of our sleep laboratory concerning profound variations in blood pressure and other vital functions during the REM state, as well as their probable implication in a number of important medical problems are now receiving ample confirmation. For example, evidence obtained here during the previous year that nocturnal angina episodes are highly correlated with REMS periods has now been substantiated by work reported elsewhere, but in one further angina patient studied here during the past year these episodes were not related to REMS periods. The anginal pain of this man generally occurred with the first few hours of the night when our previous studies have found the lowest diurnal levels of blood pressure. Additional patients with this condition are also very much sought for study, but it now appears that the pathophysiology of nocturnal angina is probably not always the same, nor always related to the vegetative disturbances of REMS.

The descriptive physiology of the REMS continues to be of great interest, as the physical reverberations of this third state seem to extend wherever they are sought. One aspect of particular attention currently is the association of the REMS with penile erection. Since one of the pioneer workers in this area, Dr. Ismet Karacan, is visiting scientist in the Section this year, further systematic study of this relationship is being pursued using normal control subjects. To date this has focused upon the normative characteristics of erection during entire nights of uninterrupted sleep in order to clarify details of temporal relationships, inter- and intra-REM period differences, or intersubject variations in this manifestation. After the normal courses of these events are better known, it is intended to use this data as a basis for testing the effects of a variety of experimental interventions, such as sleep deprivation, specific REMS deprivation, stress, etc.

Comparative studies of sleep and REMS in primitive mammals and reptiles have been virtually set aside during the past year because of lack of suitable space or facilities for carrying them further. It is hoped that they can be resumed in the future when additional space becomes available and that a collaboration might be worked out with the National Zoological Park for pursuing them more extensively in a wider variety of species and under more naturalistic conditions. One comparative question which has been answered is whether the penile erection element of REMS is entirely a human characteristic, or whether it occurs in the REMS of other mammals as well. It was demonstrated for the first time in our laboratory that penile erection during REM periods occurs in the Rhesus monkey as it does in man. That this is so offers promise that the neurophysiological mechanism of erection during REMS might be clarified, but since the Section on Limbic Integration, of the Laboratory of Neurophysiology (Dr. Paul MacLean), has already done a great deal to elucidate the central nervous mechanisms of erection in the Squirrel monkey, it would be particularly appropriate to study the mechanism of erection during REMS in that species. In collaboration with that section, therefore, present efforts are directed to study the sleep patterns of the Squirrel monkey and to determine whether penile erection is associated with REMS period of that species. At the present stage, technical problems in making electrographic sleep recordings of this primate are still troubling, and a variety of approaches to the chronic restraint which is necessary are being attempted.

Basic research in the physiology of the REM state has now entered a distinctly biochemical phase. Many lines of inquiry point to the existence of a neurohumoral mechanism responsible for the periodic triggering of this remarkable physiological condition. The effects of depriving animals of this state suggest that a hypothetical chemical substance accumulates and has the effect of producing generalized hyperexcitability within the nervous system. Direct biochemical substantiation for this hypothesis is still lacking, but evidence suggests that catecholamines or serotonin are likely involved.

Data relevant to this hypothesis is being sought by the Section on Sleep on two levels in collaboration with other research units of

NIMH and NHI. Since the Section on Experimental Therapeutics of the NHI (Dr. David Horwitz) is making clinical trials on new and potent inhibitors of catechol amine synthesis, such as alpha-methyl-tyrosine, this provides an opportunity for studying the sleep patterns of medical patients receiving these drugs before, during, and after their administration. The first two exploratory studies in this series are now nearing completion.

Another approach to this problem is being carried out in collaboration with the Section on Medicine, of the Laboratory of Clinical Science (Dr. Kopin). Rats are being selectively deprived of REMS over periods of four days, after which various structures of their brain tissue ae assayed for norepinephrine and serotonin levels in comparison with those of control groups which were not so treated. Results of this analysis are still pending, but further studies of similar nature are contemplated in which "turn-over" rates of the same substances will be studied by means of radioisotope techniques. The challenge presented by the REM state is a rare opportunity for combined efforts of scientists concerned with molar aspects of behavior and those involved in molecular physiological mechanisms. That our Section on Psychophysiology of Sleep is now sharing in such collaborations is in keeping with the unique advantages and best traditions of NIMH Intramural Research.

LABORATORY OF GENERAL AND COMPARATIVE BIOCHEMISTRY

During the last 12 months the research program of the Laboratory of General and Comparative Biochemistry has continued along the lines that have been developing in the last few years and include now the following main areas of interest: (1) mechanism and pathways of protein biosynthesis; (2) biological oxygenation; (3) the biochemistry of mental retardation; (4) biological methylation and alkaloid biosynthesis; and (5) structure-function relationships in proteins.

Work along these main lines tends to breakdown the division into three sections and collaboration between sections has been very successful and productive.

We have had no major personnel changes and have enjoyed a year of productive and stimulating research work in our chosen fields.

Section on Proteins

The research efforts of the Section on Proteins were divided between two problems during the last year as during earlier years.

The main effort of the Section, and in fact of the whole laboratory, dealt with the molecular biology of sRNA. A number of individual projects describe more in detail the multifacet approach to the structure of serine sRNA and its interaction with a number of biopolymers, such as messenger and ribosomal RNAs, the specific serine sRNA synthetase, the CMP AMP pyrophosphorylase, etc.

The second area of investigation dealt with protein structure and the correlation of molecular structure to biological function.

It is now clear that the key event in the translation of the triplet code of messenger RNA into the polypeptide sequence of protein requires the interaction between sRNA and mRNA. SRNA's biological function is related to its ability to decode the information contained in the messenger RNA, to recognize the amino acid specific sRNA synthetases, and to interact with the ribosomes, where the assembly of polypeptide chains takes place, thus, bringing together the three components of the several needed for protein synthesis.

The most significant advance in this area of work in our laboratory has been the progress in the purification of serine sRNA from yeast. We now have developed a technique for the purification of this material in reasonable quantity and in better than 90% purity. No other sRNA of comparable purity has been obtained elsewhere, to our knowledge. Improved techniques of nucleotide sequence, based on separation and characterization of oligonucleotide fragments, lead us to hope that determination of the base sequence of serine sRNA from yeast may be achieved in the next year or sooner. Elucidation of the primary structure of amino acid specific sRNA, however, is only the first step in the development of our understanding of the molecular

biology of sRNA. Much further work can be anticipated on comparative structural chemistry of sRNAs, on physico-chemical studies and on nucleic acid protein interaction.

Interest in the relationship between protein structure and biological function underlies many aspects of the work in the laboratory as it is indeed in the mainstream of biochemical thinking. Several projects represent our effort in this line of investigation. Thus, studies of the crystalline serine sRNA synthetase have disclosed that the enzyme protein only recognize the intact sRNA molecule, loss of only the terminal nucleotide from sRNA being sufficient to eliminate all interaction between serine sRNA and serine sRNA synthetase. Work on the thetin enzyme (thetin-homocysteine methylpherase), one of the favorite proteins of this laboratory, has shown that many of the structural features of this enzyme are shared by other unrelated proteins. The studies of Klee on pancreatic ribonuclease shed new light on the conformation of this enzyme. Work with the highly purified dopamine β-hydroxylase has revealed the role of protein bound copper in the hydroxylation reaction leading to the biosynthesis of norepinephrine. (This aspect of the Laboratory's effort is described below in more detail.)

Section on Cellular Regulatory Mechanisms

The research efforts of the Section were divided between two unrelated problems during the last year. The first area deals with the original, and continuing, interest in the problem of reactions catalyzed by hydroxylating enzymes. The second problem is one that was only recently initiated in the Section and is still in the preliminary stages of investigation: skeletal muscle hypertrophy.

In the area of hydroxylating enzymes, there were several significant advances. Previous work in the Section led to the discovery of a new coenzyme, dihydrobiopterin, which is an essential component of the phenylalanine-hydroxylating system. Subsequently, we demonstrated that this pteridine plays a similar role in the adrenal enzyme system that catalyzes the conversion of tyrosine to dopa. This step is the first one in the biosynthetic pathway for the neurohormones, norepinephrine and epinephrine. Work in other laboratories has shown that dihydrobiopterin functions as a cofactor in a variety of oxygen-requiring enzyme systems.

We had previously shown that one other naturally occurring pteridine, sepiapterin, shows high cofactor activity in the phenylalanine-hydroxylating system. It has now been found that sepiapterin, itself, is not active, but must first be converted to dihydrobiopterin before it can function as a cofactor. A new enzyme that catalyzes this conversion has been extensively purified from rat liver extracts. This enzyme named sepiapterin reductase, catalyzes the following reaction:

$$\text{TPNH} + \text{H}^+ + \text{sepiapterin} \rightleftarrows \text{TPN}^+ + \text{dihydrobiopterin}$$

Since it has been shown that the reaction catalyzed by this new enzyme is reversible, the discovery of sepiapterin reductase raises the question of whether sepiapterin might not occur in mammalian tissue. Until now, this pteridine has been demonstrated only in lower animals such as insects and fishes, where it is believed to function as a pigment.

In the dopamine β-hydroxylase catalyzed reaction, it has previously been shown in this Section that the enzyme is a copper protein. It was also demonstrated that the protein-bound copper undergoes oxidation and reduction during the hydroxylation reaction. This conclusion has been fully confirmed by a electron-spin-resonance measurements. Furthermore, with the use of this technique, it has been found that fumarate, an activator of the enzyme, enhances the oxidation of the protein-bound copper. This observation may provide an explanation for the activating effect of fumarate.

During the last year, a new disease has been described, which may be a variant of phenylketonuria. This new disease, characterized by moderately elevated levels of serum phenylalanine, has been called phenylalanemia.

As part of our long term interest in genetic diseases related to phenylalanine metabolism, we have recently initiated, in collaboration with Dr. Joseph Kennedy in Boston, a study of the phenylalanine hydroxylating system in liver

biopsy samples from patients with phenylalanemia.

Our preliminary findings indicate that the phenylalanine hydroxylating system is not fully active in the liver samples from these patients. The enzymatic defect is almost as severe in this new disease as in classical PKU.

In future studies we hope to be able to put on a quantitative basis the difference in phenylalanine activities in the two diseases.

In the muscle hypertrophy problem, a technique has been developed which for the first time leads to skeletal muscle hypertrophy in animals, under controlled laboratory conditions. The method gives measurable increases in muscle mass, as well as increases in muscle strength.

Chemical analysis of a wide variety of muscle constituents has demonstrated that in the hypertrophied muscle, there is an increase in content of glycogen and RNA. Time-course studies are underway to determine if these chemical changes occur before or after hypertrophy.

Section on Alkaloid Biosynthesis

The year 1965–66 has seen continued advance in several areas of sulfur aminoacid metabolism.

Studies have been initiated in collaboration with Dr. Uhlendorf of the transsulfuration pathway in mammalian cells growing in tissue culture. Factors affecting the levels of the pertinent enzymes are being explored. The long-range goal of these studies is to make available a source of tissue which one could obtain from both control patients and patients with enzyme deficiencies at the present time demonstrable only in liver tissue. This would make possible the study of a variety of genetic and metabolic problems now precluded because of lack of available material. The findings may be of general interest in relation to a great number of diseases. In addition, the findings of Eagle and his collaborators suggests that the transsulfuration pathway is modified as cells undergo "transformation" to malignancy. A more detailed understanding of this phenomenon will be of interest for the study of such transformation.

Thermodynamic studies carried out in collaboration with Dr. Klee have led to insight into the structural factors which help to confer their "high-energy" nature on sulfonium compounds. The biological methyl donor, S-adenosylmethionine is the most energy-rich such compound known. Detailed studies of its conformation in solution have been performed and led not only to a picture of the actual shape of this molecule, but also to the suggestion that most adenosine and other purine ribosides in solution have a conformation different from that described to them until now.

Since September, 1965, we have been fortunate to have as a Visiting Scientist, Dr. John Giovanelli, an expert in plant biochemistry. The results are as yet preliminary, but already a number of interesting leads have developed which it is hoped will lead to many new discoveries as they are followed up.

The human disease, cystathioninuria, a cause of mental retardation, has been proven to be due to a lack of the enzyme cystathionase. A few more detailed studies of the nature of the defect in this disease have been carried out with a view toward explaining the puzzling action of pyridoxine in these patients. Lack of material has been a limiting factor. It was also shown that these patients lack homoserine dehydrated activity, opening a new area for metabolic investigation.

Studies on homocystinuria have been continued. One of the questions raised here last year has been answered. A homocystinuric patient with an normal IQ has been found to have only 2–3% of the normal concentration of cystathionine synthase in his liver. This is a level of enzyme found in many homocystinuric patients. This finding suggests that environmental manipulation (i.e., dietary management) will almost certainly be successful in preventing the mental retardation which occurs in most patients who lack cystathionine synthase.

Section on Technical Development

During the 12 months since July 1965 the Section on Technical Development continued to fulfill its traditional role of support of NIMH and NINDB research efforts. Continu-

ing assistance was made available in shop help, special purpose instruments, and some modest instrumentation systems. The section continues to grow toward assistance in computer oriented research and digital instrumentation.

Use of the section's LINC computer has almost doubled since last year and has reached the saturation point—105 hours per week. The ability to take on new projects is now seriously limited by production runs on existing projects. Plans to relieve this pressure include:

Transfer where possible of existing production runs to CDPB, while limiting LINC functions to A to D conversion and online data editing.

Aquisition of additional computing equipment in fiscal 1967.

Help to laboratories toward setting up their own facilities where the workload justifies such a step.

The policy toward repair and maintenance of equipment has continued the same as in the previous year, with the section handling emergency problems but referring routine and long-term problems to the Instrument Branch. The electronic component stocking service continues with the occasional acquisition of government surplus components being used to supplement substantially the purchased stock.

Because of limitations in space, no significant expansion or change of plans is contemplated within the section until the move to new quarters is made sometime in fiscal 1968. At that time, it is planned to move the section to Building 36 while maintaining a small machine shop and electronic fabrication facility in Building 10. Staff will probably be expanded to include an additional technician for the Building 10 facility; and the additional space in Building 36 will probably permit the addition of a technician, a mechanical engineer, and a physicist to the staff.

Section personnel have contributed significantly to research projects in laboratories of the two Institutes. In all cases this work has been described in the reports of the laboratories involved.

A total of 164 projects were completed in the past year. A full review of all of them would be too lengthy; however, a summary list of the more important ones follows:

Projects completed

Project	*Requestor*
EEG amplifier and power supply	Dr. K. Gaarder
Pulse discriminator and counter	Dr. P. Nelson
Brain mapping impedance package	Dr. H. Rosvold
Hydrogen electrode chambers	Dr. M. Reivich
Automatic timer and pulser unit	Dr. H. Steinberg
Adjustable head rest chair	Dr. J. Silberman
Camera optical system for oscilloscope photography	Dr. D. Carpenter
Lucite prism and electrode holder	Dr. D. Carpenter
Galvanic skin response meter	Dr. P. Bergman
Automatic blood sampler	Dr. M. Reivich
Skin temperature sensing system	Dr. F. Snyder
Electrophoresis chambers	Dr. C. August Dr. M. Kies Dr. B. Rasmussen
Digital coding units for animal observation	Dr. D. Galin Dr. P. MacLean
Revolving table with automatic nipple insertion	Dr. W. Stanley
Automatic tape recorder control	Dr. D. Boomer
Conceptual training apparatus	Dr. A. Caron
Modulated oscillators (2)	Dr. P. Nelson
High impedance solid state amplifier	Dr. L. Binstock
Paper tape comparator	Dr. E. Jerome
Digital recording system	Dr. V. Carlson
Implanted heart-rate transmitter	Dr. C. Nagel
1620 computer interface	Dr. E. Jerome
Soundproof chamber for use over operating table	Dr. E. Evans
Respiration sensor	Dr. F. Snyder
Electric shutter system for carousel projector	Dr. A. Caron

Projects in process

Project	*Requestor*
Semiconductor strain gage for use with wrist flexion tester	Dr. E. Evarts
Motorized nipple assembly	Dr. W. Stanley
Constant current device	Dr. F. Snyder
Solid state pulse generators	Mr. L. Binstock Dr. K. Chandler
Programmed volume control	Dr. F. Snyder
One second timer for oscilloscope camera	Mr. L. Binstock
Audio tape clock system	Dr. R. Ryder
Automatic projector system to facilitate eye movement measurement	Dr. J. Silberman
Motorized sphere	Dr. P. MacLean
Photocell controlled dual feeding chamber	Dr. W. Stanley
Circular puppy feeding compartment	Dr. W. Stanley
Impedance mapping device	Dr. F. Snyder

Project	Requestor
Tissue section plotter	Dr. A. Coulombre
Puppy feeding nipple with vacuum sensor	Dr. W. Stanley
Programmed light intensity control	Dr. F. Snyder

Projects abandoned

Project	Requestor
Thermistor blood-flow sensor	Dr. W. Marshall
Wrist flexion and extension tester	Dr. E. Evarts

Section on Technical Development Projects

Projects initiated within the section

Project	Status
Digital tape transport system for LINC	In process
Active delay-line filter	In process
Neuron modeling system	In process
Solid state integrator	Complete
LINC low-pass filters	Complete
LINC sample and hold modification (2)	Complete
LINC rack no. 3 tape recorder	Complete
Bi-phasic constant current generator	Complete

LINC programs in current active use

Program	Requestor
Power spectrum analysis of the EEG	Dr. L. Speck
Evoked response averaging for random blocks of optical stimulus pairs. LINC sorts stimulus pairs of equal duration, averages and computes standard error for mean	Dr. L. Speck
Analysis for galvanic skin response data	Dr. T. Zahn
Preparation of programmed stimulus tapes for reaction timer	Dr. T. Zahn
Cumulative recording of food intake in a competitive situation for a social colony of squirrel monkeys	Dr. P. MacLean
Averaging of non-time-locked evoked responses by means of adaptive cross-correlation	Dr. C. Woody
Analyis of motor performance in subjects with Parkinson's tremor	Dr. J. Van Buren
On-line measures of single cell activity in auditory system of the cat	Dr. E. Evans Dr. P. Nelson

LINC pograms in preparation

Program	Requestor
Display and filming of axon model based on van der Pol equations solved on IMB 360/40	Dr. R. FitzHugh
Digitization of oscillographic traces recorded on 35mm film with optical scanner	Mr. L. Binstock Dr. R. Taylor
Analysis of evoked responses	Dr. G. Ojemann Dr. J. Van Buren
Power spectra of EEG from electrode implanted in temporal lobe during a learning situation	Dr. P. Fedio Dr. G. Ojemann Mr. W. Sheriff
Utility programs to write and read magnetic tapes compatible with the IBM 360	Mr. M. Bruce Mr. J. Bryan Mr. W. Sheriff
Activity of cortical pyramidal cells in relation to flexor-extensor activity of the arm of a monkey during a period of trained behavior	Dr. E. Evarts

LABORATORY OF NEUROCHEMISTRY

During fiscal year 1966 the Laboratory made a number of significant contributions to the general body of knowledge of how biological processes operate at the molecular level:

A molecular-level model of ribosomes was proposed on the basis of studies with the dye-stacking method developed in this laboratory. In the model, ribosomal ribonucleic acid is stretched out on the ribosome surface in a single-strand, thereby providing a relatively nonspecific matrix on which to bind messenger ribonucleic acid during protein synthesis.

A computer program for simulating chemical reactions between biochemicals was developed and applied to the problem of complex formation between nucleotide bases. All of the known properties of such complexes become explicable in terms of simple electrostatic interaction between the bases. This work represents the first successful attempt to understand how and why biological systems employ such complexes to store genetic information and to translate that information into action via protein synthesis.

Computer programs which reconstruct sequences of biopolymers automatically, developed in this Laboratory, were employed to test the consistency between proposed sequences and published fragment data as well as to design optimum experimental conditions for determining sequences. A new sequence was proposed for alanine-transfer ribonucleic acid and an optimum schedule of procedures for determining the sequences of the other transfer ribonucleic acids was developed.

A combined experimental and theoretical approach to the problem of the mode of action of non-competitive enzyme inhibitors was developed. The binding constants for chlorpromazine and serotonin derivatives on histamine methyl transferase were measured and found to agree semi-quantitatively with the values predicted on the basis of the computed electron donating abilities of the inhibitors.

Studies of the optical properties of biological polysaccharides, using optical theories developed in part in this Laboratory, showed that at least some of these biopolymers exist in helical structures just as do polynucleotides and polypeptides.

Work was begun on the isolation and characterization of cell membrane proteins, using a method of membrane isolation developed by one of the members of the Laboratory, as an initial step in determining how membranes control or otherwise affect cell differentiation and organization. At least fifteen different proteins were isolated from liver cell membranes and separated by disc/electrophoresis.

Work was continued to elucidate the nature of a fascinating biological discovery, made in this Laboratory, that chloroplasts isolated from the unicellular plant *euglena* will survive and reproduce when injected into hydra.

The new, classical theory of the interaction of light with molecular aggregates, developed in this Laboratory, was extended to include refraction, reflection, optical activity and circular dichroism of crystals and solutions.

Studies on the evolution and genetic control of immunoglobulins were pursued with vigor, using techniques for determining their structure developed in the Laboratory. Of the two genes known to be related to γG-immunoglobulin synthesis in the rabbit, the *b* locus has been shown to control at least part of the amino acid sequence of the light chains while the *a* locus controls the structure of at least part of the heavy chain. The complexity of the lemon shark, a primitive vertebrate, was shown to be comparable to that of mammals although, unlike mammals, the lemon shark lacks the other major classes of immunoglobulins.

The results of these studies have been presented to the general scientific audience in the form of seventeen scientific papers which are either in press or were published in fiscal year 1966. Fulfillment of less direct, but equally important, responsibilities of the staff to the scientific community were met by editing journals, refereeing manuscripts for journals, lecturing at universities and teaching courses in the graduate program at NIH and at local universities. Recognition of the scientific productivity of the group was indicated by offers to staff members of Chairmanships of Departments and Professorships at universities of good standing, by invitations to give lectures and present papers at important meetings and at universities, by a constantly increasing number of applications for positions in the laboratory by well qualified scientists, and by a rapidly increasing rate of requests for reprints of published articles.

During fiscal year 1966 the Laboratory devoted a considerable amount of time, energy and thought to the development of plans for the future. In order to give such plans tangible form, the staff members prepared, at no little cost in time and effort, the equivalent of research grant proposals which outlined in detail their research objectives and the means needed to carry them out over the next three to ten years.

Critical to our attempts to create plans for the future has been an attempt to evaluate the current status and probable evolutionary path of biological research and our role in that evolutionary process. At the risk of oversimplifying a vast, heterogeneous field of science, it appears possible to divide biological research into analysis and synthesis. At present there is much effort being expended to analyze biological events in terms of molecular events. Our role in this effort is clear: we are specialists in elucidating the how and why of molecular events which occur in biology in terms of the underlying physical forces involved.

When one engages in the effort to analyze biological events in terms of molecular events, one is immediately impressed by the fact that there exists a logical progression in the types of biological processes that should be studied, *i.e.*, those biological processes involving the

least number of individual molecular events should be studied first. Whether by chance or conscious design, the field of molecular biology has followed this simple logical principle. Enzyme-substrate and antigen-antibody interactions, and the storage and translation of genetic information are current foci of interest and study for the molecular biologist since they involve smallish numbers of molecular events. Our Laboratory has concentrated on these same problems. Once a body of reliable knowledge on these simple processes has been accumulated, the field of molecular biology can move progressively and with confidence toward the analysis of biological events which, presumably, involve larger numbers of molecular events. Thus, one can envisage a natural evolution of the field toward cell differentiation, growth, organization, division, aging, infection and repair and then toward organ and organism behavior. Although it is clear that powerful drugs such as LSD or biopolymers such as RNA can affect very complex processes such as memory, learning, perception and consciousness, we feel that reliable knowledge of such processes and the effects chemicals have on them will come only by the long, arduous route now being followed by the molecular biologists. In the course of this evolution, analysis will be supplemented by synthesis of individual molecular events into increasingly complex molecular systems. It is in the realm of syntheis where computers will become increasingly necessary in molecular biology, for only the computer can simultaneously retain the hard-won knowledge of each molecular event and organize large numbers of such events into an integrated system. In order to be able to continue in the forefront of molecular biological research as it shifts emphasis from anlaysis to analysis-symthesis, our Laboratory is taking advantage of every available opportunity to assist in the development of computer facilities at NIH and to devise new methods for using them to study biological events.

LABORATORY OF CLINICAL SCIENCE

The Laboratory of Clinical Science, which is conserned with the broad area which extends from the basic biological sciences into the problems of clinical psychiatry, is comprised of senior investigators each of whom is identified with a major biological discipline in addition to clinical training or interests. The research of the year now nearing completion has been concerned with an extension on the part of a number of the senior investigators of their research into areas to which they have in recent years contributed signifiant new concepts and information.

Dr. Louis Sokoloff has made considerable progress toward identifying the soluble thyroxine-mitochondrial product, which his earlier work showed to have a specific and important effect on protein synthesis, which may probably represent a fundamental mechanism of the physiological action of thyroid hormone in the body. With Dr. Krause, he has demonstrated a thyroxine effect on the synthesis of a specific, naturally occurring protein—hemoglobin—in addition to the general effects on protein synthesis in brain and liver which he has previously demonstrated.

Dr. Julius Axelrod has pursued many of the ramifications which his earlier work on catecholamine metabolism made possible. With Jacques Glowinski and Leslie Iversen, a number of studies were completed dealing with the effects of important psychoactive drugs, such as reserpine, monoamine oxidase inhibitors, imipramine, and amphetamine, which appear capable of explaining the therapeutic effects of these agents in terms of their actions at adrenergic synapses in the brain. In cooperation with Dr. Richard Wurtman, an important regulatory action of adrenocortical steroids on epinephrine formation in the adjacent adrenal medulla has been discovered with clear indications that this mechanism may be involved significantly in the adaptive response of the organism to various kinds of stress. Further studies on the endocrine function of the pineal indicated by previous work of these investigators have been concerned with the circadian rhythms of serotonin and catecholamine associated enzymes, and effects and pathways of environmental lighting in this rhythm.

Dr. Irwin Kopin has extended his earlier studies of the factors mediating and attenuat-

ing the action of norepinephrine at peripheral sympathetic nerve endings. Earlier with Musacchio and currently with Schildkraut and Schanberg, studies have been undertaken which appear capable of defining some of the regulations involved in norepinephrine synthesis at these sites. With Baldessarini, attempts are being made to develop the brain slice as a useful experimental model in factors affecting the release of biogenic amines in the brain. These investigators have continued their basic contributions to the mechanism of transmethylation in the brain. One current hypothesis has suggested an involvement of this process in schizophrenia.

Dr. Marian Kies has continued her work on experimental autoimmune encephalitis, the best defined of all organ specific immune diseases, toward an understanding of autoimmune phenomena generally and, more specifically, in the brain, the encephalitogen which she has purified from brain and shown to be identical with the basic protein extracted from pure myelin has been used in the development of an extremely sensitive test for myelin proteins. With Dr. Borriss, an inhibitory effect on lymphocyte protein synthesis has been demonstrated by purified encephalitogens. With Dr. August, a technique has been developed for detecting antigen-antibody combinations which does not require coupled biological assay and which may be useful in screening large numbers of sera for specific antibodies.

Dr. Edward Evarts has extended his studies of individual cerebral neurons in freely moving animals which made possible his earlier important contributions to the physiology of sleep. His current studies on the activity of pyramidal tract neurons in connection with voluntary movement have elucidated for the first time important temporal relationships between the activity of different classes of such neurons and conditioned reaction times which promise to provide clarification of the physiological mechanisms of sensorimotor integration. With Dr. Bizzi, an important regulation of visual sensory input has been found which appears to anticipate eye movement. This finding is the first demonstration of what appears to be an important general principle underlying central nervous organization. It is possible that whenever voluntary movements are carried out impulses go not only to the appropriate muscles but also to the sensory system, the information from which would be expected to be modified by the movement.

Dr. Jack Durell has obtained some gratifying results in his program of study of the interaction between the biological organism and the social field in two therepautic milieux which he and a dedicated staff have established. In addition to certain clinical predictors of outcome which his pilot studies have suggested, a number of interesting biochemical findings have been obtained. His studies with Dr. Schildkraut on the excretion of catecholamine metabolites, in imipramine induced and spontaneous remission in depression, have been extended with corroboration of their additional findings of a rise in the secretion of normetanephrine in association with clinical improvement. Studies have been initiated in collaboration with other investigators of the Laboratory directed at an examination of sodium movements between intracellular and extracellular compartments of the brain in association with clinical change in the manic depressive syndrome. The studies with Dr. Ryan on an alleged plasma factor in schizophrenia have been completed. They were successful in demonstrating that the increased lactate production by erythrocytes incubated with certain human plasma was mediated through hemolysis, which in turn was caused by the action of a complement-requiring antibody present in the plasma of certain individuals. They were unable to demonstrate that this antibody is characteristically found in schizophrenics. With Dr. Friedel, it has been possible to pursue earlier findings on the action of acetylcholine on phosphatidic acid metabolism in brain fractions containing synapatic elements. An hypothesis has been formulated which is compatible with many different observations in the literature and work has begun on the testing of its components.

With Dr. Guggenheim, Dr. Philippe Cardon has been conducting collaborative clinical research in a number of different areas including an extension of his earlier studies on the

differing response in various classes of individuals to norepinephrine and, in collaboration with Dr. Pollin of the Laboratory of Adult Psychiatry, on the incidence of medical illness in the families of twins discordant for schizophrenia.

With Drs. Reivich and Isaacs, the Laboratory Chief has been extending his earlier interest in cerebral circulation to examination of the regional circulation under a number of physiological states. A productive collaboration with Drs. Evarts and Sokoloff has made possible an approach to regional metabolism in various stages of sleep. The collaborative studies with Drs. Rosenthal and Wender on nature-nurture interrelationships in schizophrenia examined through study of adopted individuals is at its peak of data collection. It is hoped that results of certain of these studies will be available at the time of the next annual report.

More detailed summaries of the work of the individual sections prepared by the respective section chiefs follow.

Section on Cerebral Metabolism

The Section on Cerebral Metabolism has continued its research on the mechanism of action of the thyroid hormones, the relation of this mechanism of action to the different responses of mature and immature brain to the thyroid hormones, and the role of these hormones in the biochemical processes underlying development, maturation, and functional activity of the central nervous system.

It was work done in this project that first demonstrated that thyroid hormones stimulate the rate of protein biosynthesis. This effect of thyroxine was found *in vivo* and *in vitro* in cell-free preparations, but only in tissues which respond to thyroid hormones with increased oxygen consumption. Hypothyroidism is associated with a decrease in the rate of protein biosynthesis. Inhibition of protein biosynthesis *in vivo* by means of puromycin, a drug which inhibits protein biosynthesis at the same step or one preceding the step stimulated by thyroxine, resulted in only slight decreases in total body metabolic rate of euthyroid animals but in marked reductions of the metabolic rate in hyperthyroid animals; in fact, puromycin acutely, and completely reduced the metablic rate of the thyrotoxic animals to the level of normal or puromycin-treated euthyroid animals. These results indicated that a higher percentage of the total body oxygen consumption in thyrotoxicosis is associated with the process of protein biosynthesis than in the normal state and that the increased metabolic rate of hyperthyroidism is almost entirely secondary to the increased rate of protein biosynthesis.

For the past two years the major efforts of the Section have been directed at the molecular mechanism of the thyroxine stimulation of protein biosynthesis. Evidence has been accumulated that it is not thyroxine itself but a product of a thyroxine-mitochondrial reaction which is responsible for the stimulation. The stimulation has been localized to the step involving the transfer of sRNA-bound amino acid to ribosomal protein. It results in increased protein biosynthetic activity of the ribonucleoprotein particles, and this stimulation is independent of any effect on messenger RNA synthesis. In fact, it has been demonstrated to occur within 2 hours after the administration of thyroid hormones to an animal, a period of time far shorter than that reported to be required for RNA synthesis to be affected. Furthermore, thyroxine stimulates protein biosynthesis *in vitro* in the absence of messenger RNA synthesis and, in fact, stimulates synthetic polyribonucleotide-directed amino acid incorporation into artificial polypeptides.

Progress has been made on the isolation, purification and identification of the thyroxine-mitochondrial product responsible for the stimulation. It has been separated from the mitochondrial fraction and found to be soluble heat-stable, dialyzable, acid-labile, relatively alkali-stable, and destroyed by ashing. By means of substitution and kinetic studies, it has been distinguished from GTP, ATP, GSH, cyclic AMP, potassium, Mg^{++}, or other substances currently known or suspected to influence the rate of protein biosynthesis. Recently it has been found to be possible to remove contaminating nuceotides by charcoal treatment of the factor solution without serious loss of factor activity; this may be an important puri-

fication step and may lead to increased progress in purifying and identifying the factor.

In the previous year it was found that thyroxine *in vitro* could stimulate the incorporation of amino acids into the α and β chains of hemoglobin being synthesized by cell-free rabbit reticulocyte lysates. This was the first demonstration of a thyroxine effect on the synthesis of a specific, naturally occurring protein. Together with the observed effects in liver and immature brain, this finding in regard to hemoglobin synthesis suggests that the thyroxine effect is not limited to one or a few specific proteins but is a generalized effect on the protein biosynthetic machinery. Furthermore, both chains of hemoglobin have been well characterized, and it is known that the N–terminal amino acid (the initial amino acid laid down in the synthesis of the protein molecule) in both cases is L–valine. During the past year, N–terminal and interior amino acid analyses have been carried out, and though the studies are not yet complete, it is already apparent that thyroxine stimulates not only the completion of the chains but also the initiation of the chain synthesis. This is an important finding in that it is the first demonstration that the thyroxine effect is on de novo protein synthesis.

It was observed in earlier studies that amino acid incorporation into protein in immature brain preparations is more rapid than in mature brain preparations. Also thyroxine stimulates amino acid incorporation in the immature brain but not in mature brain. Both differences are the results of some functional dissimilarities between mature and immature brain mitochondria. Another functional difference between mature and immature brain mitochondria has now been observed. Immature brain mitochondria contain the enzyme, β-hydroxybutyrate dehydrogenase, which remains active during the period of maturation of the brain and then rapidly declines to negligible levels. Two such enzymes have been observed in other tissues, one which catalyzes the oxidation of the D-isomer of β-hydroxybutyrate to acetoacetate and the other which catalyzes the oxidation of the L-isomer. During the past year it has been clearly demonstrated that it is only the D-enzyme which is present and undergoing the changes in activity during maturation of the brain. It has been solubilized and partially purified, and evidence of a heat-stable cofactor in the reaction has been obtained. This cofactor can be partially replaced by commercial lecithin, but the replacement is less effective than the natural cofactor. The exact function of this enzyme is not known, but it is believed to be involved in lipid synthetic or metabolic pathways. Investigations are under way to determine if this enzyme plays any part in the biochemical processes related to maturation of the brain.

In the course of studying mitochondrial protein biosynthesis in brain, it was observed that impure preparations of mitichondria from immature brain contaminated with myelin and nerve endings also incorporate amino acids *in vitro* into peptide linkage in the proteolipid of myelin. This finding offers the possibility of studying the mechanisms of myelin synthesis *in vitro*, and studies along these lines are being carried on.

The studies currently in progress and being planned for next year are along the lines indicated by the open questions and gaps in our knowledge pointed out in the discussion above. It is likely that the maximum efforts will be directed at the identification of the thyroxine-mitochondrial product.

Section on Pharmacology

The direction of the Section on Pharmacology still continues along four main lines of investigations: the physiological dispositions of hormones and drugs, biochemical mechanisms of actions of drugs, enzymes involved in the metabolism of drugs and hormones and the function of the pineal glad.

Mainly through the efforts of visiting scientists, Jacques Glowinski and Leslie Iversen, considerable new information concerning the physiological disposition of H^3-noradrenaline in the rat brain has been obtained. In the past year, efforts were directed to studying the uptake, subcellular distribution and turnover of H^3-noradrenaline in different areas of the brain. H^3-noradrenaline injected into the lateral ventricle of the brain was taken up in

relatively large amounts in those areas having high endogenous concentrations of the catecholamine (hypothalamus, midbrain) and in small amounts in those areas having small amounts of the amine (cortex, cerebellum). Radioautographic studies also showed that H^3-noradrenaline was highly localized in tracts of the limbic area of the brain. Various regions of the brain showed differences in the subcellular distribution and the turnover rate of noradrenaline. Although the cerebellum contains a low concentration of noradrenaline, it appears to utilize this amine at a greater rate than any other area of the brain.

With the use of H^3-dopamine and drugs inhibiting uptake of H^3-noradrenaline, it was demonstrated that the central adrenergic neurons release noradrenaline and then recapture the discharged neurotransmitter. Marked regional differences in the effect of amphetamine and imipramine on inhibiting the uptake of H^3-noradrenaline were observed. There were also considerable differences in the ability to release the neurotransmitter by amphetamine and reserpine in various brain areas. In subcellular studies, amphetamine was found to act mainly at the nerve terminal while reserpine exerted its effects along the entire neuron and cell body. There was a correlation between the behavioral effects of reserpine and its ability to inhibit the accumulation of H^3-noradrenaline in brain neurons.

Methoxy metabolites of catecholamine (normetanephrine, metanephrine, and methoxy dopamine) enhanced the uptake of H^3-noradrenaline in certain tissues (salivary gland, vas deferens). Since normetanephrine is formed at the nerve terminal, it is suggested that this metabolite may serve a mediating role in the reuptake of H^3-noradrenaline.

Since the adrenergic receptor is the most important element in the adrenergic mechanism, an attempt to examine its biochemical properties was begun. The uptake of H^3-noradrenaline in extraneural tissues (which includes the receptor) and the effect of drugs were studied. After blocking neuronal uptake mechanisms with cocaine, it was found that α-adrenergic blocking agents had a profound effect inhibiting intracellular extraneural uptake of H^3-noradrenaline into heart muscle. β-Adrenergic blocking agents had a less marked effect. Preliminary studies suggest that uptake processes which are different from neuronal uptake are involved in extraneural intracellular accumulation of H^3-noradrenaline in the heart muscle. This study is being carried out in collaboration with Drs. Eisenfeld and Krakoff.

A study on the possible relationship between the development of hypertension and the malfunctioning of the sympathetic nervous system was undertaken with Drs. Jacques de Champlain and Lawrence Krakoff. Rats were made hypertensive with DOC and high sodium diet and the uptake and metabolism of H^3-noradrenaline were studied. There was a clear and highly significant inverse relationship between the degree of hypertension and the ability to accumulate H^3-noradrenaline in heart and certain tissues.

Dr. Eisenfeld had continued to study the properties of the binding of H^3-estradiol and target organs in the periphery and central nervous system. This uptake has been shown to be highly selective. A compartmental model for estradiol uptake by target tissues has been derived from the experimental data.

In collaboration with Dr. Richard Wurtman, it was found that the adrenaline-forming enzyme in the adrenal medulla is controlled by the secretion of corticoids in the adrenal cortex and by ACTH secreted by the pituitary gland. The corticoids act by increasing the synthesis of the protein that makes the adrenaline-forming enzyme.

Two types of ectopic tumors were characterized by measuring two unique enzymes discovered inthis laboratory. One ectopic tumor contained a methanol-forming enzyme which is found only in the pituitary and another contained a melatonin-forming enzyme which is confined to the mammalian pineal gland. The melatonin-forming enzyme was found for the first time in species (amphibians, fish) where this hormone exerts its skin blanching effects.

A new enzyme that O-methylates monophenol was found. It is present in the microsome of liver and other tissues.

Studies with the pineal showed a 24-hour

rhythm in the noradrenaline content of this gland. Unlike the serotonin rhythm, the catecholamine rhythm is completely controlled by environmental lighting. The circadian serotonin rhythm was found to be present at birth and for the first 12 days to be partly influenced by extra retinal receptors. The serotonin rhythm in the pineal appears to arise from a periodic release of the biogenic amine. The information concerning environmental lighting reaches the pineal gland gonads via the medial forebrain bundle. Interruption of the classical visual tract in the brain has no influence on the effect of environmental lighting on pineal gland and gonads. Preliminary results indicated the messages concerning endogenous rhythm are first sent through the medial forebrain bundle.

Section on Medicine

The current investigations of the Section on Medicine concern the synthesis, storage, release, metabolism and mode of action of the biogenic amines and their modification by nerve impulses, aging, drug treatment and endocrine and electrolyte status.

The rate of norepinephrine synthesis is examined by studying its formation from various labeled catcholamine precursors or by studying the rate of decrease of its specific activity after labeling with norepinephrine-H^3. Norepinephrine synthesis appears to depend on the occupation of norepinephrine storage sites in the synaptic vesicles of the neuron. Nerve stimulation releases norepinephrine from the nerve ending. A major fraction is taken back into the sympathetic nerve by an active transport system which is subject to interference by drugs such as cocaine or desmethylimipramine and by sympathomimetic amines. Portions of the remaining released norepinephrine react with the receptor, are destroyed by catechol-O-methyltransferase or enter the circulation. Such losses are replenished by synthsis. Thus, nerve stimulation or any environmental influence which results in an increased number of impulses stimulates synthesis. Drugs which block reuptake of norepinephrine released by nerve stimulation further increase norepinephrine synthesis just as tyramine depletion of norepinephrine stores stimulates synthesis of norepinephrine, as we previously observed.

Brain or heart slices concentrate several tritium-labeled birogenic amines (norepinephrine, sertonin, histamine, etc.), and electrical stimulation induces their release. Such release of norepinephrine is inhibited when calcium content is low or by drugs such as pentobarbital, chlorpromazine and desmethylimipramine. The tissue-slice technique may provide a method for studying the action of drugs, ions and hormones on the process of transmitter-release coupling.

The demonstration that labeled amines injected into the ventricles of rats may provide a valid tracer for endogenous amines stimulated the search for a simpler means of labeling the amine stores of brain. Intracisternal injection appears to be as valid as intraventricular and is being routinely used in this laboratory for such studies. Turnover rates of norepinephrine in various physiological states (sleep, REM deprivation, cold exposure, etc.) and alterations of turnover in response to drugs are also being studied. Nembutal anesthesia has been shown to elevate initial levels of several amines as well as the level of urea, but ether anesthesia does not appear to have the same effect. This may reflect cerebrospinal fluid circulatory changes or alterations in diffusion barriers in brain during nembutal anesthesia.

Studies of substances which can replace norepinephrine at its binding sites and can act as false neurochemical transmitters continue. Their possible role in disease states and drug action has been previously reported by this laboratory. The interaction of the false transmitters with bretylium, which interferes with axonal nerve impulse transduction to transmitter release, is being examined. Bretylium does not cause great release of norepinephrine, but it does very efficiently release some of the α-methylated false transmitters. This difference in action may provide a means for assessing avidity of amine binding to norepinephrine storage sites.

Investigation of familial dysautonomia has led to observations of clinical abnormalities in several sensory systems: taste, hearing, per-

ception of heat, cold and pain, and abnormalities in cardiovascular as well as peripheral reflexes. Such abnormalities are being investigated in collaboration with physicians of the National Heart Institute. Both the patients and their mothers appear to excrete excessive amounts of homovanillic acid, a metabolite of dopamine. The patients excrete lower amounts of VMA, the major norepinephrine metabolite, than do their mothers. Since no significant abnormalities in metabolism of administered catecholamines have been found, further investigation in the project will be held in abeyance until methods for assessment of receptor physiology in man can be developed.

Methylation is a major pathway for metabolism of the catechol- and other amines. The addition of a methyl group alters the activity of the compound and may result in activity increase or decrease, or it may impart entirely new properties to the molecule. S–Adenosylmethionine is the major methyl donor in mammals, and a method of assaying this key intermediate has been developed. Methionine increases SAMe levels, while methyl acceptors diminish its concentration. Levels are higher in infant liver and brain than in corresponding adult organs. The levels are elevated in leukemic white blood cells and depressed in livers of animals with a portacaval shunt, presumably because dietary methionine fails to reach the liver. The dynamics of activation of methionine are currently being studied.

Section on Biochemistry

The Section on Biochemistry has combined the techniques of immunology and biochemistry to study the phenomenon of autoimmune pathology. Because of the responsibility of scientists in the Institute to extend and develop areas of knowledge pertaining specifically to mental health we have concentrated on the brain as the experimental model. The information gained, as well as the techniques developed, are generally applicable to autoimmune pathology of other organs.

Experimental autoimmune encphalitis is the best defined of all of the organ specific immune diseases. Current studies on the etiology of this disease are based almost entirely on results of investigations of the Section on Biochemistry over the last ten to twelve years. Our group was the first to isolate a purified basic protein fraction from brain and demonstrate that the major portion, if not all, of the encephalitogenic activity of whole tissue resided in this group of proteins. Additional studies have demonstrated the immunologic homogeneity of the basic proteins as CNS specific, species nonspecific antigens. Thus, purified myelin protein antigens will soon be available for testing hypotheses of CNS autoimmunity in both clinical and experimental situations.

Our initial observation that basic proteins constitute an important fraction of myelin proteins was proven by actual isolation of the protein from purified myelin. The basic protein extracted from pure myelin was shown to be identical to the purified encephalitogen previously isolated from whole brain homogenate. An important consequence of this discovery was the fact that an extremely sensitive biological test could be added to the various chemical techniques available for studying myelin proteins. As little as 1γ of encephalitogen can be detected by bioassay whereas most chemical analyses for specific proteins require much more, e.g., a single disk gel electrophoretic pattern requires 50–100λ, at the very least 25λ. With this added analytical tool, we obtained some of the earliest definitive data on purified myelin protein-lipid complexes: the identification of some of the lipid firmly bound to proteins. Fractionation of these protein-lipid complexes by solvent distribution coupled with bioassay of the fractions provided proof of the complexity of myelin proteins (as opposed to the widespread belief that "myelin protein" was a single molecular entity).

During the past year, the work pioneered by this laboratory—demonstration that basic proteins comprised an important part of myelin—has been acknowledged by other major neurochemical groups by corroborating publications.

In a study of the role of lymphoid cells in the development of experimental autoimmune encephalitis, we have investigated the effect of purified CNS basic proteins on *in vitro* protein synthesis by guinea pig lymph node cells. Dr. Elsa Borriss, a visiting scientist in the Bio-

chemistry Section, has demonstrated that *in vitro* incorporation of leucine C–14 into guinea pig lymphocyte proteins is inhibited nonspecifically by purified encephalitogens from various species, both homologous (guinea pig) and heterologous (human, bovine). Less pure heterologous encephalitogenic preparations exhibit the opposite phenomenon—i.e., specific stimulation of leucine C–14 incorporation. The stimulation, however, is related to the presence of species specific determinants on these bovine basic protein preparations.

The demonstration that CNS basic proteins are able to affect metabolic activity of living cells *in vitro* suggests the possibility that these proteins may function as regulators of myelin synthesis *in vivo* analogous to the idea that histones are regulators of nuclear activity. (Preliminary data obtained with C. Klee support this idea.)

The long-term goal of this project is to study the role of lymph node cells in the development of delayed hypersensitivity. Experiments are in progress to isolate the newly synthesized (C–14 labeled) globulins. Immunoelectrophoresis coupled with radioautography will be used to define their location in the culture (cell bound or in the medium) and their immunologic specificity.

As an adjunct to his other immunologic studies, Dr. August has developed a technique for detecting antigen-antibody combination which may be used to study all types of binding phenomena. The prerequisite is that small molecules involved can be isotopically labeled. It possesses the great advantage that the method does not require a coupled biological phenomenon such as skin reactivity, complement fixation, hemagglutination, anaphylaxis, etc. for determination of antibody. Furthermore, many tests can be run simultaneously making it a useful method for screening large numbers of sera for specific antibodies.

The technique depends on the differential migration of large and small molecules through synthetic gels. We have used I–125 labeled antigen (the small molecule) to detect the presence of specific antibody (large molecule) in various experimental sera. With proper choice of experimental conditions and controls, the type of antibody (19s-macroglobulin or 7s-γ-globulin) can be determined, as well as information regarding the amount of antibody and the avidity of the antigen-antibody combination. Not only is the method useful for detection of antibody, it can be adapted for the study of other biological interactions such as occur in drug allergies. Another application is immunoassay of low molecular weight hormones at levels of sensitivity far below current techniques.

Finally, the laboratory has continued its study of the molecular size of the encephalitogen and characterization of its active site(s?). Fractions from serum digests of pure antigen are being investigated with regard to molecular size, encephalitogenic activity, antigenic specificity, and amino acid composition. The major constituent of the bovine encephalitogen is a cathodic protein of moleculr size ~60,000 (estimated by gel filtration on Sephadex G–75). Serum digests contain small amounts of this component plus three other UV absorbing fractions. The latter are presumably peptides of smaller size—some less than 10,000 molecular weight. At least two of the three peptide fractions have encephalitogenic activity. The biological activity of the smallest of the group is still under test.

Related studies have shown that different preparations of encephalitogenic basic protein may have widely different molecular sizes. For example, certain preparations consist predominantly of a basic protein which is one-half or one-fourth the size of the bovine preparation used for serum digestion experiments. In searching for an explanation for this variability in size, we have obtained data which support the hypothesis that variable activity of brain cathespin and/or serum proteinase present in the original brain homogenate is responsible for the size of the final purified protein.

Because of our long and varied experience in this field, we are frequently approached by other laboratories for collaboration, advice or assistance in carrying out studies on EAE. We are currently "collaborating" in this manner with Dr. M. Wolman in Tel Aviv, Dr. Martenson in Surrey, England, Drs. Amaducci and Cazzula in Italy, Dr. Waksman at Yale, Drs.

Campbell and Wolfgram in Los Angeles, Dr. Tourtellotte in Ann Arbor, Michigan, Dr. D. Heilman at the V.A. Hospital in the District of Columbia, etc.

A particularly interesting collaborative study on experimental autoimmune encephalitis is being carried out with Dr. Peter Lampert at the Armed Forces Institute of Pathology. He has studied the pathological lesions associated with the disease by electronmicroscopy. We hope to combine the techniques of the two groups in an attempt to demonstrate the presence or lack of specific antibody in the area of the lesions.

Section on Physiology

Two major problems have been investigated in the Section on Physiology during the past year: 1) the relation of pyramidal tract activity to voluntary movement, and 2) mechanisms whereby the brain coordinates and integrates eye movement information with input from visual stimulation of the retina. These two areas of investigation will be described separately.

The Relation of Pyramidal Tract Activity to Voluntary Movement

In the previous annual report, it was pointed out that it has now become possible to record the activity of individual cerebral neurons in moving animals. This new technique opens up an important area which had previously been uninvestigated: prior to the recent work carried out in the Section, there had been no studies of the way in which pyramidal tract neurons (PTNs) control and initiate voluntary movement.

In the past year investigations have been directed toward determining the precise time in a stimulus-response sequence at which the activity of PTNs comes into play. In order to determine the timing of this pyramidal tract discharge it was necessary that the monkeys make a specific hand movement in response to a stimulus. Monkeys were trained to depress a modified telegraph key until a light came on (the light being the conditioned stimulus) and then release (conditioned response) the key promptly following light onset. This stimulus-response sequence is analogous to simple reaction time tests used in man and, in fact, the reaction times of monkeys who have been thoroughly trained are the same as the reaction times of highly motivated and well-trained human subjects. This experimental situation then, allowed investigation of the central events intervening between stimulus and response in a conditioned hand movement.

The results of the study answered four major questions of pyramidal tract physiology. These questions and their answers follow.

At what point in the interval between conditioned stimulus (light onset) and conditioned response (wrist extension) does modification of discharge in PTNs occur? It was found that even in cases of minimum (180 msec.) reaction times by the monkey, the latency of the antecedant modification of pyramidal tract discharge does not fall below 100 msec. This 100 msec. latency stands in sharp contrast to the 30 msec. latency with which PTNs in the motor cortex discharge in response to a photic stimulus in animals anesthetized with chloralose. It is clear then, that for this conditioned movement, the latency of response in PTNs is delayed at least 70 msec. beyond the minimum latency demanded by the anatomical connections between the retina and motor cortex. What sequence of neuronal events takes place during this 70 msec. delay? An answer to this question would provide useful clues as to mechanisms of sensorimotor integration.

Is the occurrence of the wrist movement temporally locked to the PTN response? There was a strong positive correlation between the reaction time of the monkey and the latency of response in PTNs. Thus, in the most proficient monkeys, PTN discharge might begin 100 msec. after the conditioned stimulus; the arm muscles might begin to show electromyographic responses about 140 msec. following the conditioned stimulus; and the final response (opening the contact) might occur in 180 msec. For longer latencies of PTN response there were longer reaction times. Thus, it was found that not only did PTN activity precede the behavioral response, but also that lengthening of response latencies in PTNs was asso-

ciated with lengthening of behavioral response latencies, i.e., reaction times.

What is the relation of axonal conduction velocity of a PTN and the response which it shows in association with movement? In a previous study described in the annual report one year ago, it was found that PTNs with high axonal conduction velocities tended to be silent in the absence of movement, but to become extremely active during movement. PTNs with lower axonal conduction velocities were tonically active even in the absence of movement. The present study of a conditioned movement revealed an anlogous relationship between axonal conduction velocity and discharge properties in PTNs. Thus, units which were silent while the monkey was maintaining wrist flexion but spring into intense activity prior to wrist extension had high axonal conduction velocities. Units with low axonal conduction velocities did not show such sharp transient responses and when they were related to the wrist extension showed either a reduction in discharge frequency or an increase of what had been tonic discharge persisting throughout wrist flexion.

Is activity of PTNs related only to contralateral movements, or are there some PTNs whose discharge is related to ipsilateral movements as well? The great majority of PTNs examined were related to movements of the contralateral wrist and were relatively inactive in relation to ipsilateral wrist movements. Some units were found, however, for which the reverse was true, and this finding provides additional evidence for the role of the pyramidal tract in control of ipsilateral movement.

Mechanisms Whereby the Brain Coordinates and Integrates Eye Movement Information with Input from Visual Stimulation of the Retina

This study of the integration of eye movement information with information from the visual input to the retina grew out of a previous study of activity in the lateral geniculate nucleus during the rapid eye movements (REMs) of sleep. This study showed that each REM of sleep is associated with a volley of impulses arising in the oculomotor centers and impinging on the lateral geniculate. It was reasoned that the occurrence of this "corollary" discharge in the lateral geniculate must have significance with respect to waking as well as to sleeping eye movements, and it was therefore decided to investigate this problem in the case of the eye movements of waking monkeys. The technique of single unit recording was employed.

It was found that certain neurons in the lateral geniculate nucleus discharge in relation to eye movements, some of these neurons discharging even *before* the eye movement has occurred. Such prior discharge cannot be the result of feedback from the eye muscles and therefore proves that there may be a discharge of neurons in a sensory pathway in *anticipation* of a coming movement. This finding is the first demonstration of what will probably turn out to be a highly important general principle underlying CNS organization. It seems not unlikely that whenever *intentional* or *voluntary* movements are carried out, impulses go not only to the muscles whose contraction is necessary for the occurrence of the movement, but also to these sensory systems whose incoming information will be modified by the movement. In a sense, one may say that instead of having to wait to be told by input from the periphery that a particular movement has occurred, the sensory systems are told of the impending movement even before it takes place. These sensory systems may then interpret the input which comes to them in the light of this knowledge.

The theoretical formulation proposed above is not a new one (it was first proposed by neurologists and psychologists many decades ago). The present experiment, however, provides the first clear proof of the theory and is also the first to show corollary discharge at the level of the single neuron in association with voluntary movement.

Significance for Mental Health Research

The two projects described above involve analyses of the central events associated with voluntary movement. The study of hand movements is aimed at discovering how output information is coded, while the study of eye movements is primarily concerned with the de-

lineating events in sensory systems which are associated with voluntary movements. These two projects complement each other in that an ultimate general understanding of voluntary movement will require consideration of both of these classes of problems.

The broad aim of these projects, then, is to achieve an understanding of the central mechanisms underlying voluntary movement. At first glance it might seem that this problem bears little relevance to problems of mental health and disease. However, students of the mind have long been struck by the existence of an indissoluble relation between the movement output of the organism and the very essence of mind and thought. The greatest brain-research workers, ranging from Hughlings Jackson to Roger Sperry, have suggested that our understanding of the mind and its disorders may be facilitated by working into the system "backwards" from its output, rather than forward from its input. It therefore seems likely that the principles of nervous system organization revealed by our studies on voluntary movement may provide a deeper understanding of how the brain functions in its most complex aspects. Such an understanding would clearly contribute to our knowledge of thought processes in both health and disease.

Section on Psychiatry

The past year has been a gratifying one. The new activities and new directions of research initiated during the previous two years have begun to solidify, creating the sense that the Section is now well on the way to establishing its long-range research course. As previously stated, it is the goal of the Section to increase our understanding of the mechanisms by which disturbed behavior is generated or dissipated through a greater appreciation of the interaction between the biological organism and the social field. The major fields of observation are the two therapeutic communities which have been devised in a way which permits much data collection both of a social interactional nature and a biological nature. The longitudinal descriptive clinical ratings have been institutionalized and it is envisioned that the information thus stored will prove most instructive when analyzed several years hence. The plans to initiate controlled studies have been formalized an are now in effect. Because of the desirability of attempting to obtain a relatively homogeneous group of patients for control studies, the rate of admission of such patients to the wards has been slow but there is reason to believe that a sufficient number will have been studied within the next five years to make the results quite meaningful. An extensive follow-up study of all of the patients admitted to the therapeutic community is now under way which will provide very valuable clinical dscriptive material from which various hypotheses can be generated for more rigorous testing. In addition, the regular collection of urine and other biological samples as well as patients' participation in various physiologic procedures is now well institutionalized in both units.

In addition to the sense of smooth institutionalization of the research, there is also clear evidence of a maturing of the therapeutic team with a corresponding growth of autonomy and creativity among the therapeutic staff members at lower echelons. The psychiatric staff, as well as the auxiliary and nursing staff are able to operate with much less supervision directly from the Section Chief, while maintaining the clinical approach and philosophy which had originally been introduced by the Section Chief. In recent months this has allowed the Section Chief much greater time to employ in consolidation of the research activities and it is envisioned that the following year will be even more successful in that regard.

In addition to clinically based studies, the Section endeavors to maintain a continued interest in basic neurochemical research. The Section Chief is well aware of the danger that biological psychiatry can become both poor psychiatry and poor biology. Therefore, in addition to the large emphasis placed upon the social interactional therapeutic milieu itself, it is felt necessary to maintain a contact with basic neurochemical research so that the biological concepts employed in the psychiatric research will remain in apropriate contact with the rapidly advancing events in the neurochemical sciences.

Since a major portion of the research effort

of the Section represents long-range clinical research from which there are few results until after many years of data collection and analysis, the aforementioned sense of a consolidation in organization is a very important measure of the progress made during the year. This is not to say that there have not been a number of specific findings in the various areas and these shall now be elucidated:

The analysis of the pilot follow-up study and the relation of status at follow-up to a number of initial social parameters has been virtually completed and will soon be ready for publication. It was found, by and large, that the staff attitudes towards the patients during his first week of hospitalization could partially predict the clinical state at follow-up one year later. In addition, the discharge status was a good predictor of the status at follow up. These findings suggest various hypothesis regarding the dynamics of the therapeutic community and consideration of means of testing some of these hypotheses are now under way. The results suggest that the attitudes of staff are in fact operators, influencing whether or not a patient's outcome will be favorable. Moreover, the results suggest further that the 4 East therapeutic milieu has, in fact, achieved certain of its goals which were the carrying out of therapeutic gains into the community, thus facilitating the patient's social integration into the community-at-large.

It has been shown in a series of schizophrenic patients with remitting psychoses that depression of mood characteristically occurred following the remission of psychosis. This is significant in the clinical sense it provides a warning that the staff must be aware of possible suicidal potential particularly when psychotic symptoms first remit. In addition, the findings have provided the basis for theoretical considerations regarding the psychological and neurophysiological mechanisms concerned in psychosis and the relationship of these to possible genetic factors and to phenothiazine therapy. Studies are under way relating changes in adrenal steroid secretion to the changes in affect and to the administration of phenothiazines. Observations such as these, though open to many interpretations, help to provide some of the documented empirical basis for hypothesis generation and thus serve a heuristic purpose.

We have had difficulty finding patients with cyclical schizophreniform psychoses but have managed to study one such patient this year. There are indications that changes in excretion of catecholamine metabolites and thyroid radioiodine uptake accompanies his psychotic episodes. The results bear some similarity to results previously obtained on a patient with an alternating motility cycle while differing sharply from our findings and those of Gjessing on periodic catatonia. It appeals that we have isolated two problems of physiological change associated with two different clinical syndromes, but this conclusion requires corroboration.

Studies on the changes in catecholamine metabolism associated with the imipramine treatment of depression have continued. The earlier suggestion that a rise in the secretion of normetanephrine is associated with definitive clinical improvement with imipramine has been corroborated on additional patients. The relationship between the excretion rates of the various catcholamine metabolites are now under study as are additional patients who are being treated with imipramine or amitriptyline or without antidepressant agents to determine whether these findings are specific to imipramine.

Methods have been developed to study electrolyte metabolism in manic depressive patients and the effects of lithium and imipramine on these variables. The methods including the use of whole-body counters and probes to detect head sodium appear to be satisfactory and the data collection period is just beginning. The clinical staff has treated several patients successfully with lithium carbonate. These patients had previously been treated in a number of other ways without success and the results with lithium carbonate appear promising.

With the departure of Dr. Ryan from the laboratory, studies on an alleged plasma factor in schizophrenia have virtually been terminated. The analysis of the data from a large population study at the Rockland State Hospital gave little evidence to support an associa-

tion of the antibody affecting chicken erythrocytes with schizophrenia. There are several interesting possibilities to follow up, however, especially in that some studies of the Section on Twin Studies indicate that psychosomatic factors may operate to influence the level antibody in certain schizophrenic individuals. This is not of primary interest to this Section and it is not clear whether these studies will be undertaken.

Previous indications that there may be some elevation in the activity of the sodium pump ATPase in certain psychotic states could not be confirmed in a population study at D.C. General Hospital. Unfortunately, it was difficult to control for the influence of a number of intervening variables at D.C. General Hospital and currently more controlled longitudinal study of patients whose clinical state undergoes change is under way in an effort to determine whether there are any associated changes in the sodium pump ATPase. We have speculated that alterations in the activity of this enzyme may also be associated with the changes in electrolyte metabolism is manic depressive illness, and this problem is being approached with the use of laboratory and human physiology techniques.

Basic studies on membrane function have narrowed down to the studies of the mechanism of acetylcholine effects on phospholipid metabolism. It has been shown that the acetylcholine sensitive phosphatidic acid metabolism of brain homogenates is limited almsot completely to the subcellular fractions containing "synaptasomes". Since the arrival of Dr. Robert Friedel in our laboratory there has been an intensive interest in studying the mechanism of this process in brain synaptasomes. An hypothesis has been formulated which relates a number of observations in the literature and suggests that the primary action of acetylcholine is upon a phosphatidylinositol phosphodiesterase. The testing of this hypothesis has required the tooling up in terms of the laboratory's capacity to separate and purify phospholipids, particularly triphosphoinositide and to prepare triphosphoinositide phosphodiesterase from brain and other tisse. These methods have now been mastered but it is too early to state whether the results are confirming our hypothesis or not. The hypothesis appears to be an important one since, if correct, it may provide insights into the mechanism of acetylcholine mediated membrane depolarization and consequently synaptic transmission. Knowledge about the molecular mechanism of this reaction could be of profound significance to studies of brain function and the effects of pharmacological agents upon the brain.

Unit on Psychosomatics

Members of the Unit have continued their activities in collaborative clinical research in several different areas.

Nervous and Circulatory Systems

The postulate that responsiveness to norepinephrine may be related to patterns of nighttime catecholamine excretion could not be confirmed. The rate at which the forearm decreases in circumference in late diastole correlates moderately well with forearm blood flow through the range of flow rates measured at rest, during epinephrine infusion, and the cold-pressor procedure. Evaluation of the estimated isometric period of ventricular contraction as an index of cardiac sympathetic tone has given puzzling prelimary results: by this criterion, high "cardiac sympathetic tone" may be associated with *low* free NE excretion. Incidentally, females have been found to have longer isometric periods than males—of interest when related to the general impression that individuals with long periods may be less subject to coronary artery disease.

Diet and Catecholamine Excretion

With Guggenheim, quantitative data on effects of diet on catecholamines and their metabolites in urine have been collected. Differences between very restricted and moderately restricted diets can be demonstrated (by paired comparison) in excretion of VMA, epinephrine and metanephrine. These differences are small compared with the differences among individuals on controlled diets. Norepinephrine excretion could not be altered by very excessive intake of coffee, chocolate, bananas, fruits,

vegetables, and vanilla. These data are very helpful for planning longitudinal studies of psychiatric patients.

General Health of Families

As a contribution to studies of families and schizoprenia, Guggenheim has expanded observations on their medical health—a further thyroid disease and cancer in parents of discordant identical twins. These observations may be pertinent to the possibility that schizophrenia may be usefully viewed as one of many possible manifestations of a broader psychological disorganization and vulnerability in families.

Observations in Psychiatric Illness

With other units and sections, Guggenheim has continued studies of heart rate and blood pressure in depression, but results cannot yet be given. Baer is evaluating the response to ACTH of the adrenal glands of depressed patients, and now is starting to follow total exchangeable body sodium in depressed patients, by means of the whole body counter. This is very promising because accurate daily estimations are possible which are not subject to the cumulative errors of inaccurate urine collection.

CLINICAL NEUROPHARMACOLOGY RESEARCH CENTER

The Clinical Neuropharmacology Research Center (CNRC) is located in renovated portions of the William A. White Building on the campus of Saint Elizabeths Hospital (SEH), a large Federal mental hospital in Washington, D.C. Established by NIMH in the late fifties in accord with SEH, CNRC gradually evolved a back-bone basic laboratory program of neurological research around which clinical research activities were fleshed out when desirable, to take advantage of the opportunities provided by the hospital's environment on the one hand and of the physical and intellectual support provided by the basic research program on the other.

The experience gained during the past several years with this type of arrangement and the development by SEH since the early sixties of its own Behavioral and Clinical Studies Center (BCSC) permitted last year to more precisely define the role of CNRC intramurally and vis a vis the SEH complex of research and training activities. Accordingly, steps were taken to bring about the strengthening of CNRC basic research activities, concomitantly with a reduction in the level of direct CNRC sponsorship of those clinical studies which experience showed to be more expediently carried out by SEH staff or as a collaborative endeavor with other clinical NIMH laboratories.

Additionally, closer ties were established between the CNRC and the SEH's research and training components through courtesy appointments extended to CNRC senior investigators by the Superintendent, SEH and by the Department of Psychiatry, George Washington University Medical School, with which SEH has a close working relationship. Thus the Acting Chief, CNRC was appointed Director of Research, SEH with direct responsibility to the Superintendent, SEH for the coordination of all research activities at SEH, inclusive of those of the hospital's BCSC, now led by the former Director of Behavioral Studies, BCSC who is concurrently Director of Training, SEH. Voluntary participation of senior CNRC staff in SEH training programs for residents and medical students was facilitated by the appointment of Drs. G. C. Salmoiraghi (neurophysiology-neuropharmacology,) H. Weil-Malherbe (neurochemistry) and S. Szara (psychopharmacology) as Associate Clinical Professors, Department of Psychiatry, George Washington University Medical School.

As in previous years, much of our effort was directed toward the elucidation of the mode of action of endogenous brain substances and related pharmaca appearing to affect central synaptic transmission. Most of these studies were, by necessity, carried out in animals but some utilized selected groups of patients made available through collaborative programs with SEH and the Adult Psychiatry Branch, NIMH. Human subjects were also used in studies for the development of analytical tools to assist in the interpretation of EEG records and for the study of saccadic eye movements.

Utilizing 5-barreled glass micropipette elec-

trodes, which permit the controlled electrophoretic administration of up to 3 drugs directly at the site of extracellular unit recording, studies were carried out on the lumbar segments of the cat spinal cord to determine the properties of pharmacological responsiveness of single spinal neurons to the suspected central transmitters acetylcholine (ACh), norepinephrine (NE) and serotonin (5-HT). The results of these studies, in addition to confirming evidence for ACh-mediation of an excitatory synaptic input on Renshaw cells, yielded data highly suggestive for adrenergic synapses on motoneurons as well as on Renshaw cells and other interneurons, presumably part of a bulbospinal pathway shown by others to be composed of NE-containing nerve fibers. These studies, moreover, showed each of the three suspected transmitters to be capable of producing either facilitation or depression of cell activity, depending upon the type of cell studied.

Taken together with all other evidence that we have thus far obtained from other CNS regions, the observations now made in the spinal cord leave little doubt that the direction of a unit's response to a suspected transmitter is not exclusively determined by the latter's chemical nature—as commonly assumed—but depends also upon characteristic properties of the effector cell's receptive membrane, determining whether excitation or inhibition will occur. Hence our evidence begins to suggest that the same transmitter may be either excitatory or inhibitory for different neurons or even for different synapses of the same neuron.

To eventually test this hypothesis as well as to obtain more persuasive evidence that ACh, NE and 5-HT are indeed transmitters in the mammalian CNS, intracellular recording is required to compare the conductance changes produced by a naturally released transmitter with those produced by the extracellular administration of the suspected substance, and to investigate the effects of potentiating and blocking drugs. For this reason, a multibarreled concentric micropipette electrode has been developed and is now being tested on motoneurons. Additionally, a major effort was made to develop a preparation suitable for the study of the pharmacological properties of individual patches of a nerve cell membrane using the gastro-esophageal ganglion of a Nudibranchs which contains one very large and 2 medium-size neurons.

Since these three cells form a miniature CNS, it is hoped that this preparation will be suitable for studying morphological and/or electrophysiological changes possible relatable to learning. In any event, such a preparation will be sought as part of a program of research in comparative neurophysiology likely to evolve from current studies by the Section on Neuropsiology and from those soon to be initiated on the fine structure and the cytochemistry of peripheral and central neurons.

The metabolism of NE and other catecholamines continues to be investigated by the Section on Neurochemistry. Pursuing earlier studies by this Section on the binding and storage of catecholamines in tissues, it has not been shown that rapid freezing substantially increases the proportion of particle-bound to free NE in the brain, while incubation of brain homogenates causes rapid release of particle-bound NE. The distribution of dopamine between particulate and soluble fractions was however little affected by these procedures and it was found that the ratio of particle-bound to free dopamine in different brain regions is less variable than for NE, suggesting that the mechanism of binding is different for the two brain catecholamines.

The mechanism/s of NE binding, release and reuptake were also studied in a variety of other tissues, while the patterns of urinary excretion of catecholamines and their metabolites continued to be investigated as part of collaborative programs with NASA Manned Spacecraft Center and with the Adult Psychiatry Branch, NIMH. The latter program deals with catecholamine metabolism in depressive disorders and emphasizes the longitudinal study of individual cases. It has been found that catecholamine excretion correlates with the type of illness as well as with the changing phases of mood.

Related to these studies is the continuous scrutiny of the analytical methods available for the estimation of catecholamine metabolites with the view of improving both their

sensitivity and specificity. Particular attention was given to the method for the estimation of metanephrine and normetanephrine which in our hands consistently yielded values considerably below those obtained in other laboratories. Using labeled substrates, it was possible to pinpoint the steps where losses occurred and to introduce corrective modifications. Nevertheless, our present results are not substantially different from the earlier ones, suggesting that the difference with the values obtained by others is attributable to greater specificity of our method.

The estimation of 3,4-dihydroxymandelic acid (DHMA) also claims our attention. Earlier hopes of using a bacterial mandelic dehydrogenase for this purpose could not be realized since the enzyme was specific for DHMA having the L-configuration whereas the acid excreted in urine has the D-configuration. Attempts were made to convert the D- to L-acid by adding mandalate racemase, another bacterial enzyme. This enzyme readily reacts with D-mandelic acid; it also has some activity toward D-p-hydroxymandelic acid, but it was found to be completely inactive toward DHMA or 3-methoxy-4-hydroxymandelic acid (VMA). Therefore, another approach is now being sought for the estimation of DHMA.

Arising from this work, the properties of mandalate racemase were further characterized. It appears that this enzyme requires magnesium ions, or certain other metal ions, for activity. It was found that the enzyme is inhibited by chelators of magnesium; other anionic inhibitors, namely fluoride and phosphate, were found to compete with the substrate for the enzyme-magnesium complex. The pH-optimum and the Michaelis constant of this enzyme were also determined.

An intriguing finding was the observation that a color- or fluorescence-producing reaction occurs in about 60% of blood samples from schizonphrenics but in only about 6% of normal controls. The reacting material appears to be a porphyrin, probably formed from a precursor in red blood cells but this precursor does not seem to be identical with hemoglobin.

The metabolism and the psychodysleptic effects of certain tryptamine derivatives were studied in a collaborative program of the Section on Psychopharmacology with SEH staff. Using a double blind design, three shortacting psychotropic tryptamine derivatives N.N.-diethyltryptamine (DET), N.N.-dipropyltryptamine (DPT) and 6-fluoro-N.N.-diethyltryptamine (6-FDET) were compared in a sample of chronic alcoholic patients. With DET and DPT, the patients experienced subjective effects at the 0.7 mg/kg dose, although the observers were unable to detect any significant change. At the 1 mg/kg dose, however, significant objective and subjective changes could be demonstrated. These effects did not appear to be relatable to autonomic changes.

Supporting this view was the finding that 6-FDET reproduced some of the autonomic effects of DET and DPT without the hallucinogenic or psychotomimetic components resulting from their administration. Hence, 6-FDET may be considered as an "active placebo", permitting a more objective assessment of the psychodysleptic action of other chemically related compounds.

Parallel studies were carried out in rats trained to press a lever to obtain liquid food on a variable-interval schedule. Both DET and 6-FDET were effective but DET had a stronger action. Attempts are now being made to develop new fluorimetric methods for the estimation of 6-hydroxy monoalkyl indoleamines and other drug-related metabolites in urines and, more generally, to develop methods to objectively measure dysleptic drug effects.

In the same vein, computer methodologies for the analysis of visual evoked responses, autocorrelation and power spectra of the EEG, are being developed in collaboration with the Section on Technical Development, IR, NIMH and a laboratory for the study of fine eye movements has been established. It has now been shown that saccadic eye movements are phasically related to alpha rhythm. A study differentiating saccade-linked left and right eye movements and "on" and off" visual evoked responses is now in progress; a first study of fine eye movements in pathological population groups is about to begin.

Utilizing some of the facilities previously devoted to clinical purposes, a laboratory will

be established to be run jointly with the Laboratories of Psychology and Socio-environmental Studies and the Adult Psychiatry Branch CI, NIMH for collaborative projects and to provide other NIMH scientists with the facilities to conduct studies at SEH. Other space will be renovated to provide badly needed basic science laboratories. It is clear, however, that only a new research building could properly accommodate our program and a representation to this effect has been forwarded through channels.

LABORATORY OF NEUROBIOLOGY

The ultimate goal of the research program of the Laboratory of Neurobiology is to elucidate physico-chemical bases for various physiological and behavioral processes taking place in this laboratory can be divided into the following four general categories: (1) studies of excitable and artificial membranes, (2) investigations of physiological properties of neuroglial and ependymal cells, (3) analysis of the electric activities of the cerebral cortex of waking animals, and (4) studies of sensory mechanisms. Diverse but well coordinated work is needed in order to be able to make progress toward achieving the ultimate goal.

During the fiscal year 1966, considerable progress was made in physico-chemical analysis of artificial and excitable membranes by Drs. Tasaki, Singer, Watanabe and Kobatake. By perfusing the interior of the squid giant axon with various favorable solutions, it was shown that the protein molecules in the membrane play an essential role in the progress of action potential production. The effects of extracellular cations were studied, and it was found that excitation in sodium-free and sodium-containing media were essentially the same. Further experimental evidence was obtained in support of the "two stable state hypothesis" of nerve excitation and a quantitative theory of excitation is being developed. It was suggested that transition of the membrane from the resting state to the active state represents a sudden change (first-order phase-transition) of the membrane macromolecules.

The project on neuroglia and ependyma was pursued efficiently by Drs. I. Singer and S. Goodman. By using tissue culture material, it was shown that the activity of ependymal cilia was governed by the same physico-chemical parameters that determine excitation in squid axons. It was shown that neuroglial cells are highly sensitive to various neutral salts and pharmacological agents. Ths finding offers a new basis for interpreting the mechanism by which cilia beat, and demonstrate the wide applicability of these physico-chemical principles to biological processes.

The electrical activity of the cat cerebral cortex was studied by Dr. E. Podvoll with multiple recording electrodes chronically implanted at many levels of the auditory system. Integrated activity from the thalamus showed rises during activity was lowest during deep ("slow wave") sleep. In contrast to the E.E.G., there was no difficulty relating integrated activity to "transitional" behavioral states. The dynamic changes during sleep and waking in the thalamus was observed to a lesser degree in the reticular formation, and was not seen in other subcortical nuclei.

Drs. Podvoll and Goodman are progressing in their attempt to construct a three dimensional current vector representation of electrical activity in the auditory cortex in response to acoustic stimulation. Unique current vector patterns are being mapped out in both deep and superficial layers of the cortex and in the underlying white matter. It was shown by this method that invasion of nerve impulses into the auditory cortex generated electric currents which change their direction, as well as their intensity, as functions of time.

Dr. Goodman has also been pursuing the relationship of intrareticular evoked potentials to arousal. The data collected shows clearly that during all levels of arousal (from alert to sleeping to paradoxial sleep) there is a direct, linear relationship between the amplitude of evoked potential and the integrated activity recorded from the thalamus. Therefore, there is a functional relationship between the level of arousal of an animal and the size of the "signal" (evoked potential) in the reticular formation.

Mrs. R. Marimont continued her studies on

the Fuortes-Hcdgkin model for visual perception derived from *Limulus* eye. A second feedback loop was added to the Fuortes-Hodgkin model which greatly improves the agreement between model and system. This second loop restores the shape of the curve to approximately that of the linear system. A pedagogical manual was written, "Simple SAAMing," for the use of other NIH scientists in programming SAAM with such models.

NATIONAL INSTITUTE OF NEUROLOGICAL DISEASES AND BLINDNESS

INTRODUCTION

The Intramural Program of the NINDB has consisted of four Branches (dealing with human patients) and six Laboratories for some years. Last year the Laboratory of Perinatal Physiology in Puerto Rico was transferred to the Intramural Program.

During the 12 months since July 1965 the Section on Technical Development continued to fulfill its traditional role of support of NIMH and NINDB research efforts. Continuing assistance was made available in shop help, special purpose instruments, and some modest instrumentation systems. The section continues to grow toward assistance in computer oriented research and digital instrumentation.

Use of the section's LINC computer has almost doubled since last year and has reached the saturation point—105 hours per week. The ability to take on new projects is now seriously limited by production runs on existing projects. Plans to relieve this pressure include:

Transfer where possible of existing production runs to CDPB, while limiting LINC functions to A to D conversion and on-line data editing.

Acquisition of additional computing equipment in fiscal 1967.

Help to laboratories toward setting up their own facilities where the workload justifies such a step.

Section personnel have contributed significantly to research projects in laboratories of the two Institutes. In all cases this work has been described in the reports of the laboratories involved.

The number of research projects reported this year is 218, an increase of 26. Forty-nine projects were completed and/or terminated and 59 new projects were described. One-hundred and seven projects involve collaboration with other Laboratories and Branches within NINDB, within NIH and with outside organizations. Forty-two cross Institute lines and 49 show collaboration outside NIH.

In lieu of a recapitulation of scientific results reference is made to the summary reports by each Laboratory and Branch Chief.

In accordance with the Institute policy, the Clinical Director's report reflects only the patient care activities and not the program content. As before, it is a pleasure to thank the Director and staff of the Clinical Center without whose skilled support we could not continue to function.

OPHTHALMOLOGY

From 1 July 1965 to 20 April 1966, 146 patients were admitted to the 13-West Nursing Unit accounting for 6,685 inpatient days. The outpatient census totalled 495 patients and 1,663 visits to the outpatient. Consultation requests from other Institutes totalled 1,279, exceeding by approximately 100 the figure reported for last year. The number of major operations rose from 26 to 63. Minor surgical interventions appeared to decrease because many of these are now performed in the treatment room.

MEDICAL NEUROLOGY

For the clinical investigations, 233 patients were admitted for a total of 5,761 patient days and there were 1,290 outpatient visits. There were 300 muscle and brain biopsies obtained. The clinical neurologists responded to 384 consultation requests from other departments and performed the required myelograms, pneumoencephalograms and cerebral angiograms.

SURGICAL NEUROLOGY

During the period 16 April 1965 through 15 April 1966, 214 persons participated in the clinical investigations as inpatients totalling 7,047 patient days. 593 were examined as outpatients in a total of 754 visits. There were 154 major operative procedures, 15 minor surgical procedures and 129 physiological monitoring procedures in the new surgical suite.

MEDICAL NEUROLOGY BRANCH

Clinical Investigation Program

Introduction

Our function is to apply the most promising basic research techniques to the clinical problems of the patients. The essentiality of an inter-related multi-dimensional attack on the chosen target diseases is to be emphasized. Added to the techniques of *histochemistry* and *tissue culture* have been *biochemistry* and *immunology*, but in quite modest forms due to limitation of space and personnel. The techniques of *electron-microscopy* and *autoradiography* were achieved only on a collaborative basis, due to acute lack of facilities for these important investigations. We are very appreciative of the collaboration received in these and other techniques. It is obvious that to have a balanced clinical investigative program, each of these six techniques must be provided for more adequately.

For the clinical investigations, 233 patients were admitted for a total of 5,761 patients days, and there were 1,290 out-patient visits. There were 300 muscle and brain biopsies obtained. The clinical neurologists carried a considerable service responsibility. They provided 384 consultations to other departments, and performed the indicated myelograms, pneumoencephalograms, and cerebral angiograms on those patients.

The two-year approved residency training program in clinical neurology has continued; medical students and residents from Howard University were taught clinical neurology weekly; and investigators and technicians in neurology and especially the application of enzyme histochemistry to human neuromuscular disease.

The collaborative research program in neuromuscular disease with the Department of Neurology, Warsaw Medical Academy has continued under the P.L. 480 program. During the past year, 20 of our 40 papers published (or in press) represented collaboration between the Medical Neurology Branch and other units.

Myopathies

A monograph on *Current Concepts of Myopathies* has been published, containing the research results and opinions of our group. Six additional chapters in different symposia and monographs described aspects of our histochemical techniques and detailed results in various neuromuscular diseases. In myotonic dystrophy the following have been delineated —temporomandibular joint dysfunction; virtually diagnostic specific atrophy of histochemical type I muscle fibers; and a unique hypercatabolism of serum gamma globulin (IgG) protein (with NCI). The cardiac lesion of this disease has been successfully treated by electro-cardioversion. Two other multisystem diseases with known protein defects, ataxia-telangiectasia (β2A globulin deficiency) and acanthocytosis (β lipoprotein deficiency) were shown to have a myopathic component, increasing the number of myopathies associated with known metabolic defects. A new disease, late onset progressive rod myopathy, has been described. Similar rods were produced experimentally in tenotomized cat soleus. Contrary to other investigators, abnormalities of lactate dehydrogenase isoenzyme 5, myoglobin, and total body potassium (K^{40} method) were found to be not disease-specific in humans. In addition to biochemical changes, subtle histochemical abnormalities were found in muscle biopsies of clinically normal carriers on Duchenne dystrophy, but their interpretation must await histochemical studies of muscle of normal volunteers. Detailed correlation of electromyographic, repetitive stimulation, motor conduction velocity, and motor unit territory determinations with clinical and histochemical aspects of 500 completely studied patients having neuromuscular disease is in progress. Attempts are being made to obtain reproducible cultures of human muscle biop-

sies *in vitro* by maximow slide and diffusion-chamber techniques. In many cancer patients, generalized muscle weakness is a major cause of disability. Histochemically, we have shown carcinomatous (or cachectic) muscle atrophy preferentially to involve type II muscle fibers. The pathogenesis and prevention of type II fiber atrophy is being pursued with the hope of symptomatically benefiting patients weakened by cancer. In collagen-vascular disease (a major cause of strokes in young adults) blood vessels in muscle biopsies are being studied by histochemistry and electron-microscopy to analyse the generalized vascular abnormalities.

Episodic Weakness

Our new classification of the periodic paralyses and non-dystrophic myotonias, based on the provocative and therapeutic effects of various ionic unbalancing tests, developed last year, has proved valuable in choosing the correct therapy for these patients, most of whom have responded quite well. Histochemical studies, which now make it doubtful that structural changes in the muscle fibers are responsible for the weakness in the initial part of the paralytic attack, are being paralleled by electron-microscopic studies. Refractory period measurements of single muscle fibers and muscle fiber conduction velocity are being done between and during attacks in these patients, and we hope will soon be combined with forearm perfusion studies. In a patient with succinylcholine-induced paralysis, the low serum cholinesterase was found by electrophoresis to be associated with a newly recognized selective absence of the fastest two cholinsterase bands, and new pharmacologically atypical patterns of cholinesterase were described in her serum and the serum of her relatives.

Myasthenia Gravis

Histochemistry showed that every muscle biopsy from 45 myasthenia gravis patients was abnormal, with either denervation or preferential atrophy of type II fibers present in all. Histochemistry also demonstrated a new aspect of lymphorrhages, namely that each was around one or more abnormal muscle fibers. A detailed combined immunofluorescent and histochemical study showed that the muscle-binding factor (described by Strauss) was *not* bound to the neuromuscular junctions, contrary to previous assumptions of others. In collaboration with Strauss and colleagues (NCI), a combined clinical (including prostigmine and curare tests) and immunofluorescent study showed that 24% of patients with thymoma but without associated myasthenia gravis had the muscle-binding factor in their serum. A new toxic effect of colistin methanesulfonate (Coly-Mycin) consisting of neuromuscular blockade at therapeutic blood levels of the drug has been described in a patient with Sjogren's syndrome.

Amyotrophic Lateral Sclerosis (ALS) and Other Diseases Affecting the Lower Motor Neuron

In the clinical and pathologic spectrum of familial anterior horn cell disease, it has been demonstrated that within individual families infantile spinal muscular atrophy blends imperceptibly with the juvenile proximal form. In ALS, primary or associated biochemical and immunologic abnormalities are being sought. Distant cancer has been found in a few patients, but its possible pathogenic role remains unknown. Immunologic abnormalities of the serum could not be found by gel diffusion or passive cutaneous anaphylaxis. With a tissue culture screening system, no toxic or infectious agents in serum and spinal fluid were demonstrated. An intravenous form of the arginine tolerance test was developed and appears to be more useful than the oral test—its results in ALS are under study. With Dr. Fullmer (NIDR), abnormal collagenase activity has been demonstrated in skin from ALS patients and a few other neuromuscular diseases which, though probably abnormal, is not disease-specific. Electron-microscopic search of brain tissue for viral particles is in progress, and search for a transmittible agent is underway (these same studies are also being done in sub-acute inclusion body encephalitis) with NINDB–CFR and NCI). Contrasting with the assumptions of others, the carbohy-

drate intolerance found in about 25% of ALS patients is not disease-specific, having been found in about the same percent of patients with myotonic dystrophy, progressive muscular dystrophy, and chronic peripheral neuropathy. More detailed studies of pancreatic function are underway to further characterize these changes. The apparatus we have designed and built for quantitating muscle strength has been shown to give reproducible results in normal controls and in patients. It serves as one aspect of evaluating ALS patients who are participating in our double-blind placebo-controlled therapeutic trials. As yet no drug tested has been found of therapeutic value. RNA metabolism has been studied with its tritiated specific precursor, uridine. The autoradiographic pattern of appearance in the nucleus and cytoplasm of motor neurons has been established in rabbits. The first pilot application of this technique to human neurologic disease has indicated a normal pattern in one case of infantile spinal muscular atrophy; the patterns obtained in ALS patients are being evaluated. A histochemical study of denervated and tenotomized cat muscles has shown a number of unexpected changes and emphasized the difficulties in relating experimental animal conditions to human neuromuscular diseases. Produced for the first time in animals were target fibers, rods, type I fiber atrophy, and type II fiber atrophy. Three new histochemical changes were described in patients—"type grouping", empirically considered a sign of chronic denervation; presence of target fibers to be nearly always in type I fibers; and occurrence of central cores to be exclusively in type I fibers, which reemphasized our earlier suggestion that central core disease may be a neurogenic disorder instead of a myopathy.

In ataxia-telangiectasia the following have been demonstrated—an unusual type of diabetes mellitus with marked hyperinsulinism; unexpected presence of immunoglobulin A in bone marrow and parotid cells; a profound defect of IgA synthesis and in 2 of 5 patients concomitant IgA hypercatabolism (with NCI); and impaired *in vitro* lymphocyte transformation (with NCI).

The first two patients having a new familial syndrome of recurrent peripheral neuropathy with α-lipoprotein deficiency (Langier disease) have been discovered (with NHI). A new syndrome of familial hypertrophic interstitial neuropathy and unique cataracts has been described. Vincristine, an anti-metabolite, has been shown in patients to have a preferential effect on the fusimotor system and thus is being tried as an anti-spasticity or anti-rigidity agent (with NCI).

Methodology

The role and mechanisms of substantive tetrazolium compounds and of phenazine methosulfate in the false localization of histochemical reactions has been described. A new type of EDTA-activated myofibrillar ATPase activity in type I fibers has been found histochemically. A method for seeking the location of the pharmacologic effect (myoclonic jerks) of d-tubocurarine perfused in cat ventricles has been achieved by use of the radioactive compound, and the penetration of the various nuclear masses has been studied. The rapid monitoring of tissue being processed for electron-microscopy by simultaneous histochemistry has been developed and proved to save considerable professional time.

Neuroradiology Section

Radiographic Diagnosis

Selective arteriography in patients with spinal cord arteriovenous aneurysms has allowed the demonstration of arteries feeding the malformation in five cases. This in turn has permitted surgical ligation of the main feeders in four paraplegic patients with resulting improvement in all of them. An *Atlas of Pathologic Pneumoencephalographic Anatomy* is now in press. The "empty sella" is a condition in which the normal intrasellar subarachnoidal space fills a good part of the sella turcica. These sellae, generally borderline large or frankly large, contain a large amount of cerebrospinal fluid but normal or smaller than normal pituitary glands. The knowledge of the large empty sella is important for: a) differential diagnosis from intrasellar tumors;

b) explanation of certain hypopituitaric syndromes and certain types of spontaneous cerebrospinal fluid rhinorrhea; and c) to avoid mistakes when contemplating transsphenoidal (surgical and 90λ) or external radiation pituitary treatment. Further experience has been gained with the useful refinement of pneumoencephalography, "axial transverse encephalography". This is now an established routine diagnostic procedure. A cooperative project is now underway on the growth hormone effects in dwarfs. Repeated sella turcica measurements are being taken in the patients so treated. The reevaluation by computer techniques of the angiographic patterns of superficial cerebral veins in the two hemispheres in a large group of patients has been continued. As another aspect of studying cerebrovascular disease, the prognostic significance of parasellar carotid artery calcifications will be evaluated at the end of 1966, 10 years after the beginning of the study of the particular group of patients used for this project.

Radiation Dosimetry

The thermoluminescent LiF crystals have proven disappointing for evaluating secondary radiation from irradiated residual x-ray opaque material in the spinal canal. New attempts are being made with the recently introduced LiF–Teflon dosimeters.

Isotopic Diagnosis

The clinical comparison of isotopes for brain scanning has been expanded to now include RISA, ^{99m}Tc pertechnetate, RIAF (radio-iodinated antifibrinogen) and ^{197}Hg Neohydrin. The points which have been established with the multiple isotope-multiple scan technique are: (*a*) most space-occupying intracranial lesions can be diagnosed by several isotopes, but in some cases a diagnosis can be reached only or much better with one tracer; (*b*) RISA has a "relative" specificity for metastatic lesions; (c) ^{197}Hg Neohydrin has a "relative" specificity for glial tumors; (*d*) RIAF may have specificity for sarcomas and certain large clots; and (e) ^{99m}Tc pertechnetate is probably most useful as a quick screening tracer and it can be most effectively employed with an Anger-type camera. In over one year of extensive testing, the Tetrascanner has proven itself to be a very useful piece of equipment, combining the high resolution of the rectilinear scanners with a speed approaching that possible with stationary detecting machines (cameras). The Tetrascanner is the best available device for rapid high resolution three-dimensional brain scanning. The isotopic scanning of cerebrospinal fluid shunts is now an established procedure. Extensive additional experience has been gathered with the techniques of isotope-ventriculography and isotope-cisternography. These now may be considered as routine diagnostic procedures. The Rous sarcoma virus brain tumors in dogs, first induced in collaboration with Dr. Rabotti (NCI), are now being used by other investigators. Salivary gland scanning with ^{99m}Tc pertechnetate has been shown to be a useful diagnostic test for detecting the presence of abnormalities in the area of the salivary glands and for differentiating among salivary lesions (tumors, inflammatory processes, and primary and post-radiation atrophy). Preparation of the monograph *Brain Scanning* has continued.

Neuropharmacology Section

During the past year we have been able to clarify the ionic events which lead to contracture of slow muscle. Myogenic contracture can be initiated in slow muscle by the withdrawal of external Ca^{++}. Present evidence indicates that loss of membrane Ca^{++} is the initial ionic event, followed by increased permeability to Na^{+} which leads to internal sodium accumulation. Contracture and an inflow of Na^{++} occur concurrently. Smooth muscle develops Ca^{++} deprivation contractures without depolarization. We have been able to develop muscle models relating ionic movement to tension development without consideration of depolarization. Further experimental verification of such models is needed. The present studies have furnished evidence that (1) Ca^{++} has a dual role in the function of slow skeletal muscle; (2) there exists a Na^{+} current mechanism different from the one in fast muscle or nerves which is responsible for the

action potential because (*a*) it occurs in slow muscle that is not capable of a propagated potential and, (*b*) it is not affected by procaine which inactivates the Na^+ current responsible for the action potential; (3) there is a K^+ requirement for complete relaxation in slow muscle. This requirement indicates that part of the active transport of Na^+ in slow muscle is K^+ dependent.

The studies with fast twitch-type rat skeletal muscle have related to the mechanical properties of both the series elastic elements and the contractile components. Load extension characteristics of the elastic elements permitted calculation of kinetic and potential energy during isometric contraction. The experiments do not support the prevailing concept of a passive undamped series elasticity in fast muscle. A study has been made in the rate of the elastic and contractile properties of a rapidly (anterior tibial) and a slowly (soleus) contracting fast muscle after immobilization by joint fixation. Disuse resulted in changes in the rate of energy expenditure which produced a significant increase in the intrinsic speed of the slowly contracting muscles. The elastic properties of the more slowly contracting soleus were changed by immobilization so that the elasticity more nearly resembled that of the more rapidly contracting anterior tibial muscle.

As part of a P.L. 480 project, a serpentarium has been established at the Physiology Department of Ein Shams University, Cairo, Egypt, to maintain adequate sources of snake venom. A polyvalent antivenom from immune horses has been prepared for human use. The venom of *Cerastes Cerastes* has been found to block nerve conduction and is not reversed by KC1 or physostigmine; it has no direct effect on muscle. *Walterinessia Aegypta* venom produces hypoglycemia apparently by stimulating insulin secretion from the islet β-cells.

BRANCH OF SURGICAL NEUROLOGY

Since the last report, this Branch has conducted investigations under the following categorical titles: developmental defects, epilepsy, involuntary movements, brain tumor, cerebral edema, cerebral trauma, language and memory, effects of low temperatures, microbial analysis of neurosurgical environments, and neurosurgical monitoring. Twenty-nine reports were prepared for publication in appropriate journals.

During the period 16 April 1965 through 15 April 1966, 214 persons participated in the clinical investigations as inpatients, while 593 were examined as outpatients in a total of 754 visits. There were 154 major operative procedures in the surgical suite.

In its various investigations, the Branch has actively collaborated with the following organizations: Branch of Electroencephalography, NINDB; Laboratory of Neurophysiology, NINDB; Laboratory of Neurochemistry, NINDB; Section on Animal Behavior, NIMH; Section on Pharmacology, NCI; Clinical Center Departments of Clinical Pathology and Diagnostic X-ray; Biomedical Engineering and Instrumentation Branch, DRS, NIH; Computer Facilities, DRS, NIH; Walter Reed Army Medical Research Institute; Personnel Protection, David Taylor Model Basin, Department of the Navy; Berlitz School of Languages.

Developmental Defects

X-irradiation of freshly drawn blood lends to the perpetuation of a chromosome-type lesion in the lymphocytes which are in the pre-DNA synthesis phase (G1). Radiation of the proliferating lymphocytes, post-DNA synthesis phase (G2) lends to chromatid-type lesions which are not transmitted to the daughter cells, being generally sublethal to the affected cell. This study suggests that persons who have exposed to radiation in the past (as long as five to twelve years) have lymphocytes with a significantly greater sensitivity to chromosome damage than persons who have not suffered similar exposures.

Chromosome studies in a female who had repeated therapeutic x-irradiations during the past twelve years revealed persisting chromosomal aberrations, and also a clone of cells with a deficient chromosome in group C. About 20 per cent of all her lymphocytes in repeated cultures over two years showed this

abnormality. Recently, she gave birth to a malformed child. Although the gross appearance of the chromosomes in this offspring was normal, undetectable maternally transmitted inversion may be present.

A serine amino aciduria concomitant with amelioration of this acid level in the plasma has been demonstrated in patients with Hurler's syndrome. In similar patients, the ratio of chondroitin-sulfate-B to heparitin-sulfate was changed by administration of hormonal compounds. This finding has led to a reinterpretation of the syndrome itself and may prove of some therapeutic value. In one such case, after complete pathological examination of brain and internal organs, two major types of monopolysaccharides in various tissues were demonstrated. It was found that the brain contained an excess of acid mucopolysaccharide as well as a lipid-like substance which resisted extraction. The latter contained a small amount of sialic acid.

Epilepsy

As in the past, epileptic mechanisms in children and adults have been variously investigated in the clinic, while some electrophysiological characteristics of epilepsy have been studied in the laboratory as well.

A total of 18 children suffering from frequent epileptic attacks was investigated and treated. Seven of these received a high-fat diet for therapeutic purposes and this treatment afforded the unique opportunity to study partitioned plasma lipids and their relationship to the control of seizures. When this diet was followed by satisfactory control of attacks, there was a marked change in the value of the partitioned plasma lipids. In another group of of children, the cause of seizures was identified as hypoglycemia. This hypoglycemia was further subdivided into leucine sensitive and leucine insensitive types. Then a special dietary regimen was devised for control of the leucine sensitive hypoglycemia. Once this was achieved, the seizures stopped. It should be noted that seizures resultant from hypoglycemia in early life can be followed by severe mental retardation and paralysis unless the attacks are adequately controlled.

Seventy-one patients with cerebral seizures were studied in the operating theaters and on the wards during the period of this report. The consistency, longevity, and some clinical relationships of focal cortical discharge (ECG) have been studied in the operating theaters. It appears that focal cortical discharge (in motor cortex) is unaffected by analogous postcentral excision, but remains consistent for some time thereafter. Conversely, relatively normal electrocorticograms have been obtained over precentral cortex during clinically evident and relevant focal motor seizures. The obvious presumption is that these are due to subcortical mechanisms which do not project to somatomotor cortex, but there is no way the clinical observer can differentiate them from those of surface origin. Finally, the "focal" cortical discharge which does not disappear after relevant surface excision seems explicable in terms of the experimental lesions in monkey thalamus which were followed by persistent "focal" cortical discharges.

A group of 18 centrencephalic patients was examined by tests designed to measure the effects of spike-and-wave type of EEG activity on sustained attention, sensory input, memory, and simple motor behavior. In addition, measurements were made simultaneously of the following autonomic variables: blood pressure, heart rate, finger volume, skin resistance, esophagela and gastric motility, and respiration. A total of 1,267 bursts of spike-and-wave was recorded in conjunction with the several tests. The behavioral correlates of the bursts were analyzed from the point of view of character (form), organization, maximal discharge, voltage, frequency, length, the background activity, and several time-related variables. All of these were shown to be related to behavior on one or more of the tests. Bursts which were symmetrical, regular, and bilaterally synchronous tended to produce more behavioral deficit than other bursts; tests which required the complete attentive act were more impaired during bursts than those which required only a part of this sequence; the motor task tended to be affected least. In addition, there was a retrograde amnesic effect. The most frequent autonomic changes were arrest of respiration,

finger vaso-constriction, fall in skin resistance, and increase in esophageal motility. These were not related in a one-to-one fashion to the behavioral modifications.

These patients were imparied on the attention test in the absence of observable bursts. The behavior loss tended to lead the electrographic symptom in time; in some patients, lack of behavioral change was observed even in the presence of well-organized symmetric bursts.

Two epileptic patients who underwent temporal lobectomy revealed a transitory drop in urinary 17–OHCS, epinephrine, and norepinephrine levels one month postoperatively. However, these findings did not persist at the end of one year's study. Similarly, amygdaloid and hippocampal stimulation in epileptic patients revealed slight plasma 17–OHCS changes associated with a particular type of electrical stimulation.

In the laboratory, propagation of afterdischarge from mesial and basal amygdala to contralateral homologue has been studied. Such propagation is of considerable interest to the clinical analysis of temporal lobe seizures, particularly those arising from mesial temporal structures.

Involuntary Movements

Respiratory and heart rates, and GSR responses from the human diencephalon have been recorded in 24 consecutive patients with electrodes implanted for treatment of motor disorders. Respiratory depression followed stimulation of these electrodes in the medial frontal white matter, genu of the internal capsule, and medial portions of the ventral-lateral thalamic nuclei. The second are of respiratory depression lay on the posterior-superior border of the lateral thalamus in the vicinity of the fornix and the stria terminalis. Respiratory depression from stimulation of electrodes located in or on the border of the ventral and oral portion of the thalamus had a significantly lower threshold on the left than on the right brain. This was not true of respiratory depression evoked elsewhere. Bradycardia followed stimulation of electrodes in the posterior border of the pulvinar. Tachycardia was evoked from the superior-lateral edge of the hypothalamus. Changes of skin resistance seemed to be related to the presence of subjective sensations during stimulations.

A method of relating commissural landmarks to the skull (Hassler and Riechert) has been tested in cases when two separate pneumoencephalograms were done. The anterior and posterior commissures in the midline third ventricle of the first pneumogram were reconstructed on the second. These were then compared with the position of the actual commissures on the second pneumogram. The mean variations and the standard deviations seemed acceptable in the vicinity of the A–C P–C line and the third ventricle, but three to four centimeters away, the variations possible with additive errors assumed sufficient size so that the use of a ventricular landmark closer to the site to be localized would seem advisable.

The human sterotaxic instrument which has been in use since 1960 has been reevaluated and found to have the following features:

Localization is achieved by fractional pneumography through routine demonstration of both the anterior and posterior commissures without the use of radiopaque oils.

The instrument permits use with standard X-ray equipment.

It allows full surgical draping.

It may be aligned with the intracerebral axis.

Movement is full in three graduated planes.

It permits entry into the skull at any point (apart from the central area at the vertex which underlies the apparatus), without the use of phantom target points or recomputation.

Precise realignment for stage procedures is within its ordinary capabilities.

Single cell discharges are being recorded from cerebral cortex and thalamus of patients with motor disorders. Data have been accumulated for statistical computation which is now under way.

In the laboratory, using extracellular and intracellular microelectrodes, the spike activity from the motor cortex in response to stimulation of various subcortical and cerebellar structures has been recorded on motion picture film as well as on magnetic tape and electronic digital recorders. It was observed that "the mid-

brain reticular formation" may play a role in the transmission of impulses from cerebellum to the cerebral cortex. Transmission mechanism through this subcortical structure was found to be unaffected by barbituate anesthesia. Other observations suggest inhibition of facilitation of cortical motor neurons in response to transcallosal stimulation.

Brain Tumor

Three patients with meningeal leukemia have received approximately four perfusions each with methotrexate. Two of these have been in remission for two years after perfusion. Twelve patients with glioma have been subject to perfusion with methotrexate. 8-Azoguanine is also being tested for its destructive effect on the gliomas.

Cerebral Edema

Selectively differential behavior of the blood-brain barrier in rabbits was studied in chemical injuries produced by intracerebral injections of various compounds. Unilateral blood-brain barrier injury was produced by intracarotid injection of mercuric chloride, penicillin, or sodium acetrizoate. These regional injections were followed by stemic administration of combinations of two different fluorescent and radioactive tracers. The brain tissue was studied by fluorescent microscopy and radioautography.

Combinations of red and green fluorescent albumin showed no separation in the distribution pattern on the damaged side. Simultaneous administration of red fluorescent albumin and green fluorescent globulins revealed in numerous instances a distinct separation in distribution of protein tracers on the damaged side. In slightly or moderately damaged areas, numerous blood vessels were surrounded by only red fluorescence. The perivascular exudates and globules showed a range of color depending on relative concentration of respective tracers. Differential passage from the injured vessels was also observed in combinations of fluorescent and radioactive tracers. C^{14} inulin appeared to penetrate in damaged areas more extensively than sodium fluorescein or fluorescein labeled albumin. An interesting result o-glucose combination. Whereas in moderate or severe blood-brain barrier damage, both sodium fluorescein and C^{14} methyl-o-glucose spread intensely from the injured vessels, in very slight blood-brain barrier damage in which no abnormal passage of sodium fluorescein could be detected, there was a distinct inhibition of the normal transport of methyl-o-glucose from the blood into the brain tissue. This inhibition of normal glucose transfer from blood to brain in very slight blood-brain injuries, which are undetectable by visual tracers, may be of considerable clinical significance.

The blood pressure in cats was altered by means of hyper- and hypotensive drugs administered prior to the production of brain edema. Then edema was produced by application of a cooled metal plate to the exposed cerebral cortex. Changes of the systolic blood pressure significantly affected the rate of pression of the edema through the white matter of the injured gyrus. It was shown that the lowering of the blood pressure can almost completely prevent development of edema.

By means of these standardized methods for intracarotid injection of injurious solutes, the blood-brain barrier in rabbits was studied so that one hemisphere was subjected to the injections, whereas the other was not. Comparative assay of the chloride-in-water content in damaged and relatively undamaged hemispheres of the same brain was performed, varying the type of concentration of the injuring solute and the time interval between injury and the termination of the experiment. In most experiments, the injected damaging agent was $HCCl_2$, while Penicillin–C was administered in a smaller group of animals. Slight or moderate damage was directly related to decreased chloride content in the hemisphere showing extravascular passage of tracer dyes. This suggests a functional disturbance in exchange mechanisms operating at the blood-brain interphase and not a mere leakage through ruptured blood-brain barrier structures. On the other hand, more severe mercurial injury was followed by the development of edema which was evident grossly as well as microscopically, and as suspected was directly correlated with

an increase in both chloride and water content. The effect of penicillin is of a more complex nature. This substance, after passing through the blood vessels, appeared to penetrate quickly to the neurons as evident by the development of convulsive phenomena predominantly on the side contralateral to the injection

The enzymatic changes occurring during the development of brain edema were studied in cerebral tissue and in CSF at various stages of experimental cold injury edema. Thus, five days after production of cold injury, a six to eight-fold increase in the level of lactic dehydrogenase (LDH) was noted. At seven days, this increased activity persisted. There was no concomitant serum increase in these animals and normal animals did not show this level of activity in CSF. Similarly, brain slices from these preparations which were stained histochemically for LDH showed enlargement of astrocytes and intense staining from five to seven days after injury. Such changes did not appear either in the controlled side where one animal served as its own comparison, or in control animals. Similarly, several enzymes of CSF and sera as well as proteins in the elasmobranch species have been studied. The proteins of brain and CSF were compared by electrophoretic techniques to analogous proteins in mammalian specimens. Thus several elasmobranch enzymes were compared to mammalian enzymes, both in total activity and in isozyme patterns. These studies indicate that, in the shark, CSF protein content and the CSF levels of LDH activity are significantly higher than those in mammalian CSF.

In a further diversification of species studied, the non-neuronal elements of the elasmobranch brain have been analyzed in the laboratory during the past year. In the specimens obtained, astocytes showed a striking radiating arrangement around blood vessels and neurons. No vascular sucker-feet which are characteristically seen in the mammalian brain could be noted. Otherwise, the blood vessels of the forebrain were densely surrounded by the glial cells. A system of ependyma-lined canals was described in the cerebellum. It is assumed that these channels, as well as sacculus vasculosis, play an important role in the circulation of the SCF. Elasmobranch studies such as this are of considerable importance in broader perspectives on mechanisms of cerebral circulation and edema because elasmobranch has, as previously noted, a unique blood-brain barrier which is singularly resistant to agents which clearly damage these transfer mechanisms in the mammalian species.

Cerebral Trauma

Sufficient data on occipital blows has accumulated to establish 10, 50, and 90 per cent "dosage" curves for concussion in terms of *impulse* of blow and linear acceleration of head. These curves arrived at by probit transformation enable rigorous statistical comparison of blows to frontal and temporal positions, and are all also serving as a base line on which tests for various hypotheses of the mechanics of cerebral concussion are being made. These include the effect of a cervical collar, head fixation, increasing the head mass, shifting the center of gravity of the head, and the protective effect of various devices. The most important observation to date is the finding that a cervical collar protects against the concussive effects of impact to the head in monkeys.

Cardiovascular effects after head injury have been found to be of considerable significance. A period of arterial hypotension invariably follows a concussive blow and this is associated with a widening of the pulse pressure, a moderate increase in the central venous presure, bradycardia, and other electrocardiographic abnormalities. The latter, particularly, seem to be significantly related to the prognosis following the blow. Persisting EKG abnormalities of either rate or pattern were invariably associated with a fatal outcome after a concussive blow. The slowing of the dye-circulation noted by cerebral angiography has been further studied and appears to be biphasic in nature. Slowing is noted within the period 10–30 seconds after the blow; this is followed by a return to normal circulation after approximately five minutes, and then 15–30 minutes later a second slowing occurs, persisting for a few hours. It is out intention to pursue this aspect further with studies of flow at the times of impact.

The study of blood and C.S.F. gases and pH has revealed a considerable lag between the two compartments, particularly for pO_2. Rapid alterations in the C.S.F. compartment can be made with little or no effect on the blood. The changes in these factors following various degress of head injury are now being pursued.

Studies on spreading depression, D.C. potential, cortical electrical impedance, and response to pCO_2 alterations after experimental head injury in collaboration with Dr. Wade Marshall are in progress. To date, it does not appear that spreading depression is produced by the blow. However, there is some indication that the effect of increased pCO_2 on the D.C. potential is reversed immediately after the blow.

Language and Memory

A recent finding that certain sets of scores from the Wechsler-Bellevue Intelligence Scale correlated inversely with the extent of neurosurgery in some of our cases encouraged the development of computer programs for investigation of the efficiency of factor-weighted scores. Three factors were derived from the standardization data by Maxwell's method. The scores from Atwell and Wells' vocabulary test and the first WB factor (Verbal Comprehension) correlated with the amount of left temporal removals. The scores from Mooney's Closure Faces test and the two minor factors of the WB ("Perceptual Organization" and "Freedom from Distractibility") were affected by right temporal removals, and these impairments differed for the two sexes. These results discouraged the view that intelligence or some highest integrative process is a function only of the brain as a whole.

During performance of a standardized object-naming task, stimulation of the left superior thalamus was associated with a significant degree of anomia. In contrast, stimulation in a homologous region in the right thalamus failed to produce a similar disruption.

As previously noted, right and left temporal lobectomized patients are being subjected to a standardized language learning system which presents a maximum of 2,500 words in a foreign language over a 60-hour period. To date, four right temporal lobectomized and one left temporal lobectomized patients, all of whom are left dominant by WADA test and all of whom have undergone operation at least five years prior to this test series, have been studied. These patients are relatively well matched for age, sex, full scale performance scores, and all are incorporate in an experimental protocol which is composed of psychological tests, physiological evaluations, and which ends with examination by the Princeton Modern Language Test Service. Three right temporal lobectomized left dominant patients achieved a first year college rating on the Princeton Modern Language Series. One right temporal lobectomized left dominant and one left temporal lobectomized left dominant failed to make first year college level, but scored comparably at about a 500 word performance level. The latter finding suggests that conventional teaching with regard to effects of left temporal lobectomy in a left dominant person is not valid since such a removal is thought to affect language learning capabilities adversely and permanently. Conversely, a right temporal lobectomy in a left dominant person is thought to have little or no effect on language learning capabilities.

Frontal opercular cortex is being investigated for language representations with reference to the area called Broca. In a correlative study, electrocorticographic recording, electrical stimulation points, amplitude of speech responses, and a tape delivered sub-verbal syllable test series are correlated with recordings from two electromyographic points on right upper and lower lips in the conscious, alert patient. Myographic activity, frequency in amplitude of electrocorticographic recording and amplitude of expressed speech correlate well in this matrix.

Effects of Low Temperatures

Two separate cold perfusion systems have been developed for the purpose of creating hypothermic conditions in the brain. The first of these is dependent upon the finding that high catheterization of the brachiocephalic arch in the dog as an end point to an extracorporeal perfusion system would provide for relatively selective or regional cooling of the

carotid circulation to the brain. In such a preparation, it was possible to provide hypothermic temperatures of choice in the brain, while the body of the experimental animal remained relatively warmer and the heart continued to beat and thus provide systemic circulation. The exclusion of the cardia from hypothermic effect is of considerable importance in clinical applications of hypothermia and is of fundamental interest as well. In the second system, unilateral cooling of one hemisphere of the cat was achieved and used as a basis for electrophysiological studies both of single units and by means of macroelectrode recording. It seemed desirable to explore the prior findings which relate a critical reduction in amplitude of the surface ECG to a temperature frontier in the brain of approximately 25° C. The unilateral cooling of one hemisphere provides a potential clinical model and experimentally is being used as a means of studying effects of low temperatures on blood-brain barrier and cerebral permeability since the opposite warmer hemisphere can serve as a relative control.

Cerebral vasospasm in the experimental animal has been the subject of some study within these projects. Obviously, vasospasm or dilatation may affect the course of heat exchange procedures in the brain and have some particular importance in brain surgery at ordinary temperatures as well. Present findings indicate that gentle mechanical stimulation of the middle cerebral artery in a plane parallel to its long axis can produce a gelatinous clot which rapidly becomes adherent to the intima. This intravascular clotting is preceded by a vasodilatation and pallor of the vessel. If a similar vessel is mechanically stimulated, using microdissection instruments, in a plane transverse to its long axis, vasospasm usually results. These changes in caliber of the vessels are comparable to those seen by topical application of papaverine, which produces vasodilatation, and serotonin which produces constriction. Interestingly enough, papverine will relieve the vasostriction produced by transverse mechanical stimulation, but serotonin cannot or does not alter the vasodilatation produced by longitudinal mechanical stimulation.

In another study, the effect of urea on the type of cerebral edema produced by freezing cold was pursued. However, these experiments yielded equivocal results and it was concluded that urea did not have a predictable or consistent effect on this particular type of local hypothermis.

Neurosurgical Monitoring

Earlier preliminary analyses indicated a correlation between alpha frequency and skin temperature. This is currently being examined more thoroughly using techniques of time series analysis (auto- and cross-correlation spectral analysis) to determine the nature and validity of this correlation within finite time spans over the entire course of the experiment, and to compare the strengths of this relationship among groups of subjects undergoing different degrees of psychological testing.

An asymmetry in evoked respiratory depression between right and left thalamus has been observed, as has a difference in latency of visual evoked response between patients with adrenalin insufficiency, and either adrenalin insufficient patients under treatment or normal controls.

Microbial Analysis of the Neurosurgical Environment

The neurosurgical environment has been sampled through air-collection plates, skin culture, wound site collection methods, and collection from drapes, instruments, and various geographic locations in the system. Despite a known defect in the plenum ventilation system, counts continued to range in the order of one or less organisms per 4 cu. ft. of air per 2-hr. sample period. These results are being compared to those obtained in an experimentally designed laminar flow ventilation system as part of an investigative protocol. It is possible that laminar flow ventilation will provide for reduction and simplification of aseptic techniques with particular emphasis on those related to personnel, clothing, and patient draping.

Summary

This report marks the thirteenth year of Surgical Neurology, a Branch which was named and founded by the present Chief in 1953. Originally designed as an aggregate of physicians and surgeons with special training in anatomy, physiology, pathology, and behavioral sciences, its name was selected from the writings of Wilfred Trotter as indication of purpose. The once and future intent is contribution to knowledge of functional anatomy of the human brain, with particular emphasis on behavioral correlations. In its practical attempts to reach these idealized goals, the Branch has published 244 papers and authored or coauthored 17 books. The categories of the present report represent evidence of the contemporary effort, as well as communities of interest among the members. Thus the categorical work in cerebral edema is headed by the pathologist and joined by several surgeons. The antomist (also a surgeon) heads the projects on involuntary movements which contribute to information on basal ganglia function as well as relevant disease states. Under the Branch Chief, all collaborate in studies on epilepsy which provide physiological, pathological, and clinical information. The physician is responsible for the programs in developmental defects and makes a considerable contribution to the epilepsy studies, as well as a unique addition through various investigations in child neurology. A psychologist heads the behavioral sciences effort in which a part-time psychiatrist plays an essential role, but many other members belong to this particular community of achievement. Last but not least, the new head injury projects are directed by a surgeon with special training in chemistry and physiology.

Like the head injury projects, all categories of achievement are disease oriented and clincally structured, although based in fundamental science. Thus laboratory and ward space is required for each goal-directed program. But all are severely limited in modular allotment and bed space. For example, the head injury program is based in two beds and one and one-half modules. Moreover, the severe limitations of bed space constrain the use of the new neurosurgical suite and prevent maximum exploitation of these excellent facilities. Finally, the lengthening lists of patients seeking admission to the Branch cannot be served with desirable dispatch because of these stringent spatial restrictions.

Despite these restrictions, the present and past productivity level has been high, consistent, and generally rewarding. The Branch looks to the future with an appetite for challenge, keen interest in achievement, and a sincere hope for help.

BRANCH OF OPHTHALMOLOGY

Changes in the staff during the past year necessitated reorientation of programs in several units of the Branch. Dr. P. O'Brien was appointed to the Section on Cell Biology to succeed Dr. S. Bonting who resigned in July of 1965. Dr. O'Brien is a glycoprotein chemist who is applying his experience in this field to studies of eye tissues. Dr. A. Lasansky became Head of the Laboratory for Electronmicroscopy filling a position which had been vacant since the death of Dr. Wanko in 1964. Dr. Lasansky has to his credit important contributions to retinal research which combine morphological and physiological approaches and which place him in a unique position for participating in the program of the Branch. Other new investigators of the Staff include Dr. R. Helmsen, a Staff Fellow in the Section on Chemistry; Dr. G. Wasserman, a Guest Worker in the Section on Physiology; Dr. S. Salceda, a Guest Worker from Manila in the Section on Cytology and Hstopathology; and Dr. R. Mazlen, a Research Associate in the Section on Cell Biology. Dr. Kern terminated his assignment as Associate in the Visiting Scientist Program in September 1965 after a stay of one and a half years. Rearrangement of investigative efforts in the clinical program was also required due to the two-year turnover of clinical associates engaged in this work. The departure of Drs. Carr, Spaeth, and Green was keenly felt.

Eleven new projects are listed among the laboratory and clinical investigations and 13 are being continued. Fifteen were either terminated or temporarily inactivated.

In the period from July 1, 1965 to April 20, 1966, 146 patients were admitted to the Nursing Unit accounting for 6,685 in-patient days. The out-patient census lists 495 patients and 1,663 visits to the OPD. Consultation requests from other Institutes were answered and amounted to 1,279 exceeding by about 100 the figure of the last report period. The number of major operations rose from 26 to 63. Minor surgical interventions appeared to decrease because many of these are now performed in the treatment room.

Laboratory Investigations

Retina

The overall approach to basic studies of vision has been strengthened by the appointments of Dr. Lasansky and Dr. O'Brien. Even more than before, the research activities of the Branch center around the anatomy and function of the retina.

The most fundamental work is conducted in the Section on Physiology. It was shown for the first time that retinula cells of the Limulus eye produce a propagated potential and that the retinula cells and the eccentric cell of one ommatidium respond synchronously to a light stimulus. The train of impulses travels rapidly in the large fibers of the optic nerve, which originate in the eccentric cells, and more slowly in the small fibers deriving from the retinula cells. Therefore, the impulses arrive at a common point with different delays. The regular sequence of repeating events may be utilized in the analysis of the sensory message.

It has been concluded from previous work on time constants of responses to light that two processes of response generation exist; one controlling the rising phase of the response and the other its decay phase. Both functions are temperature dependent to the same degree pointing to the involvement of chemical reactions. This assumption was recently confirmed with the use of metabolic inhibitors. Dinitrophenol abolished the visual response before it ffected membrane potential, membrane conductance, and the nerve impulse.

In another study a comparison was made between the Hodgkin-Fuortes cascade model for visual responses in Limulus and the probabilistic model of Levinson by analyzing the responses evoked by flashes delivering only a few photons. The results obtained so far indicate that responses to absorption of a single photon fit better the characteristics of the Hodgkin-Fuortes model.

Responses of single receptor cells have been measured as a function of wavelength and energy, and action spectra have been constructed which include data from the near ultraviolet region of the spectrum. Different ommatidia seem to consist of two types of cells designated as alpha and beta cells. The alpha cells are most sensitive to light of a wavelength in the region of 525 nm. Their action spectrum resembles that of rhodopsin. The beta cells, in contrast, are equally sensitive to all wavelengths from 350 to 550 nm. Beyond this region the sensitivity falls off steeply. These cells possess a higher ultraviolet sensitivity than the alpha cells, and their action spectra do not correspond to the density spectrum of a single photo pigment.

A technique perfected for the mammalian eye permitted introduction of glass microelectrodes into the surface layer of the retina in the intact eyes of anesthetized monkeys. Positioning of the electrodes and the light stimulus is carried out under direct biomicroscopic control. The responses of single ganglion cells in the fovea and perifoveal area to very small monochromatic light spots indicated the presence of several types of ganglion cells. They differ from each other in the amount of rod and cone signals and in the degree of antagonistic color responses they receive. The analysis of a particular class of perifoveal ganglion cells, the "on-center" type, provided evidence for the first time that rod signals reach the cell more slowly than the cone signals. Differences in the light sensitivity of the receptors are associated with rod responses to dim light and cone responses to bright light stimuli. The time delays of rod and cone signals are probably determined in the receptors themselves, whereas rod and cone interaction may occur afterwards, presumably at the level of the amacrine cells.

In continuation of previous work on ultra-

fine structure and function of retinal glial cells, the barrier qualities of the retinal pigment epithelium were examined in the toad. The electrical potential and current were measured and the ionic fluxes determined by the use of radioactive tracers. It was shown that the pigment epithelium is the site of origin of the resting potential of the retina. The most important finding of the study was the demonstration of a net flux of chloride ions in the direction from pigment epithelium to choroid which accounts for a great part of the short circuit current. An active transfer of bicarbonate ion could not be proven but the experiment suggested that bicarbonate is taken up preferentially by the inner surface of the pigment epithelial cells. The distribution of bicarbonate may serve in regulation of the pH in the extracellular fluid of the retina. Work was initiated in this laboratory on the fine structure on the blood retinal barrier, but results are not available as yet.

New and promising investigations on retinal metabolism, especially on glycoprotein synthesis, are under way in the Section on Cell Biology. Early results have shown that one enzyme operating at the first step of the synthesis of an amino sugar nucleotide is present in the bovine retina. This is L-glutamine-D-fructose-6-phosphate transaminase. Most important evidence has been obtained that the retina is indeed actively engaged in glycoprotein synthesis. An enzyme associated with the particulate fraction of beef retina is able to transfer N-acetylneuraminic acid (NAN), the terminal sugar of many glycoproteins, to a non-lipid high molecular weight acceptor. Galactose, the penultimate sugar of many glycoproteins, is incorporated by the same system and the addition of UDP-galactose to the incubation mixture increases the incorporation of NAN. The stimulating effect of galactose suggests that the addition of this sugar to the endogenous acceptor provides additional sites for the attachment of NAN, and that glycoproteins may have their sugar components added one at a time rather than as a preformed polysaccharide chain.

Whether an autoimmune mechanism can be operative in acute hemorrhagic retinopathies was further studied in the Section on Cytology and Histopathology. Monkeys which had received a single dose of guinea pig cord antigen together with the complete Freund adjuvant developed eye pathology in conjunction with experimental allergic encephalomyelitis. The eyes of monkeys of four age groups were ophthalmoscopically examined by Dr. Ronald Myers of the Perinatal Research Branch in Puerto Rico before the animals died or were sacrificed. The eyes of 22 experimental monkeys and 6 controls, which had been injected only with Freund adjuvants, were examined histopathologically. Of particular interest were the eyes of those animals which exhibited the first fundus pathology one or two days before the termination of the experiment. In addition to hemorrhages in different layers of the retina and signs of breaks of the walls of fine vessels, the presence of fibrinoid occlusion of capillaries and small venules was noted which may be the first step in the sequence of pathological events. A fibrin meshwork was seen around around large vessels and aggregates of fibrin or fibrinoid occasionally occupied their walls. Later stages were characterized by destruction of the retina by the hemorrhages; some of the extravasates broke through into the vitreous or into the subretinal space. The incidence of severe vascular occlusive lesions was greatest in young monkeys but occurred also in animals one or several years old. Optic nerve pathology consisting mostly of perivascular granulomatous lesions were seen in age groups regardless of the presence or absence of retinal involvement.

Cornea and Vitreous

The Section on Ophthalmic Chemistry reported progress in the physical chemical definition of beef corneal collagen. New examinations indicate the molecular weight of collagen polymer is much higher, and that of the collagen subunit much lower, than previously thought. The polymers form aggregates of high axial ratio. Effective separation of subunits will be necessary before comparative studies on different collagens of the eye during development and disease can be undertaken.

A new project in this laboratory dealt wtih physical and chemical characteristics of proteins in the vitreous hydrogel. Immunochemical examination of the rabbit's vitreous, supports the concept that many vitreous glycoproteins derive from related serum proteins. A drastic decrease of globulin levels in the rabbit's vitreous occurs with age.

As a side issue of the research in this Section examinations were performed by the use of physical chemical methods to estimate the molecular weight of DNA extracts from purified adenovirus Type II. A value of 34,000,000 was obtained instead of 22,000,000 previously reported.

Two new experimental studies in the Section of Cytology and Histopathology dealt with corneal transplantation. The viability of the endothelium of corneal homografts after various periods of storage of the donor material was examined using the rabbit as the experimental animal. This investigation has obvious practical implications. Corneal grafts remained clear when the donor material was stored as long as fourteen days. Twenty-one days of storage resulted in transiently or permanently opaque grafts. Cytological examinations showed a progressive loss of endothelial cells corresponding to the time of storage. The surviving endothelial cells proliferated as shown by mitosis and by H^3-thymidine incorporation. Cell proliferation was still demonstrated two months after transplantation but was less marked than in the first post-operative week. Morphological changes occurred in the surviving endothelium which contained cells with giant nuclei particularly in later stages.

In another study attempts were made to use the cat for producing a corneal homograft transplant model in which rejection of the graft regularly developed without additional non-ocular desentization necessary to produce the rejection of homografts in the rabbit. The experimental plan is to attempt suppression of the rejection process by inhibitory agents. The work is in an early stage as technical difficulties in this experimental animal are great but progress has been made to overcome complications by modifying the surgical procedure.

Choroid

Occlusion of choroidal vessels, particularly those of the choriocapillaries is expected to cause damage to the structures of the retina, the nutrition of which depends on choroidal blood. By the use of a technique described last year it was shown in a sufficient number of cats that obstruction of choroidal vessels by latex spheres resulted in retinal damage of various degrees in relation to the completeness or extent of the occlusive lesion. The pigment epithelium of the retina responded in most instances to slight or moderate ischemia of the choroid by either hypertrophy or destruction. In severe choroidal ischemia the outer four layers of the retina underwent degeneration. Slight disturbance of the choroidal circulation did not seem to affect the photoreceptors.

Lens

Work on cell population dynamics in the lens epithelium of the rat was extended to studies of age effects on the growth of the lens and its epithelial population, on mitotic activity and H^3-thymidine incorporation, and on diurnal fluctuation of these two aspects of cell proliferation. A rapid increase of the epithelial area contrasted with a very small and transient increase of the number of epithelial cells. Mitoses and DNA synthesis decreased markedly with age. The extent of diurnal fluctuations of mitosis were similar at all ages, but there was a six-hour shift in the timing of the period of minimum and maximum activity in older animals. Information on these various factors is required for estimation of phases in the cell life cycle and this in turn allows meaningful studies of experimentally induced cataract.

Last year the characteristics of a new type of cataract produced in fish by prolonged feeding with thioacetamide, a rodent carcinogen, was described. This year attempts to produce the tumor-like lens damage in rats failed even when the thioacetamide feeding was continued for one year.

Intraocular Pressure

The possible existence of a blood-aqueous humor potential functioning in the process of

aqueous humor formation was reexamined in the Section on Pharmacology. Experiments did not provide data indicating the presence of a potential gradient in the ciliary body. The secretory function of the ciliary epithelium is not negated by these findings, but a blood-aqueous potential is not connected with such a function if it exists at all in the anesthetized cat.

The elaborate technique developed in this Section for measurement of the intravascular pressure and flow rate in the eye together with the intraocular pressure was employed in studies of the effects of corticosteroid preparations on various ocular structures and functions, and on the action of different pharmacological agents. When certain steroid compounds were directly infused into eye arteries the intraocular pressure rose up to 80 mm Hg. Celestamyd® was more potent than Decadron®. Interestingly the vehicle of Celestamyd® also caused an elevation of the intraocular pressure. Six other steroids, including hydrocortisone and betamethasone, did not produce high intraocular pressure. None of the tested steroids affected the caliber of iris vessels. The effective steroids inhibited the responses of iris-ciliary body preparations to epinephrine, pilocarpine, eserine and acetazolamide to various degrees and resulted in dilation of the pupils. The work is of interest at this time in view of the lively controversy concerning steroid effects on the intraocular pressure.

Clinical Investigations

Retina

Newly developed electrophysiological and psychophysical techniques were employed successfully to distinguish between tapeto-retinal degenerations and other retinopathies. The use of the Ganzfeld stimulus instead of the Maxwellian view facilitated and improved standard procedures of electroretinography. Modifications of static perimetry and the use of colored stimuli permitted recognition of incipient retinal receptor pathology at a time when other methods failed to disclose abnormalities. For patients treated with chloroquine such early diagnosis of retina dysfunction is important since in this stage the damage seems to be reversible. The elaborate testing system developed over several years in the Branch led to an increased number of patient referrals from other Institutes and Eye Centers.

Uvea

Systemic therapy with the antifolic methotrexate was continued in patients with steroid resistant anterior uveitis and was extended to the treatment of sympathetic ophthalmia. The number of patients studied is as yet too small to evaluate the effectiveness of the antimetabolite therapy. Some patients showed slow but definite regression of the peripheral fundus lesion and absorption of vitreous opacities, but the results were equivocal in other instances. One patient with sympathetic ophthalmia improved dramatically in response to the first injection of the antifolic drug, although two patients in the later stage of the same disease did not benefit from the medication.

The usefulness of chemotherapy with Daraprim and a sulfa drug was confirmed in many patients admitted to the Branch with the presumptive diagnosis of acquired toxoplasmosis. Ophthalmologists, previously critical of this therapy, have reversed their viewpoint. The failure of other types of treatment in controlling this infection was documented in a patient whose blind painful eye with secondary glaucoma had to be removed. An overwhelming number of toxoplasma cysts in various stages of viability were scattered throughout the retina. Behcet's disease, a relapsing chorioretinitis leading usually to complete loss of visual function, was studied again in several patients by adding anticoagulant therapy to the usual administration of steroids. In some patients relapses appeared to be delayed or prevented, but more cases and longer observation periods are necessary before a definite statement can be made.

Glaucoma

Most of the well organized studies on glaucoma patients or normal volunteers were carried out last year. Since final results were not

available at the time of last year's report they are briefly mentioned now.

It was shown in a study of the water drinking test that, in about 20 percent of patients, the rise of intraocular pressure precedes the fall of serium osmolality and, more important, that measurements carried out at 15 minute intervals were necessary to avoid overlooking abnormal pressure rises.

Close analysis of data dealing with effects of Dexamethasone instillation on the intraocular pressure of normal volunteers and glaucoma patients indicated that, when the drug was used for three to four weeks, the pressure increased significantly in all instances. The coefficient of aqueous outflow decreased in 56 percent of normals and 67 percent of glaucoma patients. The water drinking test became positive in about 50 percent of the normal volunteers.

Although, as a rule, systemic use of steroids does not cause elevation of the intraocular pressure, one observation showed the opposite can be true. A patient referred to NIH because of unexplained elevation of intraocular pressure was found to have applied a steroid ointment to his legs over a long period of time for treatment of a skin lesion. When the skin treatment was discontinued the intraocular pressure and outflow facility returned quickly to normal.

Hydromethylprogesterone is an anti-inflammatory steroid supposedly without effect on the intraocular pressure. In studies on 15 glaucoma and 7 normal volunteers no increase of intraocular pressure followed one month's treatment with the compound whereas the same subjects had reacted with pressure rises to local Dexamethasone medication previously.

Ocular Changes in Systemic Disorders

Crystals in the cornea are well known manifestations of congenital cystinosis. The presence of a peripheral retinopathy in these children was described for the first time. It will be of particularly great diagnostic value in cases where this lesion precedes the corneal complications. The retinopathy is characterized by a zone of depigmentation with clumps of pigment of various forms. The retinal lesion was observed in all eleven patients of the series, but was not found in adults with this disease. The histopathology in two patients confirmed the involvement of the pigment epithelium of the retina.

BRANCH OF ELECTROENCEPHALOGRAPHY AND CLINICAL NEUROPHYSIOLOGY

Clinical Diagnostic Service

This Branch has continued to provide, as in the past, a clinical-diagnostic service for all the patients in the Clinical Center requiring an EEG examination, either as part of their routine work-up or as part of specific investigative projects in which the Branch (or Institute) has no direct interest. This form of activity represents a considerable portion of the over-all activity of the Branch monopolizing more than half of the time of the technical and secretarial and about one-third of that of the professional staff.

From the time the last report was prepared (March 31, 1965) to that of the present report (March 31, 1966) a total of 1605 examinations have been carried out in the EEG Laboratory (on both in- and out-patients), following referrals from the various Institutes, and specifically:

Institute	*Number*	*Percent*
NINDB	1,025	54.0
NCI	231	14.4
NIMH	118	7.3
NIAMD	76	4.7
NIAID	87	5.4
NHI	63	3.9
NIDR	5	0.3
Total	1,605	100

The large majority of referrals has been, as always, from NINDB and, especially, from the Branch of Neurological Surgery. A number of these examinations have included repeated studies in patients with chronically implanted electrodes. To the above total one should add 20 electrocorticograms which were obtained from the exposed cerebral cortex in the course of surgical procedures for the relief of seizures.

This form of service activity provided by the Branch in the last year has not been satisfactory as in the past. Not only the monthly aver-

age of EEG examinations (about 134) has decreased by about 12 in comparision with the monthly average of the previous year, but the over-all technical quality of the records has not always been up to the standards set in the past and/or the examination procedure, occasionally, has not been carried beyond the routine stage. This situation has resulted from a series of circumstances beyond our control; i.e., an unexpected—and unavoidable—acute shortage in the technical staff. This started with the much regretted, premature death of the supervisor technician, Mrs. Maureen Berkeley who had very efficiently occupied this position in the Branch since 1955.

The situation, still critical at the time this report is prepared, is expected to improve in the near future.

Research activity

Two of the research projects which had been started during the previous fiscal year have been completed. Some of the results have already appeared in published form and the remaining ones are currently in press. These projects deal with an investigation of various electrical properties of cortical neurons in resting conditions, in the course of inhibitory post-synaptic potentials and under the effect of topically applied strychnine. Using single and double intracellular micro-pipettes it has been possible to determine the resistance and time constant of the neuronal membrane as well as to estimate its capacitance and the relative contribution of, respectively, the somatic and dendritic portions of the membrane. In the same neurons, during the peak of antidromically elicited inhibitory post-synaptic potentials, the membrane conductance was found to be increased over two-fold that observed in resting conditions. These characteristic changes in conductance appear to persist—at least in some cells—even after the voltage changes which accompany the IPSP have been depressed or abolished by strychnine. This finding is in support of previous results from this Laboratory, suggesting a more direct action of the drug upon the neuronal membrane rather than (or in addition to) the commonly accepted pre- or subsynaptic action. The same findings bear indirectly on the essence of the epileptogenic effects of strychnine and on the problem of experimental epilepsy in general. In the same series of experiments, observations were also made on the post-effects of polarizing currents applied through the neuronal membrane.

It would appear that anodal break responses are quite different from the phenomena that might occur following the gradually subsiding IPSPs; these observations would not seem to support the hypothesis of a crucial "phasing" role of the IPSP in the genesis of rhythmical activity in the CNS.

After the completion of the two preceding projects an analogous one has been recently started in collaboration with the spinal cord section of the Laboratory of Neurophysiology, NINDB. In this project the same technique and analysis are applied toward the investigation of the basic physical properties of the membrane of the spinal cord motoneurons. No major findings are as yet available.

Another project deals with the study of the electrical activity that can be recorded by means of chronically implanted electrodes from various cortical areas and subcortical structures in man. On the basis of 162 tracings obtained in 40 epileptic patients it has been possible to provide additional evidence on the great limitations of routine scalp EEG and on the distortions resulting from the latter technique. Depth or direct cortical electrography permits the identification of very discretely localized epileptiform patterns but also demonstrates the high complexity and extensive distribution that generally characterize the epileptogenic process in temporal-pararhinal structures. These and other findings have been presented—upon official invitation—at a recent International Symposium on Stereoencephalotomy and are currently in press, although the main project is not considered completed.

The other projects are not of a research—experimental nature but rather consist of critical analyses or synthetic reviews of neurological topics. The first deals primarily with a survey of the papers and monographs on epilepsy which have been published in the past year. This critical review of the world litera-

ture on this neurological disorder (in which our Institute is particularly interested) has been carried out as thoroughly as possible. The highly condensed form and rather superficial treatment of the material are the consequence of the number of publications (about 1100). This work, to be published as a chapter in the 1966 volume of "Progress in Neurology and Psychiatry" represents a follow-up of similar reviews that have been prepared in the three previous years. This is, however, the last year and there are no plans to undertake analogous projects in the future since the review of the continuously growing literature would require too much time. It should be pointed out, on the other hand, that this type of work (as unrewarding and as unimaginative as it can be) is nevertheless becoming more and more indispensible in this as well as in other fields of investigation; somebody has to do it and, to be useful, it should be complete, accurate and should be carried out by a competent investigator with good library facilities.

The second project consists of essays of a more critical-synthetic nature, always in the general field of epilepsy. The work was undertaken following formal invitations to participate, with a specific contribution, in two International Symposia. In one it was the matter of providing the physiological bases for the new classification of epileptic seizures, recently proposed by a Committee under the sponsorship of the International League against Epilepsy. This contribution has recently appeared in published form. The other deals with a survey and discussion of the basic, cellular mechanisms in the epileptic process and of the mode of action of agents and drugs commonly employed to produce experimentally epileptogenic foci in the cerebral cortex of animals. This report, based in great part on material collected from our Laboratory, was presented at the Symposium on "Comparative and cellular pathophysiology of epilepsy" held in Czechoslovakia, September 1965, and is currently in press in the proceedings of said Symposium.

The last review-type project deals with the systematic-functional organization of the thalamus and with a discussion and speculation on some of the phenomena or functions which are likely to involve (or be the expression of) thalamo-cortical integration. The thalamus represents an interesting cerebral structure which is known to suberve and/or participate in a large number of different functions. Its complexity is unfortunately enhanced by the existence of a varied and confusing nomenclature of its numerous nuclear components and it was therefore considered useful to present (and provide some rational basis for) their functional role. This study has recently appeared in published form.

Other Activities and Organizational Aspects

Training in clinical electroencephalography has been provided for one doctor. Both from a quantitative and a qualitative standpoint, this form of activity of the Branch leaves much to be desired. Some of the reasons responsible for this situation are "chronic" in character and have been elaborated upon in the past Annual Reports. In the last year the situation has worsened due to the above-mentioned crisis in the technical staff resulting in an acute shortage of fully trained personnel. Bureaucratically slow steps are currently in progress to correct this situation. The Chief of the Branch only wishes to stress once again the importance of this professional training program in clinical EEG, expecially in view of the shortage of competent electroencephalographers still affecting this country. This is a too well known fact and one of which the Branch Chief has been particularly aware as Chairman of the Board of Qualification in clinical electroencephalography of the American EEG Society; a position he has occupied for the last three years.

Other official ("extramural") positions of the Branch Chief include that of Delegate for the American EEG Society to the International Federation of Societies for Electronencephalography and Clinical Neurophysiology (position from which he has recently resigned) and that of Chief Editor for the Americas and the Far East of the international monthly publication "Electroencephalography and Clinical Neurophysiology". Besides the above-mentioned Symposia, he has been invited to participate in that organized by the Fulton Society on "Fron-

tal lobe" (Vienna, Sept. 1965) and in the Conference on Sleep organized by the Association for Research in Nervous and Mental Diseases New York, Dec. 1965). From the very beginhe has served as a consultant in the OIR International Fellowship Program.

Program Considered for the Near Future

A certain amount of modernization and expansion of equipment is under consideration, while the chronic problems of space will probably persist, unchanged, at least in the next two years. The main field of investigation of the Branch is not expected to differ significantly, at least in its broad lines, from that of the recent past.

LABORATORY OF NEUROANATOMICAL SCIENCES

Introduction

The scope of the program of the Laboratory of Neuroanatomical Sciences during FY1966 is suggested by the number of structures which were under investigation (muscle spindle, lens, vestibular afferent and efferent systems, cochlear neclei, superior olivary complex, nuclei of the lateral lemniscus, inferior colliculus, medial geniculate body, cerebral cortex, locus coeruleus, nucleus dorsalis raphes, area ventralis tegmenti, substantia nigra, olfactory bulb, smooth muscle of the vas deferens and iris, ependyma), by the number of animal types studied (rat, chinchilla, cat, rabbit, monkey, mouse, chick), by the variety of techniques which were employed (electron microscopy, scintillation counting, autoradiography for both light and electron microscopy, macro and micro surgery, spectrophotometry, electrophoresis, etc.), and by the fact that over 30 research questions were answered. Despite the scope of the program the resources of the Laboratory were this year committed in a more focused way to specific problem areas (subcellular structure, cytoarchitecture, trophic interactions, morphogenesis and regeneration). Some problems in each of these areas were simultaneously under attack in different Sections of the Laboratory, against different conceptual backgrounds and with different techniques. It is not common that single individuals, or even different individuals in the same working unit, possess simultaneously, on the one hand, the background and skills necessary for the type of cytoarchitectural information revealed by such techniques as the Golgi, the Marchi, the Nauta, and on the other hand, the battery of techniques and concepts associated with electron microscopy. This Laboratory is providing increasingly strong leadership in the field in relating the findings of both types of approach to give an integrated picture of the structure of the nervous system at all levels of resolution. This year, for example, the auditory system received attention in three Sections (from the cytologic, cytoarchitectural and morphogenetic points of view). This sharing of an object of study, without significant overlap in the mode of analysis, has provided scope for individual initiative, has led to collaboration across Section lines and will, it is hoped, prove an added stimulus to those who share common interests and attack common problems. This increase in the relatedness among groups of projects is reflected in a decrease in the number, and an increase in the complexity, of the projects reported by the Laboratory as a result of this year's activity.

Another example of the manner in which the program of the Laboratory is changing in structure is the increase of interest in, and work on, problems of morphogenesis, trophic interaction and regeneration. The Laboratory is concerned not only with the structure of the adult vertebrate system, but is also increasingly involved with the changes in structure and organization which occur during development and regeneration, and with the mechanisms which guide and channel these changes. These emerging areas of concentration are in no way freezing the program of the Laboratory. Rather, they represent a commitment of resources to present opportunities. At the level of its individual projects the program remains broadly diversified and flexible.

Investigators in the Laboratory are making increasingly judicious use of collaboration with each other, with investigators in other units of the National Institutes of Health, and

with colleagues in other institutions. Such cooperative undertakings have enabled us to tap first-rate talent for specific purposes and to use such talent for just the period of time required by our programs. Collaborative undertakings range from those involving investigators within the same Section to international collaboration. Some collaborative ventures have required less than a work-day, while others are of an indefinite duration. Such activities have proved increasingly beneficial to the programs of our Laboratory since they accelerate progress by supplementing our pool of talent when and where it is needed, and since they increase flexibility. New techniques and instruments continue to be developed as the need arises. An example of a technical advance is the application of a permanganate fixation procedure to the problem of demonstrating dense-cored synaptic vesicles in certain autonomic terminals. An example of new instrumentation is the development, in cooperation with the Technical Development Section, of the "tissue plotter" which harnesses an X–Y plotter to the stage of a compound microscope in such a fashion that objects seen at high resolution through the mocroscope can be plotted spatially relative one to another with great accuracy.

Among the honors accorded members of our staff was the conferring of the degree of Docteur Honoris Causa on Dr. Grant Rasmussen by the Free University of Brussels on 13 November 1965 in recognition of his many research contributions to the analysis of the auditory system. In addition, Dr. Kent Morest, now of the Department of Anatomy, Harvard Medical School, was honored by the American Association of Anatomists when he received the C. Judson Herrick Award for his study of the anatomy of the medial geniculate body which he undertook and completed as a Research Associate in this Laboratory.

The research activities of the Laboratory can be categorized in several different ways to bring out different aspects of the program. In the annual report for FY 1965 the program was presented in terms of the major sensory, central, and motor systems which were then under investigation. The present report distinguishes those studies which concern themselves principally with the structure of the nervous system from those studies which deal more with the dynamic interactions which occur among the components of the sensory, nervous, and motor systems. This separation is made largely for reasons of convenience since structural and functional considerations can rarely be considered separately.

Analyses of Structure

Cytology

Auditory and Vestibular System.—The auditory and vestibular systems are under intensive investigation at all levels of organization from the sensory input to ascending and descending pathways. Building upon a previous light microscopic study an electron microscopic analysis of the trapezoid body and anterior ventral cochlear nucleus has revealed two types of endings. Small, presumably efferent, endings containing small synaptic vesicles persist following destruction of the spiral ganglion. Large calciform endings which make multiple synaptic contact with the dendrites and cell bodies of the neurons of these nuclei, contain relatively larger synaptic vesicles, disappear upon destruction of the spiral ganglion, and are endings of the cochlear nerve fibers. The identification on a structural basis of two types of nerve terminals raises the possibility that in this system facilitation and inhibition are mediated by different neurotransmitters.

Olfactory System.—A hitherto undescribed synapse, the dendrodendritic synapse, has been discovered in the glomerulus and external plexiform layer of the olfactory bulb. In addition, it was discovered that some small periglomerular neurons were enveloped by the flattened soma of adjacent neurons. These observations have provided a morphological basis for such neurophysiological phenomena as lateral inhibition in the olfactory bulb. It is anticipated that similar arrangements may exist in other sensory systems.

Autonomic Nerve Endings.—The study of the ultrastructure of autonomic nerve endings on smooth muscle continues in collaboration with related chemical and pharmacological investigations in other laboratories. Modification

of a potassium permanganate fixation procedure has opened the way for the identification of individual nerve terminals bearing granular synaptic vesicles. It now appears possible to distinguish such terminals from other types. Hitherto, reliable identification of adrenergic nerves was available only at the level of resolution provided by the light microscope. The technical advance made in this Laboratory during the past year makes it possible to identify *individual* terminals at the high levels of resolution provided by the electron microscope. The new technique will permit resumption of an investigation of the structural effects of the monoamine oxidase inhibitor pargyline on the nerve endings of the rat vas deferens.

In general, work on the innervation of smooth muscle, which has been carried on in this Laboratory over the past several years, is providing detailed information concerning the types of nerve fibers which innervate smooth muscle in different regions of the body and is revealing a detailed picture of the relationship between the nerve terminal and the muscle cell.

In addition to studying the distribution of monoamine containing cell organelles in the peripheral nervous system a survey was made of the distribution of monoamines in the central nervous system. Combined electron microscopic and autoradiographic analysis of rat brains revealed that exogenous epinephrine becomes neuronally localized, but that it is not associated with any specific cell organelle. Serotonin, on the other hand, appears to localize in certain nerve endings. These findings point to possible differences in the storage forms of monoamines in different parts of the nervous system.

Pathways of Transport in the Brain for Large Molecules.—During fiscal year 1965 electron microscopic observation of the distribution of ferritin which had been experimentally introduced into the ventricles revealed the pathway by which this large molecule moved across the ependyma and became distributed among the several compartments of the brain tissue. Among other mechanisms responsible for the transport of this protein molecule pinocytosis was found to be of cardinal importance. During fiscal year 1966 the Laboratory undertook to further describe and define this important process which is responsible for the movement of many solutes across cell boundaries, not just in the brain but in nearly every tissue of the body. It was found that pinocytotic activity continues in the ependyma for a considerable period following the death of animal. Similarly, pinocytotic activity persists in living animals even in the presence of sufficiently high concentrations of inhibitors (iodoacetate and n-ethyl-maleimide) to severely damage the ependymal cells. Since pinocytotic activity continues even in severely damaged cells, it is planned to study the formation of the pinocytotic vesicles in membraneous isolates from living cells in order to determine the minimal morphological requirements for pinocytosis. Should it be possible to duplicate this phenomenon in a cell-free system the way would clearly be open for studying not just the morphological, but also the energetic requirements of this process.

Cytoarchitecture

Neuroanatomy continues to add to the knowledge of the major and minor neuronal pathways which interconnect nuclear stations in the central nervous system. Recently increasing importance has been given, in addition, to studies of synaptology. Information concerning the types, number, cells of origin and spatial distribution of synapses is increasing our understanding of the organization and functions of the nervous system.

Auditory System.—The study of the auditory-vestibular afferent and efferent systems including the receptors is still in progress in the chinchilla and cat. It is sought to establish at the synaptic level the interconnections which exist among the cochlear nuclei, the superior olivary complex, the nuclei of the lateral lemniscus and the inferior colliculus, as well as the auditory reflex connections with certain motor nuclei and the reticular formation. This is an undertaking of some magnitude and the studies and the analysis of data are not yet far enough along to warrant a report at this time. It is important to realize that our knowledge of the functional organization of

these systems, and of the interrelationships which exist between them must ultimately rest upon a detailed analysis of this structural organization.

In FY 1966, as a result of collaboration with Professor Jean E. Desmedt, Director, Laboratory of Pathophysiology of the Nervous System and Brain Research Unit of the Free University of Brussels, the Laboratory launched a detailed analysis of the ascending and descending auditory connections in primates. This work is building upon extensive past experience in the analysis of the cytoarchitecture of the auditory system in lower mammals. Thus far, strategic lesions have been placed in 30 cynomologus monkeys. The brains of these animals are currently being processed and analyzed. Collaboration with Professor Desmedt promises to be particularly fruitful since he is directing his considerable abilities and unique laboratory facilities to a physiological analysis of the primate auditory system based upon the anatomical findings. This work promises to yield the most detailed analysis of the auditory pathways yet available for a form this close to man. During the past year we have placed unusual emphasis upon studies of the auditory system. Nearly half of the professional staff the four Sections of the Laboratory contributed of the laboratory became involved in some important way in studies of this system. Three of the four Sections of the Laboratory contributed significant resources to this undertaking. During the year two of the scientists studying the auditory and vestibular systems received notable honors because of their work in this Laboratory. No other unit of the National Institutes of Health currently has a commitment this large to the analysis of form and function of the auditory system.

The Muscle Spindle.—The structure and composition of the muscle spindle began to receive attention during FY 66. The work begun by Dr. James Stephens in the Section on Neurocytology held considerable promise of revealing the fine structure of muscle spindle. Unfortunately, this work must now be suspended because Dr. Stephens, a Guest Worker, has had to return to his home institution owing to illness. The Section on Experimental Neurology recently began to apply a battery of histochemical techniques to the muscle spindle. This work will continue, and an effort will be made to determine the distribution of glycolytic and oxidative enzymes. In addition, the effects of denervation and tenotomy upon the histochemical profile will be studied. One goal of this study is to determine why intrafusal muscle in spindles is so remarkably resistant to a wide variety of myopathies.

Interactions in and among the Sensory, Central and Motor Systems

Trophic Interactions

Nerve-Muscle Relationship.—It has long long been known that most skeletal muscles require an intact innervation to maintain them at full size and function. The Laboratory has now carried the analysis of this trophic interaction onto the chemical level. Two endpoints (cholinesterase activity and the electrophoretic pattern of muscle protein) have been used to assess the effects of denervation and of reinnervation of skeletal muscle by foreign nerves. Denervation causes a rapid drop in cholinesterase activity which is independent of the level at which the nerve is transected. Physiotherapy did not retard the rate of loss of cholinesterase activity in these cases. Spinal cord transection also resulted in a significant diminution of cholinesterase activity, but the effect was less severe than after a peripheral nerve lesion. Cholinesterase activity associated with sole plate regions remains constant during growth of the muscle, whereas the activity in regions of muscle lacking sole plates increases in proportion to the growth of the muscle.

The soluble proteins of red and white muscles differ qualitatively and quantitatively. Following denervation the protein pattern of red muscle is unaltered, whereas that of white muscle assumes a pattern that is qualitatively and quantitatively similar to that of red muscle. When denervated red muscle is reinnervated by nerve fibers that normally supply a white muscle, the protein pattern becomes transformed into that characteristic of the white muscle. Tenotomy produces no change in

the protein pattern in either type of muscle. These studies and others in progress in the Section on Experimental Neurology indicate that nerve fibers exert a profound and highly specific chemical influence on the muscle fibers which they innervate. Because of their importance in understanding function and dysfunction in the neuromuscular system, the Laboratory will continue to place major emphasis upon chemical studies of trophic interaction.

Visual System.—During the past year about a fourth of the resources of the Laboratory were committed to an analysis of those factors which control size, shape and orientation of the tissues of the developing vetebrate eye. For the most part the work has been conducted with the chick embryo. It was found that the volume of the lens of the chick embryo increases exponentially throughout the period of incubation. However, from at least the 5th day of incubation onward, there is a progressive decrease in the *rate* of growth. The lens fiber mass contributes most importantly to the increase in lens volume. This increase in the volume of the lens fiber mass during development is brought about by an increase in the number of its cells, and by an increase in the mean volume of the cells. The lens fiber mass contains between 20,000 and 30,000 lens fibers on the fifth day of incubation. New lens fibers are recruited into the lens fiber mass at the equator. They are added rapidly during early development, and progressively more slo-ly at older ages. By the 20th day of incubation there are nearly 300,000 fibers in the lens fiber mass. The enlargement of lens cells to become lens fibers is brought about, at least in part, by the synthesis within them of increasing amounts of lens protein. The total lens protein increases exponentially and at a rate higher than that of the increase in lens volume. As a consequence, the amount of protein per unit volume of lens increases as development proceeds. This increase in lens protein concentration is accompanied by a progressive relative loss of water and by an increase in specific gravity of the lens.

The increase in volume of the growing lens is under the control, at least in part, of the neural retina. Removal of the neural retina from the eye early in development results in a severe depression of lens growth. Replacement of a portion of neural retina into eyes from which it has been removed sustains rates of lens growth which are closer to normal. It remains to be determined to what extent this control has been exerted by regulating the number of lens cells which enter lens fiber population, and to what extent control of growth is mediated by regulating the volume of the individual cells (e.g. by controlling protein synthesis).

A previous project demonstrated that a reasonably well formed lens can develop from lens epithelium alone when this tissue is substituted for the lens early in development. During the past year it was demonstrated that the ability of the lens epithelium to respond in this way to the eye environment decreases progressively with advancing age.

During FY 66 mathematical formulations were developed with which the shape of the lens can be quantitated. As a result it has been shown that the shape of the lens changes progressively with embryonic age in such a fashion that its posterior surface becomes more and more convex relative to the anterior curvature of the lens. This quantitative descriptive study of the development of the lens shape is preliminary to an analysis of the factors that regulate lens shape during embryonic development.

During the past year a good deal of attention was given to the lens considered as a target for influence by surrounding tissues. It is planned to continue studies of the interactions among all the tissues of the developing eye by considering each tissue in turn as both a target of influence by surrounding tissues, and a source of influence affecting neighboring tissues. As a result of this type of analysis, the Laboratory has been constructing a flow sheet which describes, in some detail, the complex chain of interactions among the tissues of the developing eye to shape it as an optical instrument. It is hoped that further studies will permit us to identify the specific mechanisms by which these influences are mediated.

Morphogenesis

Auditory System.—During the past two years the Laboratory has undertaken to determine the distribution in time and space of the terminal mitoses in 18 cell type populations in the developing inner ear of the mouse. The study involved injection of single pulses of tritiated thymidine into pregnant mice at known times during gestation, followed by autoradiographic analysis of the inner ear of the offspring after they had matured. Specially designed computer programs permitted analysis of cell population dynamics during embryological development of the inner ear. The developmental period during which the terminal mitoses occur is shorter in the cochlea than in the vestibular apparatus. Receptor cells, supporting cells and ganglion cells do not divide after the third postpartum day. In contrast, the connective tissue and Schwann cells continue to divide for at least 7 days after birth. Two spatial patterns of terminal mitosis were discovered in the cochlea. The hair cells and their supporting cells undergo terminal mitosis in a wave which begins at the apex of the cochlea on the 12th day of gestation and sweeps down the cochlea, reaching its base on the 16th day of gestation. The wave of terminal mitosis in the spiral ganglion cell population, in contrast, starts at the base on the 12th day of gestation and ceases at the apex on the 15th day of gestation. This study was conducted with genetically inbred mice. It establishes a normative base line for similar analyses of approximately 25 strains of mice, each bearing a different point mutation affecting the ear in a slightly different way. Such genetic dissections of the developing ear should tell us much concerning normal and abnormal morphogenesis of the auditory and vestibular systems. It is already possible to indicate that an insult to the auditory system during the relatively brief time during development when the sensory cells are undergoing terminal mitosis could produce extensive permanent damage. In the vestibular system, on the other hand, terminal mitoses occur over a longer period of time, and acute insult would not be expected to produce as serious a defect.

Regeneration.—The laboratory continues to study the factors influencing the rate of regeneration of peripheral nerves and degree of reinnervation of peripheral tissues. Different nerves in the same, or in different, species of animals have been found to have remarkably similar rates of regeneration. Similarly, the rate of maturation of the action potential amplitude is the same in the cat vagus nerve as in the rat sciatic nerve, and is unrelated to the action potential amplitude that the nerve is ultimately capable of achieving. In another study the micro method for the determination of choline acetylase activity is being used in an attempt to measure the density of innervation in normal, partially denervated and reinnervated muscle. Should it prove successful, this method could have both theoretical and clinical applications.

Future of the Program

The statement of our future plans which was spelled out in the FY 65 Annual Report remains valid. The programs represented by the four existing Sections will continue in their present directions. In addition, further strength will be added in the area of morphogenesis of the nervous system over the next three years.

LABORATORY OF NEUROPHYSIOLOGY

Section on Experimental Neuropathology

The Laboratory of Neuropathology, represented by the Section on Experimental Neuropathology, has been engaged in four series of investigations as part of a long term program.

Perfection of Method of Preservation

Since only perfectly fixed tissues are useful for microscopic investigation, current techniques were critically surveyed. In the course of routine preservation by the procedure of perfusion via the ascending aorta, an intracardial influx of air during the operative procedure was detected. Such air was prevented from entering the systemic circulation by filling the chest cavity and performing all operations with the heart submerged. These precautions ensure proper preservation of experimental material which otherwise might be lost. A further check of various steps disclosed that

by this submerged heart method the time required for completion of the fixation can be reduced to about one hour, while four or more had been previously recommended.

Identification of Artifactual Changes

Since in the course of histologic preparation a variety of artifactual changes are introduced, it is important to identify them so that they will not be erroneously interpreted as significant changes. A microscopic survey of paraffin sections disclosed that in otherwise well preserved tissues, the nucleoli were dislodged and displaced from their original sites in 1–2 percent of all neurons. The affected neurons were situated along the surface of the microscopic sections and the nucleoli were always expelled in the direction of cutting; therefore, the mechanism was related to the mechanical action of the microtome blade. These results invalidate theories implicating such expulsion as a mechanism by which nucleoli can participate in cytoplasmic protein synthesis.

Pathologic Neuronal Manifestations

Since clinical neuropathologic concepts are based on the study of material preserved by immersion of the brain and spinal cord in various fixatives, a series of experiments has been planned for the purpose of establishing in perfused fixed material the nature and specificity of pathologic neuronal changes. The study of serial sections of brain stem from animals with severed facial nerve disclosed in some species diminution of ribonucleic acid stainable material beginning at the periphery of the motor neuron, but such an acute retrograde change was found to be of different intensity and quality in various species. These results suggest that the reaction of neurons as manifested morphologically is influenced by several factors, among which degree of protein synthesis and intensity of cell metabolism may play a role. These species variations in neuronal reaction must be taken into consideration before a diagnosis of acute retrograde neuronal changes can be made and before the cellular changes observed with other techniques can be correctly interpreted.

Identification of Cell Types

As a basis for determining the morphologic criteria of physiologic and pathologic reactions of the central nervous system, the microglia cells and oligodendrocytes were scrutinized. The development of a quadruple staining method permitted a consistent and selective staining of these two cell types in their entirety, as well as of blood vessels, neuronal perikarya and myelinated fibers. (*a*) A study of the appearance, distribution and spatial relationship of each cell type disclosed that there are considerable regional and species variations in the morphology of microglia cells, suggestive of differences in their function. (*b*) The extraordinary shape of microglia cells in such regions as the ventricular wall was, in accordance with a newly formulated hypothesis, associated with local histologic factors which affect the development of these cells. (*c*) The nature of cell changes around encephalitic foci suggests that current concepts about the reaction of microglia in pathologic material must be revised. (*d*) The permanence of these cells with aging indicates that whatever function they may serve is not significantly altered during senescence. Although the function of microglia cells is still obscure, their affinity to neurons, myelinated fibers and ependymal cells suggests that they are concerned with the metabolic requirements of the normal central nervous system. A correct appreciation of the degree of interdependence must await the results of intensive investigations with more elaborate and sophisticated techniques.

LABORATORY OF NEUROPHYSIOLOGY

Synaptic mechanisms continue to be a major subject of study in the Spinal Cord Section. The manner in which unitary or quantal synaptic events are combined to form the larger evoked synaptic potentials has been elucidated for spinal motoneurons. Afferent fibers from muscle spindles make monosynaptic connections with motoneurons which are widely distributed over the surface of the motoneurons. Different afferent nerves may have somewhat different patterns of termination and the

interaction between synaptic activity produced by stimulation of different nerves is influenced by the dendritic distribution of their terminations. Each nerve ending may produce from one to several unitary, quantal synaptic events when the ending is activated by an action potential. Even synaptic contacts in the periphery of the dendritic tree provide a significant excitatory deplorizing effect in the cell soma near the spike trigger zone. A member of the Section on Mathematical Biophysics, NIAMD, has developed a quantitative theory of the role of neuronal dendrites and collaboration with him has contributed to this analysis of synaptic and dendritic physiology.

Experiments are beginning on the trophic effects of nerves and the long-term consequences of alteration in the activity in nerves. Chronic recording of muscle action potential has been achieved and the effects of muscle tenotomy on spinal reflex and muscle twitch time have been studied. The results are as yet preliminary.

Pacemaker activity in molluscan nerve cells has been studied using the voltage clamp technique. Pacemakers and non-pacemakers differ in their response to prolonged depolarizing potentials. The pacemaker potentials have been clearly shown to be endogenously generated in the pacemaker cell itself.

Sensory processing in the cochlear nucleus has been studied in anesthetized and in unanesthetized decerebrate cats. Relatively simple sensory mechanisms predominate in the cochlear nucleus in general, although we have demonstrated differences between the dorsal and ventral divisions of the nucleus. A marked alteration in response patterns of neurons in the primary auditory nucleus is produced by barbiturates, emphasizing the distortion in neural properties introduced by this class of drugs.

The primary events in photoreception in the *Limulus* eye have been analyzed by electrophysiologic means and described in terms of an electronic analogue. Possible relationships between the observed electrical events and various ions and photopigments have been hypothesized. Further experiments to test these hypotheses are planned.

LABORATORY OF BIOPHYSICS

Work has continued in the Laboratory of Biophysics on essentially the same basis as reported for the last Fiscal Year with particular attention to problems concerning the basic mechanisms underlying the initiation, propagation, and termination of excitation in nerve and similar irritable structures. Review of the literature makes it appear probable that the bulk of information processing in the central nervous system is related to local electronic interactions, instead of propagated activity. It would appear that the fundamental mechanisms are common and relate to the changes in the ionic permeabilities of membranes brought about by electrical potential changes and other physical and chemical agents such as mechanical displacement and synaptic transmitting substances.

Techniques for the replacement of the internal contents of the giant axon of the squid with continuous perfusion of known solutions coupled with voltage clamping procedures are being steadily improved. Some of the many possibilities for important investigations using these techniques have been worked on in Woods Hole, Massachusetts, Plymouth, England, and Vina del Mar, Chile. Of particular interest are the effect of calcium ions and the possible role of sulfhydryl groups on the sodium and potassium ion voltage dependent conductances, the degree to which other ions can pass through the sodium and potassium channels and the manner in which various substances selectively block these channels.

Evidence is accumulating that the ion selectivity and the voltage dependence are separate mechanisms. For example Chandler, Hodgkin and Meves measured the relative permeabilities of the fast channels which normally carry sodium to the alkaline earth metals and while there is a large range of relative permeabilities the time courses of the voltage dependencies are indistinguishable. Further evidence that the slow (normally potassium) and the fast (normally sodium) channels behave independently has been obtained in this laboratory with the observation that with voltage clamped squid axons perfused with ammonium and tetra-ethyl ammonium chloride

only that part of the outward current carried by ammonium ions through the slow channels is blocked.

Experiments in Plymouth, England, in collaboration with Dr. Meves (Homburg, Saarland) have shown that the prolonged action potentials which occur with high concentrations of internal sodium ions are the result of an incomplete inactivation of the sodium channels on depolarization for short times. This residual sodium conductance inactivates with a time constant of 1–2 seconds at room temperature. This slow inactivation process does not seem to be altered by changes in internal potassium, as suggested by others.

The pace of theoretical investigation in the laboratory is expected to increase with the acquisition of a fulltime computer programmer to occupy the space which will become available on retirement of the EASE analog computer.

Pioneering work has been done in this laboratory in the use of film animation techniques for the presentation of computer output to demonstrate and study excitation and propagation of nerve impulses. Visual presentation of computer output, while not new, is yet in a rather primitive state of development. Understanding of and insight into the properties of complex systems can be increased enormously. One picture is worth at least 1024 lines of printed output. Many problems remain to be investigated in which these techniques can be very useful, such as transmission in tapered fibers, spatial summation of subthreshold phenomena and decremental conduction.

The rather general kinetic model of ionic conductances developed last year yielded good fits for the potassium ion currents and is being extended to the sodium conductance.

Studies on artificial systems and the application of newer physical techniques to natural membranes—made possible by the acquisition of additional space last year—have begun to yield valuable information. At present this program has three facets: (1) thermally (and possibly hydration state) induced mesomorphic phase changes in the hydrophobic part of natural myelins studied with broadline nuclear magnetic resonances; (2) the effect of electric field strength and anionic charge density on the cationic binding to fixed negative sites on the surface of newly synthesized organic semiconductors which are also ion exchangers; and (3) projected measurements of conventional light scattering and low frequency dielectric dispersion on artificial films (air-water and water-water interfaces- of phospholipid-protein complexes for studies of fluctuations indicating possible domain type behavior.

A timely and important Conference on Physical and Mathematical Approaches to the Study of the Electrical Behavior of Excitable Membranes was held in Woods Hole, Massachusetts supported by a grant from the NIH to the University of Maryland with a considerable amount of space and services donated by MBL. Of 18 papers presented three were from the laboratory and two others by former members.

LABORATORY OF NEUROCHEMISTRY

This year the complement of Laboratory personnel is again at full strength, including four new research associates (one for each Section) and a visiting scientist from Sweden. Despite the taxing of available space, we think this a most salutary situation. Working visits from several colleagues, three summer students, and continuing collaboration with many NIH and extramural colleagues (as noted in individual project reports) have greatly enriched our programs. We have just lost one of our active collaborators, Prof. Heinrich Waelsch. He was a close friend and valued advisor to the staff of this Laboratory, and he was a giant in neurochemistry. He will be sorely missed.

Research progress continues, as indicated in the attached, individual project reports. The work by the Enzyme Chemistry Section on the Na-K-activated, Mg-dependent ATPase of neural tissues remains both informative and challenging. Essentially complete uncoupling of the initial transphosphorylation (kinase) activity from succeeding steps has been achieved by treatment of the enzyme with oligomycin plus N-ethyl maleimide (NEM). These studies provide strong support for a multistep reaction sequence in which there is in-

itial transphosphorylation from ATP to a macromolecular acceptor on the enzyme protein activated at low Mg^{++} concentrations by Na^{+}, the stoichiometry requiring 2 Na^{+} per ATP. This reversible exchange reaction, which can be so clearly demonstrated in NEM-treated enzyme, is normally inhibited at higher levels of Mg^{++} which convert the high-energy phosphorylated intermediate to a form unable to react with ADP (and hence unable to reverse). This conversion apparently involves a second site of Mg^{++} activation and may represent either a conformational change of the macromolecule or an intramolecular migration of phosphate. The effect of NEM, presumably mediated via sulfhydryl groups, is to prevent such transformations even at relatively high concentrations of Mg^{++}. Normally the final stage of the reaction sequence is the K^{+}-activated dephosphorylation of the enzyme, a process so fast that new rapid-flow techniques may be necessary for studies of its stoichiometry and thermo-dynamics. Thus these studies are gradually unravelling the complexities of the enzyme system apparently responsible for monovalent cation transport across cell membranes. As these studies progress in various centers around the world, it is clear that the work by the Enzyme Chemistry Section remains in the forefront of this field of research.

In the Section on Physiology and Metabolism the multipronged approach to the problem of functional organization of bioelectrogenic plasma membranes continues. A central facet relates to the question of protein-lipid interactions and complexing and is being approached both in terms of lipoprotein structure, synthesis and specificity as well as in terms of attempted reconstitutions of membranes from lipid bilayers (lecithin plus cholesterol) plus functional proteins (such as Na-K-ATPase). For the former, serum lipoprotein synthesis has been taken as a model system and is yielding data on lipid specificity and the nature of the protein "core" or "carrier", data which may serve as analogies to intramembranal organization. The other set of studies involve attempts to effectively solubilize various proteins destined for study in reconstituted membranes and parallel work aimed at altering in situ membrane composition (of Nitella) while monitoring impulse conduction, along the membrane. It is likely to be some time before meaningful syntheses can be expected but the data obtained enroute are both valuable and intriguing.

Another area of promise is represented by studies on fluid distribution and electrolytes from the Section on Amino Acids and Electrolytes. Data from ontogenetic and comparative studies of incubated tissue slices *in vitro* make it possible to construct a fairly detailed schema of compartmentation of fluids (and electrolytes) in cerebral tissues from both mature and developing brain. These studies have established that the swelling of cortical slices in the presence of added K^{+} or glutamate or after circulatory arrest is clearly neuronal in locus, whereas most of the so-called "preparative" swelling (accessible *in vitro* to chloride but not inulin) is clearly glial (probably astrocytic) in locus. Furthermore glial cells in subcortical white matter behave differently than glia in cerebral cortex in such respects, and there seems to be a strong indication that cortical glia normally exclude chloride *in vivo* but lose this ability under usual *in vitro* conditions. A number of interesting directions for future research are indicated by these analyses, but of immediate importance is the fact that these studies, together with those from several other groups, have contributed significantly to correction of serious misconceptions present in the field. The factor of artifacts of fixation and processing of neural tissues for electron microscopy is finally being recognized after having long been ignored or minimized by electron microscopers, and consequently the interpretations of little or no interstitial space in brain based on such electron micrographs can no longer be considered valid. Acceptance of the presence in the central nervous system of significant degrees of extracellular spaces is finally being achieved. One result of these developments is the firm establishment of the importance of transport processes in determining or influencing blood-brain barrier functions and the distribution of solutes across the bar riers and across neural cell membranes.

Customarily in these reports the importance

of concentrating on basic aspects of neurochemistry has been emphasized with the expectation that clinical relevance and applications would naturally follow. This philosophy is no better exemplified than by the clear-cut demonstrations (by Dr. Brady's group in the Section on Lipid Chemistry) of the nature of the metabolic derangements responsible for Gaucher's disease and Niemann-Pick disease. The program of the Section on Lipid Chemistry has been directed to a large extent to studies of structure, biosynthesis, maintenance, catabolism and functional significance of neural sphingolipids. Accumulations of glucocerebroside in reticulo-endothelial cells in tissues of patients with Gaucher's disease and of sphingomyelin in cells of various tissue of patients with Niemann-Pick disease have long been recognized, but largely as a result of work in the Section on Lipid Chemistry, the various possible metabolic errors could be narrowed to defects of catabolism. In order to attack these problems, it was essential to have available the appropriate, isotopically-labelled substrates and intermediates for the relevant reaction sequences. The P.L. 480 project with Dr. David Shapiro (at the Weizmann Institue, Rehovot, Israel) has proved to be invaluable in providing a number of the necessary key compounds. Consequently the presence in normal animal and human tissues of glucocerebrosidase (splitting glucocerebroside to ceramide + glucose) and sphingomyelinase (splitting sphingomyelin to ceramide phosphorylcholine) has now been demonstrated. When spleens from patients with Gaucher's disease were examined, glucocerebrosidase activity was found to be markedly attenuated (15% or less of normal). This finding has been independently confirmed and extended to brain-tissue of patients with the infantile form of the disease. Similarly when livers from patients with Niemann-Pick disease were tested, sphingomyelinase activity was either absent or greatly attenuated, again a finding confirmed independently. These metabolic blocks in the catabolism of the respective sphingolipids appear to account specifically for the accumulation of the excess. lipids in these diseases. From the nature of the defect in Gaucher's disease, it is postulated that the normal source of the glucocerebroside is as a degradation product of either ganglioside (in the central nervous system) or globoside (systemically). Globoside is the major stromal glycolipid release from senescent erythrocytes and structural considerations suggest that other, analogous diseases may arise along the degradative pathway of globoside. In fact Fabry's disease seems to be just such an example. The achievements summarized briefly here have been recognized in the recent Superiod Service Award to Dr. Brady. It is anticipated that the much more difficult problem of Tay-Sachs disease will prove amenable to similar assaults and that therapeutic approaches to these conditions will prove more rationally feasible.

The Laboratory of Neurochemistry, IR, NINDB has now been in existence for 5 years. In retrospect these have been productive years and have witnessed a considerable degree of maturation especially in terms of training neurochemists. The immediate future involves some uncertainties. A successor to the present Associate Director for Intramural Research remains to be found so that any proposals for future moves of the Laboratory to Bldg. 36 and hopefully more adequate facilities must await the programs of the new director. The present limitations on CORD deferments for Research Associates pose problems for continuities in the training program, for the size of the available recruitment pool, and eventually for output of trained personnel in the field. It is to be hoped that consideration will be given to the eventual nationwide impact of any prolonged hiatus in the influx and outflux of research trainees. Despite such problems of the moment, the Laboratory seems to be well found and embarked on a number of important and rewarding ventures.

LABORATORY OF MOLECULAR BIOLOGY

Structure and Alteration of Nucleic Acids

Use of Transforming DNA for the Classification of Mutagenic versus Inactivating DNA Alterations

Mutagenic DNA alterations are defined as those changes of DNA which do not prevent

replication but occasionally or always give rise to a change in the sequence of bases in some of the progeny DNA. Inactivating DNA alterations, in contrast, do block nucleic acid replication, except when they are repaired or occasionally overcome otherwise. They either involve drastic changes of one or more bases, rendering impossible the pairing with a complementary base, or they interrupt the continuity of the information by crosslinking or breakage of the sugar phosphate backbone. These alterations are rarely mutagenic but lead to chromosomal breaks, large chromosomal alterations, or induced recombination. In transforming DNA, mutagenic alterations are emasured by the induction of mutations, using the method of linked mutation induction, whereas inactivating alterations are measured by the inactivation of a particular transforming marker and the simultaneous absence of a significant mutagenic effect. These quantitative determinations permit one to explain the biological effects of different agents and enable one to predict the relative frequency of pointmutations versus large chromosomal alterations.

In the past the distinction between mutagenic and inactivating DNA alterations has not been possible, because an observed lethal effect could be caused either by an alteration of DNA itself or by a reaction of some other cellular component. Only one of the two types of alterations could therefore be measured at a time, either mutagenic alterations by the induction of mutations or large chromosomal alterations by cytological observations. Many agents such as peroxides which exhibited a weak mutagenic effect actually are strong inactivating agents with pronounced ability to induce chromosomal breaks. It is noteworthy that all strong carcinogens which can chemically alter DNA are inactivating rather than mutagenic agents.

The possibility to distinguish experimentally mutagenic and inactivating alterations in transforming DNA was first realized by the use of hydroxylamines. At a 1 M concentration hydroxylamine is predominantly mutagenic, by attacking cytosine. At concentrations of 10^{-2} M and smaller, however, hydroxylamine exhibits only an inactivating effect on transforming DNA and is no longer mutagenic. This inactivating effect depends on the presence of oxygen and it has now been shown that the reaction of hydroxylamine with oxygen produces hydrogen peroxide. The hydrogen peroxide is destroyed again by a second reaction with hydroxylamine; its concentration is thus kept at a constant level. This finding explains the initially strange observation that high concentrations of hydroxylamine were less inactivating than low concentrations: at high concentrations of hydroxylamine the oxygen becomes limiting in the solution so that hydrogen peroxide is much faster destroyed than produced and consequently the inactivating effect is virtually absent at a concentration of 1 M hydroxylamine.

A strong inactivating effect on transforming DNA has been observed for several other compounds containing a free NOH group as well as by some hydrazines. Since these reactions depend on oxygen and give rise to hydrogen peroxide, it seems likely that all chemicals having these reactive groups will produce hydrogen peroxide and inactivate DNA. Most of these agents are carcinogenic.

The Effect of Hydrogen Peroxide on DNA and its Components

Having observed that many inactivating agents give rise to hydrogen peroxide or the actually reactive OH radical, a systematic study of the reaction of hydrogen peroxide with DNA and its components was launched. It was found indeed that hydrogen peroxide has a drastic effect on DNA. It decreases the melting temperature of DNA and after prolonged treatment causes the complete separation of the two DNA strands at temperatures which are far below the ordinary melting temperature of DNA. When the reaction with the DNA nucleotides is measured, one observes the destruction of thymine, cytosine and guanine bases, which is noticed by a rapid decrease of the UV absorption of these nucleotides. The adenine deoxynucleotide releases the free base adenine which subsequently can further react, giving rise to a new UV absorbing compound. Experiments with radioactive compounds are under way

which allow one to determine the decay products of the above reactions.

The Effect of Combining Mutagenic and Inactivating DNA Alterations

When DNA harbors only mutagenic DNA alterations, its progeny produces mixed clones , which consist half of mutant and half of nonmutant organisms. This phenomenon can best be observed in phage T4, in which the mutagenic alteration, by 1 M hydroxylamine, gives rise to mottled r-plaques. If this mutagenic Effect is superimposed by an inactivating effect, either low concentrations of hydroxylamine or ultraviolet light, many pure nutant clones are produced. This conversion of mixed into pure clones is not observed in a mutant of phage T4, which is deficient in its ability to repair ultraviolet induced lesions. Therefore, the conversion apparently is caused by a repair mechanism by which the inactivating alteration is cut out of DNA and the repair process copies the other mutated DNA strand, thus giving rise to a DNA molecule in which both strands are mutant.

Genetic Analysis of Virus Induced Mutants of Escherichia coli

The properties of virus μ1, which lysogenizes *E. coli* bacteria and simultaneously mutates them, has been further investigated, using lactose nonfermenting mutants. Linkage tests have suggested that in such mutants the prophage is linearly inserted into the specific structural gene for either β-galactosidase or galactoside permease. The inserted phage apparently is under the control of the β-galactosidase operon, because addition of the inducer of β-galactosidase, isopropylthiogalactoside, increases the release of phage by a factor of 20.

Studies on the Structure of Chromosomes

The DNA pieces which one isolates from cells must have been attached lengthwise to one another in chromosomes because genetic markers are one-dimensionally arranged. It is not known, however, whether this attachment involves only DNA itself or whether there exist any non-DNA links. The following experimental observation suggests ways in which this problem can be approached biochemically. When DNA was isolated from calf thymus nuclei in a glycerol-phosphate buffer, it showed a certain molecular weight distribution. But when the nuclei were first treated by saline EDTA at 4°, before the DNA was isolated, the molecular weight of the subsequently obtained DNA increased with the time of treatment. It will now be attempted to obtain similar results in extracts of thymus nuclei and to identify the components required for the apparent polymerization of DNA.

Control Mechanisms and Differentiation

Differentiation in higher organisms comes about by a multitude of individual biochemical reactions, each of which is subject to a specific control mechanism. The way in which a particular cell develops depends on its previous history, on its environment, and on its genetic constitution. In order to find out how these different factors interact and which molecular principles are involved, one has to investigate a simple system of differentiation for which both biochemical and genetic studies are feasible. Bacillus subtilis provides such a system for which mutants can be easily isolated and genetically characterized. One can study the induction and repression of individual enzymes such as alanine dehydrogenase and one can follow sporulation and germination. The sequence of the developmental processes during sporulation corresponds to a well-controlled sequence of functional steps by which the expression of individual genes is switched on; mutants blocked at different stages of sporulation also seem to be unable to perform any of the enzymatic reactions that occur later in the normal development. Specific results obtained in the last year with this bacterial system will be described below.

Depression of Alanine Dehydrogenase in Bacillus Subtilis by Internal Induction

Alanine dehydrogenase (AID) is induced by L- or D-alanine and several alanine analogs. This enzyme is also increased by starvation of nicotinic acid, riboflavin, and pantothenate in mutants lacking the capability to synthesize

these vitamins. Initially, this effect suggested a decrease in the concentration of an internal repressor whose synthesis or function might require the product of the vitamins as cofactors. It has been found, however, that in all three mutants the internal concentration of L-alanine increases severalfold during the vitamin starvation. This increase is not caused by the increased alanine dehydrogenase itself because it has also been found in a mutant unable to produce alanine dehydrogenase. The apparent derepression of A1D by vitamin starvation seems to be therefore actually caused by an internal induction with L-alanine. This finding raises the question whether in other systems in which an apparent derepression has been observed the effect is not also caused by an actual internal induction. Experiments are planned to check this possibility.

Lack of Sporulation in Cytochrome a*-Deficient Mutants of* B. subtilis

Cytochrome *a* is normally present in vegetative and sporulating cultures of B. subtilis. By mutagenic treatment 11 cytochrome *a*-deficient strains have been obtained. The cytochrome *a* concentration in these mutants is less than 15% of the parent strain. All of the mutants are either completely asporogenic or oligosporogenic, whereas the rates of exponential growth in sporulation medium are generally similar to the wild type. Revertants from the sporulation-minus character to a sporulation-plus character appear occasionally and are always accompanied by the restoration of cytochrome *a* activity to normal. These results show that in B. subtilis cytochrome *a* is not required for rapid exponential growth but seems to play an essential role in sporulation. The gene(s) for cytochrome *a* formation seems to be therefore a typical developmental gene, required only during differentiation.

A New Lysyl sRNA Produced During Sporulation

Sporal transfer t-RNA can function as a substrate for amino acid activating enzymes from vegetative cells. Whereas the ability to accept several amino acids is significantly smaller in sporal than in vegetative RNA, the acceptance of lysine is more than twice that observed with vegetative RNA. Comparison of the MAK column elution profiles of lysyl transfer RNA from spores and vegetative cells shows that the spore preparation contains a species of lysyl t-RNA that is either absent in vegetative RNA or present only in very small amounts. This new lysine RNA appears late in sporulation, at the time at which the spore wall is synthesized and the prespore becomes refractile. The physiological role of this new species of lysine t-RNA will be examined.

Initiation of Germination

The germination of Bacillus spores is specifically initiated by L-alanine. This process occurs in the presence of actinomycin D or chloramphenicol and therefore does not seem to require either nucleic acid or protein synthesis. In order to elucidate the mechanism of spore germination, mutants have been isolated which can no longer germinate in the presence of alanine alone. One of these mutants could germinate when, in addition to alanine, autoclaved glucose was added to the medium. It was eventually found that the germination of this mutant could occur when both fructose and glucose were added in addition to L-alanine. This finding indicates that germination is not merely caused by the allosteric change of a protein but requires a series of enzymic reactions. During sporulation sucrose presumably accumulates and can subsequently be broken down to glucose and fructose during germination. These compounds are then further utilized for energy-producing reactions. In the deficient mutant, the enzyme which breaks down sucrose presumably is no longer present. This possibility is under investigation. It can now be understood why different sporulating organisms isolated in nature have different germination requirements. They are blocked at different steps of a complex network of reactions.

LABORATORY OF PERINATAL PHYSIOLOGY

The Laboratory has acquired the island of Desecheo lying 12 miles off the west coast of Puerto Rico. This island, approximately 600 acres in size, is of a mountainous, forbidding

terrain and is covered with thick vegetation. It is currently being developed as part of the primate ecology program. An integrated social group of approximately 70 monkeys will be transferred from Cayo Santiago to Desecheo. This group of monkeys is currently under intensive study on Cayo Santiago with reference to social organization, dominance hierarchy, and reproductive behavior. After transfer to Desecheo, this group again will be observed for changes in social structuring which may result from the sudden transport of the group into an alien environment. Particular attention will be paid to whether the group's integrity will be sustained or whether the group will fracture off into smaller subgroups. Attention will be paid to the reproductive periodicity and to the dominance hierarchy to ascertain possible effects of the disruption in life pattern brought about by the transplant.

Detailed accounting of the pattern of reproductive activity and of birth on the islands of Cayo Santiago, La Cueva, and Guayacan has continued in an effort to characterize the pattern of cyclic reproductive activity as expressed in these three separate island circumstances. Of particular importance is the fact that La Cueva and Guayacan share the same climatic and ecological environment although they remain entirely separate as ecological niches. In contrast, the island of Cayo Santiago, located at the opposite end of Puerto Rico, represents a different environment having an annual rainfall of 60 to 80 inches per year, in contrast to the dry, arid climate of La Cueva and Guayacan which enjoy only 20 inches of rain per year. The seasons of reproductive activity and reporoductive yields are settling down to a more stable pattern on a year by year basis in La Cueva and Guayacan as the monkey populations there achieve a greater degree of stability. In the past year the breeding and birth seasons on La Cueva and Guayacan have become closely similar in their timing and in the characteristics of their normal distribution. This is in contrast to the breeding and birth seasons on Cayo Santiago where the peaks of activity occurred three months earlier. Continuing statistics of this sort over the next three to five years are required in order to more definitely determine the validity of the proposition which is suggested that breeding cyclicity in the primate may relate to environmental factors. The addition of Desecheo as a facility for free-ranging monkey studies will add further dimensions to the analysis of the factors defining breeding periodicity.

Earlier studies carried out on Cayo Santiago have indicated that the tests of the male rhesus monkey undergo cycles of hypertrophy and atrophy paralleling the cyclicity inherent in the breeding season. Currently, studies are being initiated of the ovaries and uterus of the female to determine whether menstrual cycling occurs throughout the year in the free-ranging circumstance as it does in the caged colony and whether the ovaries undergo changes in morphology in relation to the breeding season.

In the experimental enclosures on La Cueva studies involving manipulation of social structure are in progress with replacement of the dominant male by another strange male for specified periods of time. Induced changes in the social rank of the adult females quickly occur under this circumstance with loss of rank of the consort of the removed dominant male and increases in rank of less dominant females who initiate a consort relationship with the new dominant male. Reversal of these circumstances can be seen with replacement of the previously dominant male. Interestingly, the rank changes of the adult females are accompanied by changes in rank of their offspring. It is seen that the offspring participate actively in displays of aggression against others with increase in rank and share in the reception of aggression on fall in rank. Another study utilizing connecting tunnels between experimental enclosures has indicated that aggressive displays between animals of independent social groups occur primarily between dominant animals. Parallel studies on bands of free-ranging monkeys support the conception that significant interactions between bands occur primarily through the high-ranking members.

A wide range of studies has been carried out

by guest workers on Cayo Santiago including studies of differences in behavior of young males of dominant mothers and those of subdominant mothers, studies of the importance of novelty in the expression of curiosity by the monkey, the dynamics of interband encounters, and long-term studies of social behavior in a specific monkey band.

In the central laboratory in San Juan, studies have been carried out of the mating behavior of caged Rhesus monkeys for the purpose of better understanding primate reproductive behavior itself, for ultimately investigating the neural mechanisms underlying various aspects of reproductive behavior, and finally as part of an effort to increase reproductive yields of the breeding caged colony. A count is taken of the menstrual cycle of individual females in the breeding colony. The individual females are then exposed for a two-day period to males starting on the 11th day after the beginning of the last menstrual flow. The behavior of the animals is observed through closed circuit television. Specific compatibilities or affinities exist between specific individuals while antagonisms occur between others. These compatibilities or antagonisms are so important that breeding succeeds or fails in relation to them alone. Exposure of specific individuals may yield only aggressive behavior. The maturity and experience of the individual males is another important factor in determining reproductive success and in expressing reproductive behavior. Males born in the laboratory and cage-reared males exhibit impairments in the capability and the expression of reproductive behavior as compared to males of comparable age raised in the wild. Mating behavior is usually initiated by the male in the caged circumstance. However, initiative is occasionally taken by receptive females when paired with caged-reared males. Grooming activity is prominent between female and male in relation to reproductive activity. The great majority of grooming activity is female grooming male. In cages reproductive activity and conceptions occur throughout the year in contrast to the marked cyclicity observed in the free-ranging colonies.

In physiological psychology progress has been impaired by the lack of skilled technical personnel for the development of solid state control systems for the behavioral situations. However, progress has occurred in several areas where direct testing of animals was possible. The deficit following lesions of the prefrontal cortex in the monkey has been in delayed response performance. Classically, delayed response testing has been carried out with a pair of identically appearing visual objects with choice of correct response in relation to spatial rather than object cues. Considerable study and effort has been expended in an effort to determine the nature of this deficit in delayed response. In current studies the cue which tells the animal of the location of the reward is an object rather than spatial cue in that two objects, distinct and different in appearance, are utilized. One is rewarded depending upon the cue afforded the animal with each presentation. Lesions of prefrontal cortex disturb object-cued as much as spatial-cued delayed response performances. The essence of the deficit with prefrontal cortex lesions is one relating to delay in responding to an appropriate cue as such rather than a defict having relation to spatial set as such.

In studies of commissure function it has been found that the localization of transfer of training between the hands of learned motor skill is coextensive with that of the transfer of training between the hands of sensory discrimination tasks. This result indicates that the problem of learned motor skills is one of parietal lobe physiology primarily rather than one of physiology of frontal lobe and emphasizes the importance of sensory feedback from the extremities in relation to the motor act at the level of acquisition of motor skills. These findings fit with the suggestion from clinical experience that lesions of parietal lobe are more disturbing of the skilled motor act than are lesions elsewhere of the cerebrum.

Studies have been initiated attempting to define the neural mechanisms underlying vocalization in the monkey. Progress to date has confirmed the possibility of bringing vocalization of the monkey under environmental control by means of a behavioral test situation rewarding the animal with food upon vocali-

zation. In these studies investigations will be carried out of cortical and subcortical mechanisms and their relation to vocalization.

In the area of comparative neurology studies have been completed on the ascending spinal projections in the lizard. The most interesting finding is that few fibers from the spinal cord terminate as far rostrally as the thalamus. The great majority of fibers end in the lower brain stem and in cerebellum of the same side. Interestingly, no fibers end in the optic tectum as has commonly been ascribed for lower forms. Also, in carnivores and primates fibers from spinal cord fail to terminate the superior colliculus. These findings cast doubt on the theory that superior colliculus is a nodal point for reception and interpretation of sensory input from diverse modalities in lower forms. These studies are currently being extended to other lower vertebrate types in an effort to define the phylogenetic evolution of the spinal projections to the brain. Other studies currently in progress include comparative studies of retinal projection in fish, amphibians, reptiles and mammals. In lower forms there is, in addition to projections to the optic tectum, multiple other projections leading to hypothalamus, ectomammillary nucleus, and to other foci. These latter projections, when fully analyzed for various forms, may prove of considerable importance to understanding of some of the reflex and autonomic aspects of light stimulation.

Efforts are being made in the area of neuroanatomical studies to develop further the techniques for investigation of fiber degeneration in the nervous system. Specifically, some success has already been obtained in using tinctorial stains for the demonstration of degenerating fibers. This technique may prove more successful than the silver techniques currently in use which are difficult to achieve uniform success with and which require careful individual handling of the tissue sections.

In the area of fetal physiology studies have been completed on the potential value of chemical analysis of amniotic fluid as an indicator of the fetal circumstance *in utero*. If the fetus *in utero* suffers embarrassment in relation to its acid-base status of if the fetus dies *in utero*, there may be critical alterations in chemistry of the amniotic fluid reflective of these alterations. The oxygen tension of amniotic fluid has been proposed as a possible indicator of fetal circumstance. However, on theoretical grounds, namely, because of the poor diffusibility of oxygen, it has suggested that oxygen tension may be a poor index of fetal circumstance. On the other hand, carbon dioxide, because of its good diffusibility, might be a more accurate indicator. For these reasons studies of amniotic fluid chemistry were carried out. The lactate concentrations in amniotic fluid show extreme variability in healthy fetuses at different gestational ages and also at the same gestational age. This degree of variability of lactate concentration in apparently healthy fetuses suggests that lactate levels in amniotic fluid bear a poor relationship to fetal circumstance. A separate study has determined and characterized as curves the pH, the bicarbonate concentration, and the partial pressures of CO_2 at different gestational ages. The variability of these values in different fetuses of the same gestational age has been relatively small. Furthermore, studies of concentrations of these substances following experimental fetal embarrassment and/or fetal death *in utero* has indicated these values shift in relation to fetal circumstance but with a time lag of 15 to 30 minutes. Hence, although determination of these substances in the amniotic fluid gives a measure of fetal circumstance, the considerable time lag makes it of a lesser practical clinical importance. Of interest is the fact that the values of these substances in the amniotic fluid follows their values in the maternal bloodstream only after long time delays of up to 12 to 24 hours (in the presence of a dead fetus). Hence, amniotic fluid is in slow equilibrium with the fetus. Alterations and adjustments of the acid-base status of the amniotic fluid occur through the fetus and in turn through the umbilical circulation. Some practitioners assert that high concentrations of oxygen given to the mother during parturition or under clinical stress have a deleterious effect on fetal welfare by virtue of vascular constriction within the utero-placental circulation. Such a circumstance is believed to occur in

retrolental fibroplasia in relation to hyperoxygenation of the fetus. In a series of experiments involving chronic catheterization of fetal as well as maternal blood vessels it was determined that high concentrations of oxygen given to the mother in no circumstance produced deterioration of the fetus in terms of cardiovascular or acid-base status of the blood. It was interesting that babies in the best circumstances were the ones receiving the greatest effect on these functions whereas fetuses who have already undergone deterioration in acid-base and cardiovascular status benefit least from oxygen administration to the mother.

There has been increasing interest in the relation between birth weight and gestational age in the human. It has come to be recognized that there are babies who are too small for their gestational age at the time of delivery. These "small for dates" babies exhibit different mortality-morbidity statistics than do premature babies of the same weight. Generally speaking, such babies represent far better risks for survival but have medical problems of their own including increased incidence of pulmonary hemorrhage, tendency toward hypoglycemia during the perinatal period with consequent higher risk of brain damage, etc. It has been suggested that "small for dates" babies are associated with placental insufficiency. A study has been initiated on the consequences of placental insufficiency in the monkey by ligating fetal umbilical and placental vessels at different gestational ages. Such devascularization procedures result in considerable morphological changes in the placenta which are being analyzed by a placental pathologist within NINDB for correlation with placental pathology in the human. The results of this study have clearly indicated a relationship between the extent of placental insufficiency as produced by varying degrees of devascularization and the degree of growth retardation exhibited in the offspring. Growth retardation up to 45% has been exhibited by animals who have had ligation of fetal placental vessels. Lesser degrees of growth retardation have been seen with lesser degrees of devascularization. In addition, several instances of mild brain damage have been exhibited by animals in this series. No relation has been found between the degree of growth retardation and the occurrence of minimal brain damage. The conclusion of this study has been that "small for datesness" can be directly related to placental insufficiency.

Abruptio placentae is of major concern as a cause of fetal death or severe fetal brain damage. Little experimental work has been done on the problem of experimental abruption. A program of investigation of abruption as experimentally produced in the monkey has been initiated in the laboratory. Abruption of placentae beyond 25% to 40% of total volume results in immediate death of the fetus. Lesser degrees of abruption are tolerated and lead to brain damage. Interesting morphological changes occur as a result of the devascularization of the placenta from the maternal side. Within the study of placental abruption and placental insufficiency there are available pathological specimens of the placenta representing all varieties of devascularization from both the fetal and maternal sides. These studies together are yielding a better understanding of pathological changes of the placenta. It may be concluded that there exist only minor degrees of redundancy with regards to the placental tissue in relation to the support of fetal growth and well-being. The degree of redundancy diminishes with gestation.

An investigation of the pathology of the stillborn has been initiated with the focus of attention being primarily upon the problem of maceration. It is noted that stillborns can be divided roughly into those undergoing varying degrees of maceration or post mortem liquefaction and those in whom there is a relative preservation of morphology of organs despite prolonged death and incubation at body temperature *in utero*. The study of the pathology of the stillborn will be assessed primarily in relation to the thesis that maceration of the fetus occurs following sudden death of the fetus *in utero* whereas preservation of morphology with ultimate mummification relates to circumstances of gradual death of the fetus with gradual supervention of arterial hypotension. At a later time this thesis will be attacked

experimentally. The pathology of the brain of the stillborn fetuses has yielded some interesting patterns of pathological change. Among these patterns the most prominent has been that of perivenular hemorrhage into the white matter. This pattern has also been seen in fetuses dying of asphyxiation at term.

Studies of the neuropathology of asphyxiation at birth have continued with the monkey. The studies have emphasized both the characterization of the morphological changes in the brain in relation to asphyxiation but particularly also the correlation of patterns of neuropathological change seen with patterns of functional derangement produced in the fetus during asphyxiation itself. Generally speaking, the pathology of perinatal asphyxia produces a reliably reproducible pattern of clinical and neuropathological change. Clinically the animals exhibit profound sensory changes over the distribution of trigeminal nerve and throughout the body. They show difficulty with sucking and swallowing, alterations in voice, and ataxic changes secondary to the sensory losses. Pathologically the animals exhibit destruction in specific brainstem centers such as the sensory trigeminal nucleus, the vestibular nuclei, the basal cerebellar nuclei, and in certain thalamic nuclei, most particularly the posterior and lateral ventral nuclei. The pathological changes vary from mild chromatolytic changes in neurons to areas of focal necrosis and/or hemorrhage. However, other patterns of pathological changes are seen such as laminar necrosis, brain swelling, and perivenular hemorrhage in the white matter. Laminar necrosis has commonly been associated with brain swelling and has been seen in severely acidotic fetuses. A single case of severe cortical atrophy with ulegyria has been seen with neonatal asphyxia and was associated with severe bilateral retinal hemorrhages and increased venous pressure. Further studies utilizing asphyxia at term along with studies of blood pressure, heart rate, acid-base status, electrolyte distribution, lactate and other moieties of the blood will hopefully yield a greater insight into the relation between functional embarrassment during asphyxiation and patterns of neuropathological change.

During the course of studies with asphyxiation at term a remarkable difference was witnessed between babies delivered by cesarean section under local anesthesia versus those delivered by cesarean section under deep nembutal anesthesia. Those delivered under local appeared far more vulnerable to the onslaught of asphyxia and showed far greater incidence of neuropathological change with a given degree of asphyxiation. This important finding is being further investigated.

It was early recognized that the studies of brain damage should not be restricted to the perinatal period but should be extended into early and mid-gestational periods as well. Toward this end techniques of fetal surgery have been developed so the fetus may be removed from the uterus, vessels catheterized, studies of cardiovascular physiology and blood chemistry carried out along with other manipulations, the fetus returned to the uterus and brought to term. The first has involved compression of the cord for measured lengths of time at different gestational ages in the hopes of producing patterns of pathological change. The complete spectrum of alteration has been produced from no effect on cord compression for between 20 to 35 minutes depending upon gestational age to a circumstance of severe central nervous system damage with longer periods of compression. In between has been lesser degrees of brain damage with production of clinical neurological states resembling human cerebral palsy. The animals with lesser degrees of clinical and pathological involvement of the nervous system with cord compression have yielded disease states resembling those produced by asphyxiation at term. The most severe types of brain damage including destruction of all neurons in the neuraxis have not been seen with asphyxiation at term because of the difficulty of survival in the nursery. The most interesting point has been the similarity in distribution within the brain stem of the pathological changes with both cord clamp at different gestational ages and with asphyxiation at term.

Study of human infant brains exhibiting signs of severe brain damage has suggested that various types of pathology including por-

encephalic defects may relate to vascular occlusive phenomena occurring during the gestational period. Other pathological states such as hydranencephaly have been thought due to occlusion or compression of the major vessels in the neck. In an effort to experimentally study these phenomena the carotid arteries have been ligated in the neck at different gestational ages. Bilateral carotid artery ligation has failed in the majority of instances in producing CNS disease either clinically or pathologically. Examination of the vessels at the base of the brain has indicated that anastomotic channels between the carotid and the basilar system have been adequate to carry the necessary volumes of blood to support development and maintenance of structure. However, when both the carotid arteries and the jugular veins have been ligated, pathological states are produced. In most instances infarction occurs with cyst formation in the region of distribution of the anterior and middle cerebral arteries. There remain only thin membranes where the anterior portions of the cerebrum had existed. In only one instance has the pathological appearance of hydranencephaly occurred in an animal with bilateral carotid and jugular ligation who had in addition profound fetal anemia and also possibly fetal compromise. A continuing effort is being made to reproduce this important and interesting pathological state.

Earlier work in the laboratory has indicated that the combination of perinatal asphyxia plus hyperbilirubinemia is required for the production of kernicterus. Infusion with bilirubin at high concentrations alone does not result in evidence for brain damage. It has become clear that the pattern of staining of the nuclei in the brain stem in experimental kernicterus in the monkey coincides with the pattern of neuropathological change occurring in relation to asphyxiation at the time of birth. Present studies are extending these investigations in an attempt to produce animals with chronic neurological syndromes. Present studies have attempted to investigate some of the binding assays which have been utilized in an effort to determine the extent of dissociated bilirubin present in the serum and available to produce CNS damage. Results of these studies indicate that the presently proposed laboratory tests for bilirubin dissociation are poor indicators of the production of kernicterus.

It has been possible to reproduce both the clinical and the neuropathological sequelae of lead intoxication in the newborn monkey. Newborn monkeys have been fed lead chloride in their milk formula. After a period of 1–3 weeks intake of lead the infants have exhibited increasing irritability, anorexia, vomiting, convulsions, ataxia, and finally depressed consciousness, and death. Pathologically the changes have included capillary endothelial hypertrophy and proliferation, reactive glial changes simulating the findings of lead intoxication in the human, granulomatous infiltrates with tumescence and petechial hemorrhages. A completely satisfactory paradigm for the study of lead encephalopathy is present in the monkey.

Studies have continued on the problem of experimental allergic encephalomyelitis as induced in the monkey. In the last year there has been a study of the susceptibility of all age groups to this disease. The most susceptible age groups are the 3 and 9 month olds whereas the year olds and adult animals are more delayed in the production of neurological symptomatology. The age group showing the most delayed symptomatology after innoculation with Freund's adjuvant and spinal cord material are those of the newborn period. Premature animals produce signs of CNS disease with a still longer incubation period. Thus, although animals of all ages have regularly produced severe and usually fatal CNS disease when charged with the spinal cord antigen, those which have been most delayed in development of disease have been those in the earliest age groups. There were interesting differences in distribution of lesions with a tendency for animals of the earlier age groups to develop lesions in the brain stem and cerebellum, whereas animals of one year or older tend to produce lesions more prominently in the forebrain. The disease is fulminant with an incubation period of from two weeks to two to three months. The lesions develop over a 2–4

day period and tend to be granulomatous, hemorrhagic, and sometimes necrotizing. They are associated with infiltrations of both acute polymorphonuclear leukocytes and later with infiltrations of mononuclear leukocytes. One of the most interesting aspects was the associated extensive hemorrhagic retinopathy which resulted in severe changes in the optic fundus and was usually associated with pathological changes also in the primary optic pathways of a hemorrhagic, infiltrative, inflammatory nature.

In the past year studies have been initiated in a broad area relating to the regressive changes occurring chemically and morphologically within neural tissues in relation to circulatory failure and to death. These studies assume that these changes are similar to if not identical with the changes occurring in relation to circulatory and anoxic damage occurring in animals still surviving. Progress to date has indicated that with sudden cessation of circulation, neural tissue incubated at body temperature undergoes rapid dissolution with an obscuration of morphology, disintegration grossly of the tissues, and imbibition of fluid with leakage of electrolytes into the surrounding fluid. On the other hand, tissues similarly incubated and observed of animals who have undergone gradual death with gradual supervention of arterial hypotension have failed to show evidence for rapid dissolution of the tissues grossly or microscopically and have failed to imbibe fluid to the extent true of animals suffering sudden circulatory failure. The various functional parameters that relate to the presence or absence of these dissolutive changes and the biochemical alterations occurring within the tissues accompanying these alterations have yet to be determined.

Within the breeding colony and nursery 180 conceptions resulted from 1156 matings. The total number of deliveries was 181, 82 by cesarean section, and 99 by the vaginal route. Additional work within the breeding colony consisted of 1156 sperm tests, 1949 palpations for pregnancy, 530 blood counts, 96 urine analyses, and 152 fecal examinations. Forty animals asphyxiated at birth required intensive care in the nursery. Six animals with lead poisoning and 24 with experimental allergic encephalomyelitis created special feeding and handling problems for the nursery staff. Nine thousand routine dental, weight gain and lymph gland examinations were conducted on the infant and juvenile populations. The current inventory of animals is 65 adult males, 325 adult females and 66 juveniles within the caged colony in the central laboratory. Ninety new monkeys were acquired from various sources during the year.

Studies carried on within the veterinarian area have included studies on the pathogenesis and transmission of pulmonary acariasis. The availability within the laboratory of wild-reared animals of Indian origin, Cayo Santiago reared animals and laboratory cage-reared animals has made possible the study of the transmission of this common pulomonary disease of the Rhesus monkey. Animals brought in from India have manifested almost 100% infestation of the lungs. This results in chronic scarring and in some instances parenchymal fibrosis. The common use of the Rhesus monkey as laboratory animal makes it of concern to know more about this important disease of the Rhesus monkey. Studies of laboratory reared animals up to 4 years of age has revealed no gross or microscopic evidence of infestation with pneumonyssus simicola. Cayo Santiago reared animals are variously infested but it is not possible to determine trends because of the smallness of the sample from this group. From these provisional findings it would appear that a close and prolonged association of infected and non-infected animals is required for the transmission of the disease.

Studies have been carried out of mycoplasma antibody titers in the sera of normal and clinically ill Rhesus monkeys in the caged colony. Mycoplasma has been incriminated as a cause of primary atypical pneumonia in man and also in certain other genital infections in man and animals. The studies of antibody titers in the monkey have been directed toward determining the possibility of infection of this species with this organism.

Studies are in progress of the birth weight of the Rhesus monkey in relation to gesta-

tional age and with reference to cesarean section versus vaginal delivery. One of the most important points emerging has been the great variability in the birth weight of animals delivered under a similar circumstance. There is a slight trend for the animals born by cesarean section to be slightly heavier than animals delivered vaginally.

The embryonic tooth development is being followed in the Rhesus monkey in cooperation with the National Institute of Dental Research and with the Cleft Palate Research Center. The anatomical materials are supplied by our laboratory to outside centers for morphological investigations.

DIVISION OF BIOLOGICS STANDARDS

INTRODUCTION

The National Institutes of Health and its precedent organizations have had the responsibility, since 1902, for the control of biological products. Early in the history of these institutions, the regulatory function was carried out by a relatively small group of scientists, administered as a "division" or laboratory" depending on the organizational structure. These scientists performed their own research on vaccines, serums, and antiserums, blood and blood derivatives, etc., and their control activities, within the same environment as the other scientists in the small enclave of the Hygienic Laboratory or on the large campus of the National Institutes of Health. Collaboration within the small organization was the rule, and the regulatory function was carried out with the aid of scientists not directly involved in it. Conversely, the research activities of the staff involved in control work contributed to the vigor of the entire organization.

Ten years ago, in recognition of the advances being made with vaccines, of the burgeoning of virology, and of the growing complexity of immunology and hematology, the Division of Biologics Standards was established as a part of the National Institutes of Health with a status equivalent to that of other Divisions and Institutes. Division scientists still profit from collaboration with colleagues in other Institutes and still contribute to the intellectual vigor of the NIH.

This is highly salutary. It is, indeed, hard to imagine how the regulatory functions of the Public Health Service Act could be carried out except in such a climate. The Division is always confronted with problems that involve requirements for the most sophisticated data of modern virology, oncology, and molecular biology. We cannot forget "old" products such as smallpox and yellow fever vaccines; and we must be continually cognizant of advances in microbiological and pharmaceutical research which affect the standards and the techniques of immunization. We must also be prepared to fill gaps in our knowledge of immunization against old and new agents, to set standards for relatively rarely used but highly important materials such as antivenins and antitoxins; and we must strive to maintain high standards in the distribution of blood and the separation and purification of blood products. The expertise involved in all these functions can be provided only in a vigorous research environment.

While the research of the Division is directed to a large extent toward these goals, we would indeed be remiss in meeting our responsibilities if we did nothing more. We cannot be a completely passive agency, waiting for results obtained by other organizations, either public or private, that we can apply. The Division must be aware of the moving front of biomedical research, not only to be prepared for new demands for standards of safety, purity, and potency of biologics being developed for use in the prevention or treatment of human disease, but also to single out and investigate new problems gouged out by the moving glacier of advancing knowledge.

As an example of the last point, the Division has the responsibility for investigating the relation of recently described avian leucosis agents, found in eggs, to the safety of yellow fever vaccines which have been produced in chick embryos for many years. While the chances appear small that such agents have a deleterious effect on human beings vaccinated with attenuated yellow fever virus grown in chick embryos, we would be derelict in our duty did we not approach the problem from

a control and an investigative standpoint. We thus become involved in research of both a fundamental and practical nature.

Other examples are the elucidation and identification of covert viruses in tissue cultures which may be used in the manufacture of vaccines, the investigation of chemical and physical factors involved in the inactivation of vaccines, and the study of the ingredients added to injectable products purported to make them more effective for longer periods. With certain agents, such as influenza viruses which vary widely in successive epidemics, the Division staff must keep contantly alert to the epidemic intelligence provided by health agencies around the world, so as to be able to recommend new formulations of virus strains in vaccines for each season.

These functions, obviously, cannot be carried out by an organization dedicated solely to the routine examination of products destined for human use. They involve far more: Constant attention to developments in the fields of microbiology, hematology, allergology, and immunology, and research directed toward the problems which arise with new information concerning biological products.

This year's report reflects the diverse nature of the work of the Division. Those functions of the Laboratory of Control Activities, which in former years were reported as research projects, are now summarized as control work. The index, however, will show that scientists in this Laboratory are also active in research collaboratively with personnel in the other Laboratories of the Division. Similarly, the control and service activities in other Laboratories, previously described as projects, are now included in the Laboratory Chiefs' summaries. There remain to be reported as research projects a wide range of studies bearing on many factors involved in the development and control of effective and safe biologics.

RESEARCH AND DEVELOPMENT

The following summaries of the programs of the Laboratories present in more detail the research and development activities of the Division. As has already been mentioned in regard to the research work of personnel of the Laboratory of Control Activities, there is considerable inter-Laboratory collaboration within the Division. For example, the rubella studies in the Laboratory of Viral Immunolgy involved also Dr. Ruth L. Kirschstein, Chief of the Laboratory of Pathology and Mrs. Hope Hopps of the Laboratory of Virology and Rickettsiology. The work now to be described will be, for convenience, identified only with the Laboratory of the principal investigator. However, the individual project reports will identify the other individuals and groups who have contributed to it.

Section on Experimental Virology

The continuing investigations of this Section relate especially to the question of viral oncogenesis, and to the circumstances under which tumor-formation occurs or can be suppressed by ocogenic viruses.

Following reports that certain human adenoviruses were oncogenic for hamsters, adenovirus type 12 and simian virus 40 were used in studies to determine the possibility of preventing tumors in hamsters by means of vaccines. Repeated large doses of homologous type virus given to hamsters which had been infected neonatally either with adenovirus type 12 or simian virus 40 resulted in suppression of tumor-production. Many hamsters, and in some experiments all the animals, failed to produce tumors. Inactivated homologous virus failed to produce this effect.

Suppression of more tumor-production occurred also when simian virus 40 was inoculated into newborn hamsters and adenovirus 12 was injected in repeated doses later. Here an interesting phenomenon was observed: Two strains of adenovirus 12 were used. These are indistinguishable by many tests, such as virus neutralization and cross protection. However, they were found to differ in their tumor-suppressing capacity in hamsters that had been infected with simian virus 40 when newborn. The strain which exerted the protective effect had a history of having been propagated in monkey kidney epithelium cultures where it could have been in contact with simian virus

40. The strain which was not protective had been isolated and propagated in human cell cultures and never in monkey kidney cell cultures. Simian virus 40 could not be recovered from the protective strain; and according to tests carried out in the Laboratory of Infectious Diseases, NIAID, no simian virus 40 or tumor antigen could be demonstrated either by complement fixation or by immunofluorescence.

This program is of major significance in developing a full understanding of hazards that may exist in proposed live virus vaccines and in providing information on the importance of the immune state in relation to viral oncogenesis.

LABORATORY OF BACTERIAL PRODUCTS

Cholera Vaccine and Related Studies

Field trials of potency of cholera vaccines in East Pakistan showed significant protection lasting for as long as two years. However, in the Philippines, no protection was apparent after less than six months except when the vaccine was used with an oil-in-water adjuvant. Abscesses following administration of this type of adjuvant preclude further use, but aluminum adjuvants may be able to effect longer persistence of immunity without such side effects.

Recent results suggest that cholera in the suckling rabbit may be due to toxin (choleragen or Craig's skin toxin). It appears that the passive protection of the infant rabbit described in last year's report may have been due to antitoxin. Because of the current interest in cholera toxins, studies are being directed towards detoxification, assay of toxin and toxoid, and the role of the toxoid in assay procedures.

Laboratory personnel are heavily engaged in the cooperative work on cholera being sponsored by WHO, the Pakistan-SEATO Cholera Research Laboratory, and the U.S.-Japan Cooperative Medical Science Program. The Chief, Laboratory of Bacterial Products, Dr. Margaret Pittman, has continued to serve as the NIH Project Officer of the Pakistan SEATO laboratory and as a member of several advisory committees. Dr. John C. Feeley spent two months as a travelling consultant for WHO reviewing potency trials. Dr. Feeley is also a member of the U.S. Panel on Cholera of the U.S.-Japan Cooperative Medical Science Program.

Pertussis vaccine

Two candidate preparations of pertussis vaccine have been characterized and tested in comparison with the Japanese toxicity reference and vaccines used in a clinical trial in Great Britain. The toxicity assay results obtained by the collaborators, with one strain of mouse, differentiated the two vaccines according to a clinical reactivity which was significantly different. In these tests a common strain of mouse was used. Our study indicates that different strains of mice, and conventional versus germ-free mice differ significantly in toxic reactions to pertussis vaccine. These factors will be considered in the clinical study now in the formative stage and in the specifications for freedom-from-toxicity testing of pertussis vaccine.

In order to determine the antigenic pattern of U.S. vaccine production strains of *Bordetella pertussis*, and of strains isolated from occasional cases of whooping cough, and to have typing antisera produced for reference purposes, a contract was initiated in June 1966 with the Michigan State Department of Health. This will provide needed information on changes in antigenic pattern of *B. pertussis* in the United States, to compare with reports from England and Canada.

Tetanus Toxoids

In the cooperative study on the prevention of neonatal tetanus in the Territory of New Guinea, the results indicate that if women of child-bearing age were vaccinated against tetanus and given a booster injection of either plain or adsorbed tetanus toxoid, they would respond with antitoxin titer levels sufficient to protect the newborn child. Adsorbed toxoid induced higher titers and would be preferable for booster as well as for primary immunization as previously reported.

The study on the assay of the unitage of the adsorbed and plain toxoids used in the field

trials indicates that the animal-measured unitage was correlated with duration of antitoxin levels. The two types of toxoids differed about 6-fold in unitage. Although primary response to each toxoid was similar, at the end of two years the mean antitoxin level of the women who received the adsorbed toxoid was about 6-fold greater than of those who received plain toxoid.

The investigation of the cause of abscess-formation following the injection of two lots of tetanus toxoid in water-in-oil adjuvants is showing promising results which indicate that certain toxoid preparations (as well as cholera vaccine which also caused a high incidence of abscesses in the Philippines) and other antigens are capable of causing Arlacel A, the emulsifier in the adjuvant, to break down into free fatty acids. Oleic acid, an impurity in Arlacel A, was incriminated some years ago as the cause of abscess-formation following injection of influenza virus vaccine. It now appears that even with purified Arlacel A certain antigens will cause release of fatty acids from the emulsifier. Analyses of the free fatty acids released and their effect on tissue are being made.

Pleuropneumonia-like Organisms and Mycoplasma

A transitional L-form of *Streptococcus sanguis* has been isolated from the lesions of 21 patients with recurrent aphthae and from lesions of only one of six non-aphthae patients. Aphthae patients also gave a positive skin reaction to a polysaccharide extracted from the streptococcus. These results suggest a causal relation of *S. snaguis* to recurrent aphthae.

The *Mycoplasma orale* isolated by direct culture procedures from the blood and bone marrow of 4 of 10 leukemic patients and not from 10 non-leukemic patients has stimulated much interest on the possible etiological or associated role of *Mycoplasma* in leukemia.

Tuberculin

The significant work on the separation of tuberculin into protein and carbohydrate fractions, each of which is capable of eliciting delayed reactions in sensitive guinea pigs, has been extended to show that these fractions in Freund's Adjuvant are capable of inducing passive cutaneous ananphylaxid antibodies, also complement-fixing and precipitating antibodies. An interesting observation has been made to the effect that high concentrations of Old Tuberculin tend to suppress the response to lower concentrations of material when injected in parallel positions on opposite sides of sensitized guinea pigs. This has implications in regard to potency tests. It has been shown that, at concentrations suggested by WHO for potency testing of the International Standard, the reaction to the U.S. Standard Old Tuberculin was suppressed.

Poison Ivy

The finding obtained in one contract project that urushiol was present only in extract of freshly collected leaves and that the commercial poison ivy extracts used in another contract project did not contrain urushiol opens the need for an investigation on whether or not such extracts would be capable of desensitizing posion ivy sensitive persons. The clinical contract project results indicate that the degraded catechols of urushiol do not desensitize.

LABORATORY OF BIOPHYSICS AND BIOCHEMISTRY

Microbial Biophysics

Theoretical investigations of problems dealing with mechanisms and kinetics of viral inactivation were facilitated by a contractual arrangement with Electronic Associates Inc. of Rockville, Maryland, which provided analog computer rental and engineering services. A set of differential equations was devised to express the survival ratio of photosensitized T_2 coliphage as a function of time, light intensity, oxygen tension, and three parametric rate constants representing the inactivation rate, O_2-diffusion rate, and rate of utilization of O_2 within the phage particle. The computer was used to solve these equations simultaneously and to plot any selected variable as a function of time. The solutions allowed the selec-

tion of critical conditions for laboratory experiments. It was found that oxygen concentration in the photosensitized phage particle decreases as it is used up by the photochemical reaction and as it diffuses into the medium. For significant photodynamic inactivation (90% or more) to occur, the velocity constant for diffusion must be less than 10% of the velocity constant for inactivation.

Photodynamic inactivation curves for T_3 coliphage sensitized with toluidine blue and irradiated under a variety of conditions were found to fit closely to theoretical curves for a model in which each of four critical sites must be destroyed in order to kill the phage.

Theoretical curves on the effects of aggregation on the inactivation of virus suspensions were calculated from published data on distribution of aggregates of various sizes in vaccinia virus preparations. Apparent velocity constants for inactivation of highly aggregated preparations are about 25% less than the values for monodisperse suspensions of single particles.

These studies on the mode of action of viral inactivating processes are of continued value to the Division in providing fundamental information for the control of viral vaccines.

Analytical Chemistry

An original method for measuring mercurial preservatives in biological products, employing the atomic absorption spectrophotometer to determine the amount of mercury, has been developed to the point where it can now be evaluated on various vaccines. The spectrophotometer has also been used to detect an easily measurable amount of thallium in an experimental *Mycoplasma* vaccine purported to be thallium-free. Analytical methods for phenol in the presence of high concentrations of protein, and for benzethonium chloride, have been perfected. An electrolytic apparatus to measure moisture in dried biologics has been put into service to be evaluated in relation to conventional methods.

Virus Characterization

Adenovirus 4 particles were found to have a density of 1.34 gm/cm^3 in cesium chloride. A small DNA virus, designated adeno-associated virus or AAV, symbiotically associated with one of several adenovirus 4 strains investigated, has been shown to have a density range overlapping adenovirus 4. Separation of adenovirus completely free of AAV by density gradient centrifugation would therefore be impossible. However, since some AAV particles have densities ranging up to 1.44 gm/cm^3, it is possible to separate AAV completely free of adenovirus by centrifugation in cesium chloride. Preparations of AAV thus purified have been used to prepare antisera against AAV, and these antisera have provided means to rid adenovirus of AAV. The diameter (18 mu-21 mu) and fine structure of AAV have been determined; it appears to be the simplest possible icosahedron, having a reovirus-type net-like capsid. AAV is structurally unrelated to its associated adenovirus. AAV is more resistant to detergents than adenovirus.

AAV-antibody reactions have been observed in the electron microscope in a manner which permits visualizing the attachment of gamma globulin molecules upon the viral surface. AAV appears to be antigenically distinct from adenoviruses in neutralization, agglutination, and complement fixation tests, although both grow to high concentrations in the same cell cultures. Measurable replication of AAV was sought in cell culture systems, but was never oberved in the absence of adenovirus. It is therefore concluded that AAV requires adenovirus as a helper.

The studies are especially relevant to the production of viral vaccines, in which the occasional presence of adventitious agents has constituted a serious control problem. The capability of detecting contaminating viruses by direct physical observation, and promptly and definitively characterizing new viruses is regared as an important asset to the DBS program.

LABORATORY OF BLOOD AND BLOOD PRODUCTS

The research program of this Laboratory has included studies of the stability of blood products, the development of methods and

standards for these products, the investigation of red cell antigens, plasma antibodies, and the clotting and fibrinolytic systems, and the examination of proteins of body fluids. These various fields of interest have as their goal the improvement of procedures used for the control of safety, purity and potency of biological products derived from blood. Thus the projects are directed toward improving existing control tests, developing new ones, obtaining stability data leading to more realistic dating periods, and providing the professional staff with constantly updated information necessary in evaluating new products and procedures.

Results obtained to date indicate that the amount of trichloroacetic acid-soluble material in immune serum globulin increases progressively during storage. Concomitantly, material with a sedimentation coefficient of approximately 3S appears in these preparations, and the amount of such material increases with time. Starch gel electrophoresis has demonstrated an increase in the number of protein components during the storage period.

To develop methods for measuring the physical and chemical interaction of a perservative and surface antiseptic with a plastic which may be used as a packaging material for biologicals, a study of the pilot system, benzalkonium chloride + nylon-6,6, has been undertaken. Measurements made thus far appear to confirm the initial hypothesis that the diffusion, permeation, and solubility coefficients depend on concentration, temperature, and time. Other factors that must be considered are the cationic characteristics of benzalkonium and its critical micelle concentration.

To evaluate the effect of high molecular weight additives on blood typing serums, various concentrations of polyvinylpyrrolidone (PVP) have been added, in the presence and absence of bovine serum albumin (BSA), to systems containing group O, Rh_0 (D) positive cells and anti-Rh antiserum. It was found that a thousand-fold increase in titer (compared with controls containing no PVP but equivalent levels of BSA) could occur when agglutination was carried out in the presence of 1% PVP and 5% Bsa. PVP in the absence of albumin caused no rise in titer.

Basic research in the Laboratory has included investigations on various phases of the clotting and fibrinolytic systems and studies of the proteins of body fluids.

When fibrinogen rich in antihemophilic factor (AHF) was clotted by thrombin, most of the AHF activity was lost. Similarly, even in the absence of active thrombin (i.e., after inactivation of the thrombin with diisopropylfluorophosphate) polymerization of fibrin by calcium was accompanied by significant losses of AHF. By addition of 0.3 M glycine ethyl ester to the clotting medium, however, it was shown that in the presence of both calcium and active thrombin 80 to 90 percent of the fibrinogen could be clotted with a loss of less than 10 percent of the total AHF activity.

Investigation of an increase in antihemophilic factor activity in the plasma of mice bearing plasma cell tumers was performed. Of 20 lines of mouse plasma cell tumors tested, the only line that caused an increase plasma level of antihemophilic factor also caused the accumulation of a macroglobulin in the plasma. This macroglobulin was therefore isolated and injected into mice without tumors. Subsequent to the injection there was a 2-fold increase in antihemophilic factor activity which lasted approximately 48 hours; appropriate controls showed no increase.

LABORATORY OF PATHOLOGY

The Laboratory of Pathology has now completed its first full year as a separate entity. Until this year, the Laboratory's largest project has been the performance of neurovirulence tests of lots of Poliovirus Vaccine, Live, Oral. However, with the great decline in poliomyelitis in the United States and in the world, the number of lots submitted for testing has markedly decreased. As a result, the Laboratory has been able to turn its attention to other problems related to the pathogenesis of infectious deseases and their prevention or control. In addition to its own research and control activities, the Laboratory has had an active collaborative research program with members of the staffs of other Div-

ision Laboratories and with members of other Institutes as well.

Neurovirulence Testing

Several Type III Live Poliovirus vaccines were tested repeatedly and the reproducibility of the test system could be demonstrated. The data presented led to development of an international study involving the comparative testing of vaccines of varying degrees of neurovirulence. This study is now is progress.

Secondary and primary seed lots of Yellow Fever Vaccine have been studied in rhesus monkeys so that, in the event that a new seed lot is needed, the neurovirulence level of the vaccine will have been established.

Oncogenesis and Pathogenesis of Infectious Agents

Studies on the comparative oncogenic effects of polyoma virus strains, simian virus 40 and adenoviruses have been continued. It has been found that polyoma virus produces tumors only in mice, hamsters and mastomys, SV40 in hamsters and mastomys, adenoviruses in hamsters, mastomys and certain inbred strains of mice. The oncogenic potential of these viruses can be enhanced by decreasing the immunologic capability of these animals; this can be accomplished primarily by performing thymectomies on newborn animals. In addition, studies of a "hybrid" virus of adenovirus 7 and SV40 (strain E46) indicate that the genome of either the adenovirus portion or the SV40 portion have specific oncogenic effects under varying conditions. Studies on the pathogenesis of tumors produced by clones of various SV40 plaque sizes are of different virulence and oncogenic potential.

Studies on cells *in vitro*, either derived from tumors, or "transformed" by oncogenic viruses, have been performed. The cellular transformations have characteristics of malignancies and have varying growth potentials when inoculated into animals, depending on the species.

Studies of the pathologic reaction in muscles of monkeys injected with tetanus toxoid emulsified in mineral oil adjuvant reveal that the emulsion tends to localize in pockets throughout the muscle and to cause severe reactions in the muscle as long as 6 months after inoculation. It appears that this material may remain in the muscle forever and continue to act as an irritant. Studies of other adjuvants are planned so that information will be available when they are proposed for use.

The Laboratory has assumed a major responsibility for a contract on the testing of various vaccines and viruses for oncogenesis. This contract has just been let, but staff personnel have spent several months determining proper dosages of materials for baby hamsters and in organizing the program.

Neuro-Anatomy

Studies on the neuro-anatomical development of various animal species have provided the baseline information for work on the immunologic and allergic response of the central nervous system in infection or immunization.

LABORATORY OF VIRAL IMMUNOLOGY

Rubella

A major research emphasis of the Laboratory has been development and clinical testing of an attenuated rubella virus suitable for use as an immunizing agent. This work was initiated in order to "tool up" the competence of the Dvivision in anticipation of the need to test candidate rubella vaccines. However, as a result of tissue culture studies on the rubella virus, it became apparent that attenuation could occur in high passage levels.

In vitro and *in vivo* techniques were devised capable of signaling if and when modification of cell culture-propagated rubella virus had occurred. High passage levels of the virus were found to exhibit marked differences in behavior when compared to virulent low passage virus in RK_{13} continuous rabbit kidney tissue cultures. Of even more interest was the observation that the high passage strain evoked the production of increased amounts of interferon in various types of cell cultures.

The rhesus monkey was used to compare the characteristics of infections produced by

the virulent and the modified high passage virus. These animals served as an ideal experimental model since the virologic events associated with virulent rubella virus infections in monkeys closely simulated the circumstances observed in man. The tests with the high passage strain provided the final clue to the fact that laboratory manipulation had, indeed, attenuated the virus. The animals inoculated with virulent rubella virus developed infections characterized by viremia, virus shedding from respiratory and rectal secretions, and communicability to uninoculated cage contacts. Monkeys infected with the high passage strain developed antibodies but did not exhibit viremia or significant virus shedding, and uninoculated controls remained free of infection.

The high passage strain was used to prepare an experimental live rubella virus vaccine. This material was inoculated into 8 rubella-susceptible girls under carefully supervised conditions of isolation. None of these children showed any symptoms of illness which could be attributed to the vaccine, yet all developed neutralizing antibodies indicative of immunity. Viremia was not detected. Although small amounts of virus were obtainable from the pharynx of several vaccinees, the material was not communicable, since none of 8 rubella-maintained in close association with 22 susceptible contacts. Again there was no clinical disease and no evidence of communicability.

This work has major scientific import for the following three reasons: (1) It provides the first evidence that rubella virus can be attenuated by laboratory manipulation; (2) it provides the first "marker" techniques that are capable of distinguishing between virulent and attenuated rubella viruses short of clinical trial and (3) it provides an attenuated strain of the virus that is promising for further investigation in the search for a vaccine.

Viral Genetics

In other investigations this Section again turned to the highly sensitive qualitative complement-fixation techniques which in the past year's annual report had been shown capable of detecting subtle antigenic differences between closely related viruses. The current work with the technique has demonstrated that the Freund and Rauscher mouse leukemia viruses appear to be antigenically identical.

Mumps

Two new procedures have been developed for detecting mumps virus antibodies. Both of these methods (a modified hemagglutination-inhibition test and a plaque-reduction neutralization test) are more sensitive than the conventionally employed antibody assay techniques. These new procedures were developed in anticipation of future needs in research and control obligations concerned with the prevention of mumps.

Vaccinia

Rhesus monkeys have been used to study the cutaneous and immunologic response to inoculation with vaccinia virus. Interest has been focused on determining the importance of the route of inoculation and virus dose on immunity. Animals have been vaccinated by (1) the multiple pressure technique, (2) jet inoculation, (3) intradermal inoculation, and (4) subcutaneous injection. Inocula have contained from 10^2 to 10^8 $TCID_{50}$ of vaccinia virus.

By varying the virus dose and route of inoculation, it was possible to evoke antibody production without eliciting a dermal response. This simulated the type of human response observed during clinical investigations with dilutions of smallpox vaccine given by jet injection.

LABORATORY OF VIROLOGY AND RICKETTSIOLOGY

Arboviruses

The newly established arbovirus collection has been greatly expanded to include other viral (and mycoplasma) antigens and antibody reagents. This was a result of the decision to establish an immunoserology unit to serve as a nucleus for a major service and research component of LVR. At the time of this writing (April), 184 agents are in the working stock of 300 frozen lots of "master", "working", and

"vaccine" virus pools, 112 antigens for complement fixation and hemagglutination-inhibition and 122 antibody reagents (hyper-immune ser and ascitic fluids). Very recently 65 new arb virus prototypes were received from Ya Arbovirus Unit; master and working poo will be produced shortly. Techniques of imm nodiffusion and agar gel diffusion have be added to the already routinely performed qua titative complement fixation, hemagglutinatio inhibition and immunofluorescence techniques

A new study on the electromicroscopic morphology of certain viruses was undertaken in collaboration with Dr. K. O. Smith, LBB. An early result was the determination of the myxovirus nature of the Kemerovo virus prototype (isolated by Soviet workers from ticks and humans). The agent was definitively identified as Newcastle disease virus by J. Casals and ourselves. Human epidemics due to Kemerovo virus have presented a serious public health problem in Siberia.

Avian Leukosis

Owing to the Division's increasing responsibilities related to *avian leukosis viruses*, a major build-up of the required facilities has been accomplished during the year. The combination of RIF tests in tissue culture, serological COFAL tests, and immunofluorescence covers the gamut of techniques necessary to cope not only with the potential RIF contamination of egg-produced vaccines, but prepares us for the future should the virus of Marek's disease of fowl prove to be a tumor virus potentially hazardous to humans receiving egg-produced vaccines. (A reference collection of candidate Marek's virus agents is being set up.) Such experience will serve as a base for other oncogenic virus work.

Four Rous sarcoma virus tumors (Bryan, Zilber-Carr, Prague and Armstrong) have been maintained in hamsters by transplantation; recently the tumor cells of all four were successfully adapted to tissue culture opening up a new area for research and development. A new and important method for determining fowl maternal RIF antibody (or virus) in egg yolk by immunofluorescence has been worked out. This should allow ready monitoring of chicken flocks and preparation of RIF-free cell cultures from chick embryos taken from eggs with yolk known to be negative by this specific immunofluorescence test.

Adenoviruses

Current studies on adenovirueses serve to accumulate information which will be of help in determining the safety of candidate adenovirus vaccines for use in man, and, in addition, will be of assistance in understanding the biology of adenovirus infection. During the past year, the early findings of Huebner and associates on the "hybridization" of adenovirus type 7 and SV40 were extended by showing that SV40 genetic material is apparently also enclosed within adenovirus type 3 capsids. Related to this are current studies on the occurrence of SV40 antibody in the sera of people vaccinated with the adenovirus lots known to contain SV40 genetic material and on the possible serological response to the "adeno-associated virus" particles known to be present in many adenovirus vaccines.

Influenza

The Section on Respiratory Viruses continues to develop information which should be of assistance in improving the effectiveness of influenza virus vaccines. For instance, evidence has accumulated to indicate a marked recent shift in the antigenic composition of some of the contemporary strains of influenza B. This affects the Division's plans for the construction of the next vaccine formula.

Important data on the relationship of serum neutralizing influenza antibody levels and resistance to subsequent influenza virus challenge were obtained from a human volunteer study. Even more important information on the dynamics of continual change in the antigenic composition of influenza viruses may be obtained from a field study of immunologically virginal populations of several Pacific atolls. Some of the island groups experienced a severe epidemic of influenza in 1964; their antibody responses to epidemic infection are being followed and the response to monovalent vaccine will be examined. An additional group of

nearly 1,000 people, without influenza A or B antibody, who had escaped infection during the 1964 epidemic, offers a unique opportunity to evaluate immunological responses to monovalent influenza virus vaccines.

Rickettsiae

Studies have concerned interferon production, parasite-host-cell relationships, comparative electronmicrosopic morphology, and genetic relatedness of rickettsiae, mycoplasmas, and viruses. Interferon production does not seem to be a prominent feature of rickettsial proliferation *in vivo*. On the other hand, the level of interferon production may serve as a reliable marker for rubella virus attenuation. Careful collaborative electromicroscopic studies of mycoplasmas, larger viruses and rickettsiae showed that the 3 groups of organisms are morphologically distinguishable from one another.

Studies on the characteristics of virulent and avirulent rickettsiae (typhus, spotted fever and scrub typhus) are continuing because of the need for a better understanding of the phenomenon of attenuation in relation to live rickettsial vaccines. Certain fractions of the killed typhus rickettsiae (soluble antigen, cell wall and intracellular protoplasma) are being compared with the intact organism to define their relative immunogenicity as a step toward more potent, purified vaccine. As a refinement rickettsiae grown in cell culture rather than in eggs are now being tried. Initial steps toward developing a vaccine potency test using monkeys, rather than guinea pigs, have been taken by determining the susceptibility of rhesus and cercopithecus monkeys to epidemic typhus rickettsiae.

The Laboratory now possesses a competency to perform studies on the genetic relatedness of rickettsiae using the DNA homology techniques. The initial studies employed the DNA from *R. quintana*; the technique will be applied to other rickettsiae, mycoplasmas and probably viruses.

Tissue Culture

In an effort to establish a back-up cell line for BC–C–1, particularly as needed for measles vaccine testing, a second line of cercopithecus kidney cells has been under test—this is designated BS–C–2 (MA–134) cell line. Extensive comparative studies indicate that BS–C–2 can satisfactorily replace the standard BS–C–1 line.

The *Tissue Culture Section* continues to supply the entire Division with large numbers of primary and continuous cell cultures from verifiable sources and of high quality. Some 400,000 cell cultures in tubes and 150,000 in bottles of different sizes were issued during 1965. This service activity occupied practically the entire time of the Section's personnel.

However, new emphasis on research and development has resulted in the introduction of several new methods into production; these have improved the quality and quantity of cell cultures at sizable savings. New research projects deal with studies on malignant cell transformation *in vitro*, using human diploid cells (WI–38 cells), which are of particular concern to DBS, having been proposed for use in human vaccines. This possibility has stimulated another project, the purpose of which is to determine the presence of blood group or tissue transplantation antigen in cultured WI–38 cells, because of a potential hazard of sensitization of vaccine recipients.

Paolins

Studies of the antitumor effects of clam extracts, presumably containing the active principle (paolins), have been extended to include adenovirus 12 tumors (virus-induced or transplanted) in hamsters, mouse leukemia (L–1210), mouse sarcoma 180, and mouse melanoma (S–91). Preventive treatment of newborn hamsters soon after inoculation with oncogenic adenovirus 12 seemed to inhibit tumor formation. Weaned hamsters with transplanted solid adenovirus 12 tumors shed the tumors and survived indefinitely when the clam extract was injected into and around each tumor. Mice with otherwise lethal melanomas, leukemias and sarcomas responded to clam extract treatment, sometimes in a most dramatic fashion. To allow general confirmation and extension of these challenging results, a large quantity of clam extract has

been prepared by a commercial laboratory on contract with the Division. The material will be used to supply the demands of other investigators at NIH and elsewhere and for further attempts at characterization, isolation, and identification of the active principle in clam extract.

Hepatitis

The A–1 agent described some years ago as a virus recovered from an icterogenic human plasma pool, has now been definitely identified as a strain of *Mycoplasma gallisepticum*. Serological tests, by plaque reduction and growth inhibition techniques, of sera from various series of human cases of hepatitis, indicate some relation of this *Mycoplasma* to the disease. However, more work is necessary to rule out anamnestic responses in the human cases tested and to be sure that significance can be attached to small rises in titer. Moreover, it has not been possible to re-isolate the *Mycoplasma* from the icterogenic pool. The new approach to the A-1 agent suggests a large number of additional studies.

CONTRACT OPERATIONS

The Division has utilized contracts on a relatively small scale for several years, for such purposes as the development of sources of snake venoms, improvement of standards of potency of blood-typing sera, and the performance of studies on the hemagglutination level of pooled human plasma. These have been summarized in past annual reports. In fiscal 1966, the Division received an additional appropriation from the Congress, together with the charge to investigate problems of oncogenesis associated with viral vaccines and other products.

Procedures for the initiation of contractual projects and for the dual review of contracts have been set up. A great deal of effort has been expended in obtaining information on contractual operations of other Institutes or Divisions within NIH that bear on the questions of viral and chemical carcinogenesis. The DBS Contracts Committee, consisting of the Assistant Director, the Laboratory Chiefs, and a few other people, has been apprised of this information. We have, therefore, gained the necessary background to orient our own efforts in directions that are especially important to the Division's mission. Although the section on contracts does not yet reflect this progress, because the contracts are not yet formally let, we have developed a sizable program. A contract for the testing, in baby hamsters of the oncogenetic potentialities of a variety of viral vaccines or possible "candidate" vaccines, is being established at the time of this writing. Other contracts for the procurement of intermediate products in vaccine preparation are in the process of development. These materials will be tested in the baby hamster system, probably with the establishment of another contract. Still additional projects on the chemistry, toxicology, pharmacology, and oncogenicity of various inactivants, stabilizers, and preservatives used in vaccine manufacture, and of mineral oils and emulsifiers proposed for use as adjuvants in allergenic products and vaccines, have been advertised to potential contractors.

It is noteworthy that the development of this contract program, which is now gaining momentum, has proceeded in what we consider an ideal way. Contractual operations should be, properly, extensions of the activities of the Division staff. The contract on baby hamster testing has been developed as the result of considerable activity on the part of Dr. Amos Palmer of the Laboratory of Pathology and Mrs. Claire L. Cox, recently assigned from the Laboratory of Bacterial Products to the contracts program. These scientists, under the supervision of Dr. Ruth L. Kirschstein, Chief of the Laboratory of Pathology, have been engaged for two months in preparatory work on product-testing in baby hamsters, on the development of coding systems, and the procurement of products. Because of their efforts, we are now prepared for the initiation of operations by the contractor and for the proper supervision of his activities. Another example of the participation of staff in the contract effort is the determination of what additional materials, for tests in hamsters, will be produced within the Division or procured

from outside sources. As a result of special meetings, Division personnel have established the need for outside procurement of only 50 per cent of needed materials; the rest will be furnished by staff members who are especially competent to produce them. An additional example can be cited: The DBS Contracts Committee was interested in the development and testing of yellow fever vaccine freed of avian leucosis agents. As the result of committee meetings held by Dr. Nicola Tauraso, the appointed project officer, it has developed that there is enough interest and enthusiasm among many staff members to provide for the performance of most of the necessary work within the Division; only a small fraction, if any, will have to be done by contract.

This, indeed, signalizes the value of scientific program coordination. We expect, confidently, that the contractual operations will continue to be closely related to the activities of Division staff. Some contracts which were in effect for other purposes have been re-oriented to fit into the oncogenesis testing program. For example, the University of Texas contract on plasticsbiologics interaction is being oriented, beyond toxicology, to the study of oncogenic effects. This is the result of the interest of Division staff members in certain observations noted by the contractor.

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