NATIONAL INSTITUTES OF HEALTH

Review of INTRAMURAL RESEARCH 1962

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

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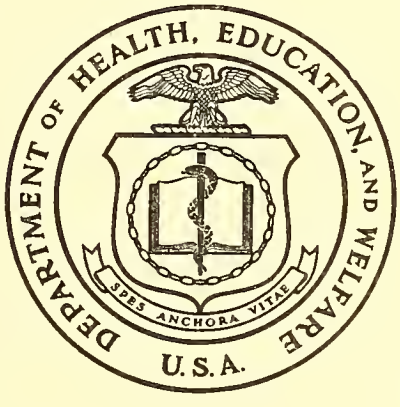

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FOREWORD

The National Institutes of Health utilizes extramural and intramural programs in fulfilling its mission of conducting and supporting research, research training, and related activities in the biomedical sciences. The extramural activity, administered from Bethesda, affects most biomedical research institutions in the United States, and is now reaching into areas throughout the world. The intramural operation is carried out in the laboratories and clinics in Bethesda and seeks primarily new information in biology and medicine. This volume—which, it is believed, serves a number of scientific and institutional needs—summarizes the work done and knowledge gained in NIH's own laboratory and clinical facilities during calendar 1962. The reports are essentially in the form prepared by their originators, with only the most general guidelines, and without appreciable editorial modification. The reader may thus savor the diversity of outlook and attitude prevailing among NIH scientists, while noting that such diversity in no way inhibits pursuit of the common goal of understanding and mastering disease processes.

NIH's intramural programs embrace challenging scientific opportunities and major social responsibilities. During years of rapid growth and change in both substance and dimension of the health-related sciences, it is a matter of pride that NIH continues to meet these opportunities and responsibilities with undiminished standards of excellence.

James Q. Shannon

JAMES A. SHANNON, M.D.,
Director, National Institutes of Health.

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NATIONAL CANCER INSTITUTE

INTRODUCTION

The major studies of the clinical branches of the National Cancer Institute are an evaluation of chemohterapeutic methods for the treatment of neoplastic disease; the use of radical surgery in carcinoma of the uterus and head and neck; a study of the natural history of neoplastic diseases, particularly as modified by various therapeutic procedures; studies of the biochemical and physiological changes produced in the host by a tumor, and studies involving the use of experimental animals as a supplement and complement to the studies carried out in man.

SPECIFIC HIGHLIGHTS

Specific highlights in the last year are an increase in the remission rate in choriocarcinoma to 75%; the introduction of methylglyoxal-bis-guanylhydrazone (the first drug to be active in a large percentage of patients with acute myelogenous leukemia); and the introduction of vincristine (a new agent that is active in the treatment of acute lymphatic leukemia). These are the only new agents to be introduced into the therapy of acute leukemia in the last ten years. A very active urological service has been developed which provides urological consultation within the National Cancer Institute and throughout the other institutes. In addition, a stimulating and productive urology research program has been initiated. A monkey colony has been established, under contract, for pharmacological and carcinogenesis studies.

NATURAL HISTORY OF THE DISEASE

Acute Leukemia

The principal complications of acute leukemia are infection and hemorrhage. The procurement of platelets and their transfusion in large quantities represents a major research and therapeutic effort. It has been shown that platelets can be procured, can be transfused in large quantities and that bleeding complications can be averted. Further studies in platelet storage are essential to make this technique more widely available in clinical medicine. The use of white cells derived from patients with chronic myelocytic leukemia has resulted in a very marked reduction in the mortality associated with *Pseudomonas* septicemia, thus averting another one of the principal causes of death in acute leukemia.

Multiple Myeloma

A review has been completed of the natural history of this disease. Of particular interest was the inability to correlate the clinical findings with the type of myeloma protein produced. There was no difference in the median survival of patients with gamma myeloma, $Beta_{2A}$, or Bence-Jones protein producing myelomas. There were statistical differences in the level of serum protein, the extent of bone disease and clinical symptoms between the gamma myeloma protein producing and the non-gamma myeloma protein producing patient groups. Studies are being carried out on the effect of chemotherapy in experimental animals with a plasma cell tumor. Cytoxan and 5-Fluorouracil were shown to be effective in producing an improvement in survival and elimination of serum protein abnormality. Other agents produced prolongation of survival time but only infrequently was there an improvement in the serum protein abnormality. Urethane improved survival time but was without effect on the serum proteins. Large doses of androgens have been used in multiple myeloma. In four of five patients there was an increase in the rate of formation of red cells. There also appears to be some increase in the white blood cell count. L-phenylalanine mustard was shown to be effective in approximately 40% of 40 patients with multiple myeloma, with an increase in hemoglobin, improvement in the bone marrow and bone healing. Patients with multiple myeloma showed an increased susceptibility to infection due to impairment of antibody

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production. There was no correlation between protein types and degree of immune deficiency. Significant advances were made in the ability to diagnose by immunochemical means the types of proteins produced and to identify the cells producing these proteins.

Head and Neck Cancer

A review of 42 patients dying of epidermoid carcinoma of the head and neck region disclosed that 55% had distant metastases, almost entirely to the lung. This high incidence of metastases is primarily due to a prolongation of life by control of the primary lesion as a result of radical surgery. There appears to be a good correlation between epidermoid cancer of the mouth and pharynx with combined alcohol and tobacco consumption.

Cervical Cancer

Two hundred patients with a diagnosis of carcinoma of the crevix have been followed for at least one year after therapy. Fifty-two percent of these patients had received previous X-ray therapy or surgery; 39 of these patients were treated by radical hysterectomy; 25 had anterior pelvic exenterations, and 71 had total pelvic exenterations. The survival rate of five years was 57% for both radical hysterectomy and anterior pelvic exenteration groups, and only 16% for the total pelvic exenteration group. The overall survival rate was 37% at 5 years.

Infection in Surgery

Clinical surgery continues to be concerned with post-operative infections. The infection rate following extensive cancer surgery decreased from approximately 30% in 1956 to a low of approximately 10% in 1962, but the percentage of infections that were staphylococcal increased from approximately 35% in 1956 to approximately 70–80% in 1962. This decrease incidence was brought about by the introduction of both a planned program of antibiotic prophylaxis and increased awareness of the staff to this problem.

Theory of Basal Cell Cancer

A tentative definition of the biogenesis of basal cell carcinoma has been developed. These cells fail to keratinize. Because of this, the mechanisms by which the cell is enabled to move from the basal layer to the surface is lacking, causing the basal cells to accumulate as a tumor mass. This is an interesting concept requiring further investigation.

THERAPY

Evaluation of uretero-ileostomy in extensive pelvic surgery, studies of circulating tumor cells and wound seeding continue to be major efforts.

The effects of a single dose of radiation therapy of the order of 2,000–2,500 Roentgens for treatment of patients with primary cancer of the mouth, pharynx or larynx have been investigated. There was immediate disappearance of the primary cancer in 18 of 20 patients. At 2,000 R, mild local reactions were observed. At 2,500 R, there were severe local reactions in all of the 11 patients treated. There has been one recurrence of the primary lesion at primary radiation site. This study indicates that 2,500 R, in a single dose, exceeds tolerance . The tissue reactions at 2,000 R were those which were expected.

Chemotherapy is a major clinical endeavor of the National Cancer Institute. The principal findings in the last year: (1) demonstration that methylglyoxal-bis-guanylhydrazone is effective in the treatment of acute myeloblastic leukemia; (2) the findings that vincristine was effective in the treatment of acute lymphoblastic leukemia; and (3) the demonstration that a combination of methotrexate and Actinomycin D can raise the remission rate to 75% in choriocarcinoma.

The primary treatment of non-metastasizing trophoblastic disease with chemotherapeutic methods has resulted in complete remission in 13 of 15 patients studied, thus preserving the uterus for further child bearing. In two patients complete remission was achieved by hysterectomy after chemotherapy. A combination of four agents (vincristine, folic acid antagonist, 6-mercaptopurine, prednisone) with the provocative initials VAMP, has produced early remissions in acute leukemia. Other new agents that have been tested are: hydroxyurea, nitrosourea, ethiochloanolone, azauridine, and terephthalanalides.

BIOCHEMICAL AND PHYSIOLOGICAL STUDIES

Immunological

A number of studies are immunologically oriented. These include determination of the chemical structure of the gamma globulins by means of enzymatic and chemical degradation, and a comparison of the three types of gamma globulin associated with myeloma and macroglobulinemia.

Patients who were incurable by conventional modes of therapy but in good general condition have been immunized by intradermal and intramuscular injections of their own frozen killed tumor in Freund's adjuvant. In two of the seven patients studied, there was an inflammatory response with degenerative changes in the tumor cells. The response has also been evaluated by means of skin tests, immunofluorescent studies and the use of various hemagglutination and cytotoxicity methods. Tumor from 10 patients with mycosis fungoides has been injected in a horse and the serum harvested. This serum showed a reaction to mycosis fungoides tumor tissue by immunochemical tests. When this serum was given to 3 patients intratumorally there was necrosis of tumor at the site of injection but no comparable reaction was observed when this material was injected into normal skin of the same patients.

Steroid Chemistry

Gas liquid chromatography has been used for the development of a rapid and reliable quantitation of the chief urinary 17-ketosteroids. It is anticipated that several problems concerning ketosteroid excretion can now be studied. Gas liquid chromatography has also been used to study the excretion of dehydroepiandrosterone. A fluorometric method for the determination of testosterone in small quantities has been developed which makes it possible to measure testosterone secretion rates. A simplified method has been described for the clinical determination of urinary corticoids.

Pyrimidine Metabolism

By measurement of the rate of excretion of pseudouridine and the conversion of Carbon ^{14} labeled orotic acid to pseudouridine, an estimate of total pyrimidine metabolism can be made. This method is comparable in its application to the measurement of urinary uric acid excretion to quantitate purine metabolism. These studies have shown a disproportionately increased pyrimidine synthesis in chronic lymphatic leukemia. 6-Azauridine produces both a large increment in total pyrimidine synthesis and an apparent increase in the synthesis of decarboxylation products from orotic acid.

Pharmacology

To support the chemotherapeutic efforts, a number of pharmacological studies have been carried out. These consist of both toxicological and biochemical pharmacology. Several of the newer agents have been prepared in radioactive form and their disposition in experimental animals and man, and in some cases metabolic fate in terms of conversion to other chemical compounds studied.

Normal Skin

Psoriasis has been shown to be due to a ninefold accelerated proliferation of the epidermal cells, and is due mainly to an expanded population of germinative basal cells per unit area of surface epidermis, rather than due to an increased mitotic rate of the normal complement of germinative cells. In the normal and psoriatic epidermis mitosis is confined to cell layers in immediate proximity to supporting connective tissue. In the hair matrix and in basal cell tumors, mitosis occurs in cells more or less randomly distributed in the epithelial population and in areas comparatively distant to supporting connective tissue.

Lymphangiography

The injection of a contrast medium into the lymphatics of either the foot or the hand and serial X-ray examination of regional areas has provided a technique for visualization of lymphatic channels and lymph nodes in over 200 patients. A high degree of accuracy in the prediction of lymph node involvement in cancer and in the diagnosis of enlarged nodes in patients with lymphoma has been achieved. This technique has proven to be of sufficient value that we are recommending to the Diagnostic Radiology Department that it be adopted as a procedure to be available routinely in their department.

White Cells and Platelets

White blood cells and platelets are being used in large quantities. Studies of methods of pro-

curement and storage are necessary. A centrifuge is being developed to separate white cells and platelets from red cells. A working model has been built and a considerable purification has been achieved. Additional working models are to be constructed and it is hoped that a centrifuge can be built that will provide for the efficient inline extraction of white cells and platelets from whole blood.

Carcinogenesis

Carcinogenesis has been studied in new born rodents and monkeys.

METABOLISM SERVICE

Amino Acid Transport

Studies of amino acid transport in the kidney serve as a model for investigating the problem of cell membrane transfer or transport. The kidney is a convenient tissue to use since transport is a prominent process occurring in this organ.

To develop an animal model system for the study of aminoaciduria, the ability of rat renal cortex slices to concentrate a variety of amino acids has been investigated. Steady state uptake curves for glycine, L-glycine, and alpha amino-isobutyric butyric acid have been fitted to a three compartment model system by means of a digital computer. These three compartments are medium, extracellular space, and intracellular space. Influx and efflux rate constants between these three compartments have been calculated and estimates of the rate of protein synthesis and conversion to carbon dioxide have been made. From these studies it was concluded that the rate of incorporation of those amino acids studied into protein does not depend upon equilibration with the intracellular amino acid pool. Growth hormone did not affect amino acid uptake or protein synthesis. No difference was observed in kidney tissues derived from normal and hypothesectomized rats.

Maleic acid, a compound which has been noted to produce aminoaciduria, glycosuria and phosphaturia in intact rats has been studied by *in vitro* techniques. Results of these *in vitro* studies corroborate *in vivo* findings. This suggests that maleic acid interferes with intracellular energy yielding processes required by active transport systems, rather than by competing with amino acids for membrane transfer. The effect of maleic acid could be prevented by the addition of sulfhydryl groups. Although sulfhydryl groups had this effect, evidence for maleic acid exerting its effect by sulfhydryl binding could not be obtained and to the contrary—the maleic acid effect could be reversed by transfer to media not containing the acid. With labelled maleic acid, specificity appeared to depend upon its failure to penetrate the cellular compartment in either muscle or intestine.

A patient with elevated blood histidine and histidiunria level failed to excrete increased quantities of any other amino acid in the urine. The other members of the family who were studied had completely normal urinary amino acid excretion. These studies indicate that histidine failed to compete with other amino acids for tubular reabsorption.

Gamma Globulin Structure

Most of the known functions of the immune system are effected by gamma globulin molecules. The structure of gamma globulin molecules is being investigated in an effort to relate molecular structure to molecular function and to the genetic and biochemical properties of plasma cells. The gamma globulins, however, are unique among protein systems in being very heterogeneous. Therefore, careful separation of the major normal gamma globulins subgroups, study of the less heterogeneous myeloma proteins and Bence-Jones proteins, and dissection of purified proteins into the substituent polypeptide chains was undertaken. Because of the complexity of the gamma globulins, terms such as 6.6Sγ, β_2A and γ_1-microglobulins are used to describe different classes of globulins and S and F pieces and "L" and "H" chains to describe subunits.

Subunits of gamma globulin molecules were obtained by enzymatic fragmentation (S and F pieces) and by chemical reduction and alkylation ("L" and "H" chains). "L" chains represent approximately 25% of the gamma globulin molecule. Polypeptide chains of the "L" type were identified in all four classes of gamma globulins, i.e., in the 6.6S gamma globulins, β_{2A}-globulins, and γ_1-macroglobulins of serum and in the gamma-microglobulins of normal urine. In addition to normal gamma globulins, all of 140 myeloma proteins, macroglobulins and Bence-Jones proteins

formed in malignant plasma cells were found to have typical "L" polypeptide chains. Whether a protein was a 6.6S gamma globulin, a β_{2A}-globulin or a γ-macroglobulin was determined by the properties of the H chains, represented in the F piece.

The Bence-Jones proteins were shown to be antigenically and electrophoretically identical to "L" polypeptide chains obtained from myeloma proteins or macroglobulins of the same patient. Other subunits of myeloma protein, represented in the H chains and F pieces, however, were unrelated to Bence-Jones proteins, and it appears reasonable to regard Bence-Jones proteins as excessive quantities of "L" type polypeptide chains.

Two types of "L" chains were identified in all classes of normal gamma globulins. The Type I and II "L" chains differ markedly in antigenic determinants and indicate differences in structural configuration of these two types of polypeptide chains. Approximately 65% of gamma globulins have Type I "L" chains, and 35% have Type II "L" chains. Bence-Jones proteins are either Type I or II polypeptide chains and, similarly, myeloma proteins and macroglobulinemic macroglobulins have either Type I or II, but not both types of "L" chains. Type I and II molecules appear to be synthesized in separate cell clones.

The observations on gamma globulin structure, especially on Bence-Jones proteins and myeloma proteins, indicate several characteristic features of plasma cells. Plasma cells normally synthesize polypeptide chains in two forms ("L" chains and "H" chains). In man, two types of "L" chains and three types of "H" chains have been identified. Clones of malignant plasma cells synthesize only one of the two types of "L" chains and one (or none) of the three types of "H" chains. No malignant plasma cell clone forms all types of polypeptide chains. It seems likely that normal plasma cell clones are similarly limited, and that the normal plasma cell population is heterogeneous in respect to its capacity to synthesize gamma globulin molecules.

Gamma Globulin Metabolism

A combination of factors made possible a number of studies of gamma globulin metabolism. These were (1) the capacity to prepare purified gamma globulin fractions (i.e., normal 6.6S gamma globulins, normal 18S gamma$_1$-macroglobulins and Bence-Jones proteins), (2) the capability of measuring the serum levels of individual groups of gamma globulins, (3) the capacity to label purified proteins with I^{131} without damage, and (4) availability of patients with a wide variety of changes in the gamma globulin components and of inbred strains of mice with protein producing plasma cell tumors.

The metabolism of 6.6S gamma globulins (mol. wt.=160,000) was established in subjects with normal gamma globulin levels. Forty-four percent of the total body 6.6S gamma globulin was intravascular and the total body gamma globulin content was approximately 1.22 grams per kilogram of body weight. The mean half time of gamma globulin survival ($T\frac{1}{2}$) was 23 days and the fractional degradation rate was 3.0 percent of the body pool per day. The turnover rate for 6.6S gamma globulin was 0.036 grams per kilogram of body weight per day, i.e., approximately 1/7 that of albumin.

In patients with multiple myeloma and large amounts of gamma-myeloma proteins the rate of gamma globulin catabolism was increased (with reduction in $T\frac{1}{2}$ to 11 to 17 days) in 7 of 11 patients, indicating that increased catabolism contributed to the hypogammaglobulinemia of these patients. In six patients with macroglobulinemia and four patients with Bence-Jones proteinuria, serum β_{2A}-myeloma protein or no anomalous protein, the serum and total body gamma globulin levels typically were reduced. The survival of gamma globulin was normal or prolonged (mean $T\frac{1}{2}$=30.5 days) and the calculated synthetic rate was reduced (mean value=0.024 g/kg/day) indicating that impaired gamma globulin formation was the major cause of hypogammaglobulinemia in these patients.

Two characteristic features of gamma globulin metabolism were defined by studies in mice and in man. Gamma globulin catabolic rate was shown to depend on the serum gamma globulin level. The fractional rate of gamma globulin catabolism decreased as gamma globulin levels fell, and increased as gamma globulin levels increased. A maximum rate of gamma globulin catabolism was approached as gamma globulin levels rose above 3 grams percent in both mouse and man, indicating a limit to the flexibility of gamma globulin catabolic processes. The factors determining gamma globulin catabolism were shown to be highly selective, i.e., specific for 6.6S

gamma globulin and not responsive to changes in the serum levels of the closely related proteins formed in plasma cells (gamma$_1$-macroglobulins or β_{2A}-globulins) or of serum albumin. These observations indicate that a specific site on the gamma globulin molecule is responsible for removal of 6.6S gamma globulins. They also indicate that a specific homeostatic mechanism determines gamma globulin catabolism and contributes to the maintenance of normal serum gamma globulin levels.

Albumin Metabolism

There is little definitive knowledge of the factors that control the rate of catabolism of the serum proteins. Conflicting evidence suggests that the gastrointestinal tract, the liver, or even the kidney and reticuloendothelial system play the major roles in the catabolism of albumin. An increase in metabolic rate, fever, and cortisone administration accelerates the catabolism of albumin while a diet extremely deficient in nitrogen prolongs albumin survival.

The distribution and metabolism of the serum proteins depends on both host factors and the nature of the protein. In an effort to evaluate the importance of host factors, the metabolism of a single preparation of I^{131} labeled mouse albumin was studied in mice, rabbits, dogs and sheep. The mean survival T½ of mouse albumin was 1½ days in mice, 6 days in rabbits, 10 days in dogs, and 15 days in sheep, equivalent to the survival of autologous albumin in these species. This suggests that host factors play the major role of albumin survival in these species.

The catabolic rate of a given protein can be modified without altering the survival of the remaining proteins, suggesting that the individual proteins have unique catabolic pathways. Specifically, it was shown that the survival of normal 6.6S gamma globulin was 22–26 days T½ in control subjects and 30–70 days in patients with agammaglobulinemia, while the survival of I^{131} albumin was 17–20 days in both groups. A comparable situation was seen with serum albumin. In two analbuminemic patients, there was a markedly prolonged survival of I^{131} albumin but a normal survival of I^{131} gamma globulin.

In addition to the specific routes of catabolism for proteins nonspecific factors such as loss into the gastrointestinal tract appear to be a major factor in the normal catabolism of the protein, as well as the pathogenesis of the hypoalbuminemia seen in association with a variety of disorders. Intravenously administered Chromium^{51} labeled albumin has been developed as a technique for the demonstration of gastrointestinal protein loss. It was shown to be a simple technique of value in the quantitation of protein loss, detection of its site and the early determination of therapeutic effectiveness of agents for the treatment of gastrointestinal disorders. The major disadvantage of the technique lies in the short intravascular survival of the Chromium^{51} labeled albumin. Of advantage over I^{131} PVP are the nonabsorbability of the label, the ability to label a variety of serum proteins, and the greater ease of differentiation of normals from patients with excessive gastrointestinal protein loss. The use of I^{131} albumin in conjunction with oral amberlite IRA 400 resin has been shown to be inadequate since the basic assumption of this technique that iodine be lost into the gastrointestinal tract only bound to albumin is not met. When NA I^{131} was given intravenously and the resin and Lugol's solution by mouth, 40–50 percent of the injected dose appeared in the subsequent stool collections. Turnover studies using I^{131} labeled 6.6S and gamma macroglobulins indicate that these proteins are lost into the gastrointestinal tract in those patients with albumin loss into the gut. In the case of 6.6S gamma globulins as with albumin, there is only a relatively small increase in the synthesis rate of the protein despite exceedingly low serum concentrations. Gastrointestinal protein loss has been demonstrated, in cooperation with physicians of NHI and NIAMD, in patients with Whipple's disease, giant rugal hypertrophy, sprue, constrictive pericarditis, and congestive failure, agammaglobulinemia in chronic pancreatitis, celiac disease, and nephrotics as well as in two disorders previously described in idiopathic hypercatabolic hypoproteinemia. A syndrome including intestinal protein loss has been noted in six children with extreme eosinophilia and an allergic diathesis. Intestinal X-ray abnormalities were not significant. Intestinal biopsy examinations showed only eosinophilia of the mucosa. Two of these patients were given steroids and had a complete remission. Two additional patients not treated with steroids responded completely, or in part, to elimination of dietary allergens, presumed to be milk proteins. In a

majority of the patients that were previously described as idiopathic, hypercatabolic hypoproteinemia abnormalities of the intestinal lymphatics have been shown to be the major associated factor. This disorder of the lymphatics appears to be generalized rather than limited to the intestinal tract since some of the patients had associated chylous effusions and lymphedema, or abnormalities of the lymphatic channels as shown by lymphangiography. The abnormalities demonstrated by lymphangiography included the apparent absence of lymph nodes in the abdomen of one patient with an 11-year history of chylous ascities beginning at birth.

The differentiation of gastrointestinal protein loss from failure of protein synthesis appears to be exceedingly important since in 23 of the 50 cases studied, there has been a reversal of the intestinal protein loss on appropriate therapy. Specifically, surgical resection has been shown to be of value in pericarditis and by others in patients with gastric carcinoma, giant rugal hypertrophy, and one case of intestinal lymphangiectasis. Corticosteroids have been shown to be of value in the treatment of celiac disease, regional ileitis associated with agammaglobulinemia, and the allergic disorder associated with eosinophilia, while oral antibiotics were of value in patients with Whipple's disease and agammaglobulinemia and a patient with chronic salmonellosis.

Immunological Studies

Two lines of laboratory work have been developed. The first was the preparation of purified normal gamma globulins of each class, i.e., 6.6S gamma globulins, β_{2A} globulins, and γ_1-macroglobulins from normal serum and gammamicroglobulins from normal urine, as well as the purification of Type I and II Bence-Jones proteins, gamma-myeloma proteins, β_{2A}-myeloma proteins and gamma$_1$-macroglobulins. The purified proteins have been used for a variety of metabolic and structural studies on gamma globulins. The purified proteins made possible a second line of laboratory work, i.e., the preparation of polyvalent antisera reacting with all gamma globulin groups and of specific antisera reacting only with 6.6S gamma globulins, β_{2A}-globulins, gamma-macroglobulins and Type I or Type II gamma globulins. The availability of specific antisera has made possible:

(1) Simplified and more rapid diagnostic tests for multiple myeloma and macro-globulinemia.

(2) Identification and morphological characterization of the cells forming 6.6S gamma globulins, β_{2A}-globulins or γ_1-macroglobulin levels.

(3) Quantitative methods for the specific measurement of serum 6.6S gamma globulin, β_{2A}-globulin, and γ_1-macroglobulin levels.

Clinical studies in patients with macroglobulinemia and multiple myeloma revealed both groups to have an increased susceptibility to bacterial infection. The multiple myeloma patients had a significantly higher rate of infection than was found in the macroglobulinemic patients but no differences were found among myeloma patients forming gamma-myeloma proteins, β_{2A}-myeloma proteins or Bence-Jones proteins. Both myeloma and macroglobulinemia patient groups were equally poor in their capacity to produce antibodies in response to antigen administration, and all had reduced levels of normal gamma globulin components reflecting generalized impairment of normal immune mechanisms.

Further study of the usefulness of plasmapheresis in the therapy of hyperglobulinemic hyperviscosity syndromes revealed that arithmetic increases of serum macroglobulin level were accompanied by logarithmic increases in serum viscosity. This laboratory evidence provided a theoretical basis for the rationale of intensive plasmapheresis in patients with macroglobulinemic (or other) hyperviscosity syndromes.

Nucleic Acid and Pyrimidine Metabolism

Little knowledge was available until recently concerning quantitative aspects of human pyrimidine metabolism. This laboratory has been engaged in efforts to utilize urinary pseudouridine as a tool for investigation of pyrimidine metabolism, in a fashion somewhat analogous to uric acid in purine metabolism.

The rate of pyrimidine production was estimated to be increased 2–3 fold in patients with chronic myelogenous leukemia. These patients were then given 6-azauridine and C^{14} carboxyl-labelled orotic acid at various times after initiation of the therapy. By determining radioactivity in respiratory CO_2 and urinary orotic acid it

was shown that the rate of pyrimidine production was further increased several fold. The data suggest there may actually be an increase in the production of uridine nucleotides and that a block in conversion from orotic acid to uridine nucleotides might not be the only site of action of 6-azauridine. Gouty patients who were given labelled orotic acid showed no abnormality corresponding to the early purine shunt seen in this disease. C^{14}-labelled orotidine was prepared and evidence obtained that it is metabolically inert in man.

Urinary pseudouridine excretion was measured in patients under dietary control and compared with urinary uric acid and creatine. There was inconsistent creatinuria in both chronic myelogenous leukemia and chronic lymphocytic leukemia patients. In agreement with reported data, the urinary uric acid levels were normal in patients with chronic lymphocytic leukemia and increased in chronic myelocytic leukemia. However, the chronic lymphocytic leukemia patients showed a very substantial elevation of urinary pseudouridine. Isotopic studies with orotic acid indicate this excessive pseudouridine excretion is a reflection of generalized pyrimidine overproduction by these patients. Isotopic studies with uric acid further indicate that the low urinary uric acid is not caused by an increased degradation of uric acid. Measurements of acid soluble and insoluble nucleotide containing compounds of lymphocytic leukemia lymphocytes failed to indicate a chemical preponderance of pyrimidines to account for the urinary findings.

The enzyme systems converting thymidine monophosphate to triphosphate have been investigated further. Although unusual mechanisms for this conversion have been suggested, our results tend to indicate that the conversion proceeds through the thymidine diphosphate and a separate enzymatic activity is responsible for conversion to the triphosphate.

An enzyme system incorporating uridine triphosphate into acid insoluble polynucleotide material from ribosomes has been identified. This material has been solubilized, partially freed of nucleic acid, and purified about 20-fold. Two additional enzymes from the cytoplasm of E. coli have been identified and separated. Each has been freed of the bulk of the contaminating nucleic acid and purified approximately 20-fold. One incorporates guanosine triphosphate into polynucleotide material, the other will incorporate uridine triphosphate into polynucleotide material in the presence of either DNA or RNA.

In relation to studies on protein synthesis some purification has been achieved of the rather unstable enzyme from mammalian cytoplasm of rat and rabbit (liver and thymus) converting guanosine triphosphate through the diphosphate to the monophosphate. There is a possibility that this enzyme is related to the early stages of protein biosynthesis but this situation remains to be clarified.

Studies performed with HeLa cells indicate that under very precise control conditions, it is possible to obtain a stimulation of uptake of radioactive phenylalanine into protein by exposing the cells to polyuridylic acid.

Studies of Erythropoietin

An improved and more sensitive assay system for erythropoietin utilizing radioiron incorporation into red cells of the polycythemic mouse has been developed. Studies of erythropoietin have been directed toward the following: factors stimulating production of erythropoietin, its chemical nature, site of production, and metabolic rate. In mammals, bleeding, anoxia, and cobalt administration are potent stimuli for erythropoietin production. The mechanisms of action of the fundamental stimulus to erythropoietin production remains unknown. Mice given intraperitoneal injections of homologous red cells and large quantities of dextran showed a simultaneous increase in the total red cell volume and reduction in the peripheral hematocrit and a marked depression of erythropoiesis. This suggests that the total body content of red blood cells rather than the concentration in the peripheral blood is a controlling factor in erythropoietin production. In the frog bleeding of one-third the blood volume resulted in a marked stimulation of erythropoiesis. When frogs were maintained in a hypoxic atmosphere, there was no stimulus to erythropoiesis, indicating that in this species hypoxia is not a potent stimulus of erythropoiesis. The frog did not respond to cobalt or to human erythropoietin. However, the frog does produce an erythropoietinlike material since serum from anemic frogs contains a factor stimulating erythropoiesis in recipient frogs.

The erythropoietin activity in the serum of anemic subjects from a cerebellar tumor and renal

cysts was nondialyzable, was not soluble in lipid solvents and was inactivated by trypsin and sialidase. On electrophoresis the activity migrated with the alpha$_2$ globulins. The molecular weight was estimated at 25–30,000. The erythropoietic activity was neutralized by antibodies produced in the rabbit to a human erythropoietin extract. These studies indicate that these erythropoietins derived from different sources were similar.

Measurable levels of erythropoietin were generally found in patients with a hemoglobin content less than 8 grams percent except for those with extreme renal damage. Erythropoietin was not found in patients with polycythemia vera or relative polycythemia. It was found in 50 percent of patients with polycythemia secondary to anoxia and in 30 percent of patients with polycythemia secondary to tumors. The rate of catabolism of erythropietin in man was measured by determining the rate of decrease of erythropoietin activity following transfusions of aplastic anemic patients to hemoglobin levels sufficient to decrease erythropoietin production. In general, it has been found that at hemoglobin levels above 10 grams percent that there is no detectable serum or urinary erythropoietin. When patients were transfused to this level, the half time of the rate of decrease of erythropoietin activity varied from 11 to 35 hours in five patients, and in one patient was approximately 100 hours. Loss of erythropoietin activity in the urine did not contribute to the decline in erythropoietin serum titer.

The adrenal cortical tumor 494 carried in Osborne Mendel rat is associated with an increase in hematocrit in 60–70% and 100–200% increase in total circulating red cell volume. A saline extract of the tumor produced a small but statistically significant erythropoietic stimulation in the mouse. Administration of androgenic cortical steroids and an extract of normal adrenal gland did not produce such stimulation.

Effects of Metabolic Rate on Erythropoiesis

The effect on erythropoiesis of changes in the metabolic rate was studied in the dog, frog, and alligator. In the hypermetabolic dog, there was a 25% increase in the total red cell volume and rate of red cell synthesis, but no change in the red cell life span. In the hypothyroid dog there was a 40% decrease in the total red cell volume with a concomitant decrease in the rate of synthesis of the red cell, but no change in red cell life span. Thus in the dog the effects of changes in metabolic rate are in the rate of erythropoiesis and total red cell volume and not on the red cell life span. In poikilothermic animals, there was a significant alteration in the red cell life span. In the frog taken from 20° to 4° C., there was an increase in the red cell life span from 125 to 250 days accompanied by a considerable decrease in the rate of erythropoiesis. A similar change was seen in the alligator again with a marked decrease in the rate of erythropoiesis.

Means of Measuring Red Cell Life Span

Chromium ^{51} is widely used to measure red cell survival. The chromium content of the blood decreases for two reasons: (1) elution of isotope from intact surviving cells; and (2) removal of senescent cells. Measurement of the rate of elution of chromium in patients with hematologic disease showed that this varied from 0.57 to 2.27% per day. This wide variation in the elution rate makes the use of Chromium ^{51} as a means of measuring red cell life span less satisfactory than isotopically labelled diisopropylfluorophosphate. The reticulocyte count was compared to the rate of production of red cells as measured both with radioactive iron and by measurement of the life span of the red cell. A coefficient of variation of 0.70 was observed between reticulocyte count and rate of formation of red cells. However, in various specific instances, the deviation of the reticulocyte count from that predicted was large. It was concluded that the reticulocyte count remains a clinically useful tool but cannot be used to predict the rate of formation of red cells.

Diisopropylfluorophosphate labelled with both P^{32} and tritium can be used to measure the red cell lifespan. The use of the same chemical but with different isotopic markers permits the measurement of the survival of two populations of red cells and thus the differentiation of intra- from extra corpuscular factors leading to premature cell death. In the normal individual, the red cell life span of *in vivo* labelled and *in vitro* labelled donor cells was normal although the survival of the autochthonous cells was slightly longer than the homologous cells. Two types of variations were observed in disease. In some patients, the donor and recipient cells were removed at approximately equivalent rates. In other patients, the

recipient cells were removed at an accelerated rate while donor cells had a normal lifespan. In the former group there was some extracorpuscular factor leading to red cell shortening, and in the latter group, some intracorpuscular factor.

Ineffective erythropoiesis can be defined as the production of red cells which are either destroyed in the marrow or very shortly after arrival in the blood. For some time it has been known that following the administration of isotopically labelled glycine there was a peak of incorporation of isotope into bilirubin at 3 to 5 days, and again a second peak at 120 days. In the normal, the second peak represents the bilirubin derived from the catabolism of the hemoglobin in senescent cells. The source of the early peak was unknown, but thought to be associated with erythropoiesis. In two patients with failure to produce red cells, this early peak was almost entirely absent indicating that in large measure if not entirely, this early peak is associated with erythropoiesis.

Porphyrins

Studies of the biochemical lesions of porphyria in patients and in experimental animals have been continuing. The major goal of this project is to find the biochemical defect causing porphobilinogenuria in both experimental animals and in human acute intermittent porphyria. Four major metabolic pathways have been studied which could, in theory, cause an increased excretion of porphyrin precursors. The only one of these which appeared to be involved in experimental porphyria was the oxidation of glycine to carbon dioxide. Glycine oxidation can occur by at least four well defined pathways: (1) conversion to delta aminolevulenic acid; (2) conversion to aminoacetone; (3) conversion to serine, and (4) conversion to glyoxalate. Certain aspects of the first three pathways have been studied.

The synthesis of ALA has been shown to occur in very small quantities in isolated mitochondria from normal liver. This activity is markedly increased in experimental porphyria. This pathway, therefore, cannot explain the decreased oxidation of glycine. Aminoacetone is formed from the condensation of glycine and acetyl CoA. The enzyme catalyzing this reaction is normally present in such quantity that the capability for formation of aminoacetone from glycine greatly exceeds the capability for formation of ALA. A deficiency of this enzyme in porphyria might explain many of the chemical phenomena of porphyria. The amount of this enzyme present in the livers of porphyric rats is normal. Another source of aminoacetone which is probably a more important source than glycine is thereonine. This enzyme pathway is not altered in experimental porphyria but is markedly decreased in the liver of tumor-bearing rats.

In experimental porphyria, diet has a profound effect. This has been demonstrated in man by alteration in the excretion of amino-levulinic acid and porphobilinogen. The mechanisms involved are not known. Administration of estrogens increases porphobilinogen excretion in patients. In several female patients porphyria attacks could be related to the menstrual cycle. These patients have been treated successfully with estrogenic and androgenic materials.

A continuing search for the mechanism of decreased amino-levulinic acid dehydrase in livers of tumor-bearing animals fails to reveal conclusive evidence of a material in tumors which can be extracted and produce this change in recipient normal animals.

The mechanism of the lowering of liver catalase is different from that of lowering ALA dehydrase since "toxohormone" does not cause a decrease of ALA dehydrase and a decrease in catalase may precede that of ALA dehydrase. Injection of cortisone causes a decrease in hepatic ALA dehydrase activity and adrenalectomy causes an increase. The possibility that the decreased ALA dehyrase in the livers of tumor-bearing animals might result from increased adrenal activity was investigated by growing tumors in adrenalectomized animals. Adrenalectomy did not prevent the decrease of hepatic ALA dehydrase produced by tumors. The oxidation of thereonine to aminoacetone is profoundly decreased in the livers of animals bearing large tumors.

DERMATOLOGY BRANCH

The clinical and laboratory research activities of the Dermatology Branch continue to be concerned with two major areas; namely, (1) study of normal and abnormal growth and differentiation of the epidermis and related epithelium and (2) study of the lymphomatous disease mycosis fungoides.

1. Epidermal Growth and Differentiation

Recent work in this Branch has established that mitotic division of epidermal cells is normally *confined* to the single layer of cells in direct contact with the underlying connective-tissue corium. *In vivo* studies measuring the time of appearance of C^{14}-glycine in proteins shed at the skin surface have indicated that the epidermal turnover time is 28 days. Since the epidermis is approximately 28 cell layers in thickness, the inference is made that the basal cell layer reduplicates itself one-fold each day, and that the transit time of a newly-formed cell, from its site of production, to the epidermal surface is also approximately 28 days. During its transit at least three distinct fibrous (keratinous) proteins are produced, each at a different level within the epidermis since these proteins, labelled with C^{14}-glycine reach the surface at different times. (See Project #3602, 1962.)

In *psoriasis*, the germinative basal-cell population has been found to be increased to three cell layers, and is further increased by another factor of 3 by folding of the epidermal-dermal line. Thus the theoretical transit time of newly formed cells, from the basal zone to the epidermal surface in psoriasis may be mathematically calculated to be 3–4 days. *In vivo* studies, measuring the time of appearance of C^{14}-glycine-labelled proteins at the epidermal surface confirms this. The epidermal hyperplasia in psoriasis therefore seems mainly due to a ninefold expansion of germinative basal cells, rather than due to increased mitotic rate of a fixed number of basal cells. Faulty maturation of epidermis in psoriasis seems secondary to marked decrease in time allowed for maturation of cells before they are shed.

In the *normal hair root*, the entire root matrix is comprised of germinative cells, which in large scalp hairs may constitute as many as 30 cell layers. Daily reduplication of this large cell population accounts for the rapid growth "rate" of hair.

Further studies in this Branch indicate that a *basal cell tumor* arises because of inability of its constituent cells to produce normal fibrous keratin proteins. A major protein, found in normal epidermis, is not found in this tumor, a finding corroborated by our earlier studies and recently by an electron microscopic study from another institution which has shown the absence of structural fibrous proteins in basal cell tumor cells. A basal cell tumor hence forms from basal cells which are unable to pass through the normal epidermis via the process of keratinization, and which, by retaining their normal capacity for mitotic division, enlarge into ever increasing masses below the epidermis.

The role of normal connective tissue environment in maintaining normal biologic behavior of epithelial cells, and the influence of altered environments, have been further analyzed. Human epidermal cells, removed from their normal environment and cultured *in vitro* (Laboratory of Biology, NCI) appear to have lost normal behavior capabilities (or gained abnormal capabilities). Freshly removed epidermis or epithelium, autoimplanted to new sites in the integument, responds in predictable normal patterns. Implanted culture cells, on the other hand, reveal no evidence of differentiation into any of the normally expected patterns and have histologic appearances of undifferentiated cancer cells.

2. Mycosis Fungoides

Evaluation of the status of immunologic reactivity of patients with mycosis fungoides has continued.

Skin tests with a series of antigens have demonstrated that a significant depression of delayed sensitivity is characteristic of mycosis fungoides only late in the course of the disease. The homograft rejection mechanism is intact. In patients who have well-defined areas of cutaneous involvement a provocative pattern of acceptance and rejection of *autografts* has been noted. Normal-to-normal, involved-to-involved, and normal-to-involved transplants take in the usual fashion. Approximately 75% of involved-to-normal transplants are rejected in what appears to be a typical delayed reaction characteristic of the response seen to homografts.

A horse has now been injected 15 times with homogenized tumor from 10 patients with mycosis fungoides. Serum from the horse has a band on Ouchterlony plate against mycosis fungoides tumor tissue. There is also a band against serum from normal patients and to serum from patients with mycosis fungoides. Complement fixation tests against mycosis fungoides tissue from three different patients are posistive; normal skin does not absorb out this antibody with techniques employed thus far.

When the horse serum is run against normal human serum in immunoelectrophoresis there is a good band to albumin, alpha$_2$ and beta globulins and a poor band to gamma globulins.

Three patients with mycosis fungoides have received intratumoral injections with this serum. In each there has resulted necrosis of tumor at the site of injection. No such reaction occurs in uninvolved skin of these patients when injected with the serum, nor in normal skin of patients without mycosis fungoides.

ENDOCRINOLOGY BRANCH

This year has permitted the Endocrinology Branch to fully set up an independent research program for two recently acquired senior investigators. Moreover, these individuals have been effectively integrated into our patient-care program in such a way that each of our senior investigators now accept major clinical responsibilities for only 4 months at a time and then has 8 consecutive months for continuous research effort. Meanwhile, the overall coordination of clinical and research functions continues to be worked out through daily informal discussions and through our weekly grand rounds.

It is gratifying to note that whereas only one of last year's clinical associates elected to remain for a third year, two of this year's group have already requested a third year in research. We anticipate that these third year men will greatly enhance our research effort in coming years and will provide potential recruits for future staff positions.

Two highly practical accomplishments in the field of the chemotherapy of choriocarcinoma and related trophoblastic tumors in women seem to stand out for this year. The first is the successful application of chemotherapy as a primary form of treatment in nonmetastatic trophoblastic disease. It may be anticipated that thousands of young women in coming years may be spared the loss of their reproductive capacity by this form of nonsurgical treatment. Moreover, in principle, this represents the first instance of the substitution of chemotherapy for surgery in the effective and definitive management of malignant disease.

The second noteworthy development is the statistically valid establishment of the effectiveness of Actinomycin D in the methotrexate-resistant patients, thus increasing the expected complete remission rate from 47% to 76%, as manifested by the last 38 patients with metastatic trophoblastic disease admitted for study.

At a more basic level, the studies of our steroid group have provided exquisitely sensitive tools for the isotopic analysis of the metabolic behavior of numerous vital steroid substances whose role in malignancy and in related disease processes has hitherto remained obscure. This newer methodology may be expected to elucidate many of the problems in this area.

The development of isotopic methods for tracing one of the most potent of biologically active trace elements, namely biotin, may also be regarded as a significant methodological accomplishment in a most difficult area of immediate pertinence to the cancer problem.

Basic studies on the endogenous and exogenous factors involved in hormone-induced tissue growth provide essential background data for new approaches to the control of normal as well as neoplastic growth in hormone-sensitive tissues. The development of newer pharmacologically active agents which can alter such growth processes continues to demand our best efforts.

On the whole, it may be concluded that the free pursuit of basic information combined with a readiness to exploit such data in a practical manner may be expected to provide research developments of value and interest.

MEDICINE BRANCH

A comprehensive nationwide approach to the study of the treatment of acute leukemia has been undertaken by the Leukemia Task Force. The emphasis as regards chemotherapy is in the following areas: the rapid introduction into the clinic of agents which show significant activity in transplanted rodent tumor systems; the effective performance of Phase I therapeutic trials in acute leukemia; quantitative clinical trials to determine the relative efficacy of agents as regards their ability to induce and maintain remissions; and a comprehensive analysis of animal screens to determine which correlate most closely with the clinical data. The Medicine Branch has participated in a major way in the planning, execution, and evaluation of a number of these studies.

New agents which have proven effective in the treatment of acute leukemia during the past two years include vincristine, Cytoxan, and guanylhydrazone. Vincristine produces remissions

rapidly in 50–60% of children with acute lymphocytic leukemia. A coded study wherein vincristine is compared to a placebo in the maintenance of vincristine induced remissions is nearing completion. Preliminary analysis would suggest that vincristine is not effective in maintaining remission (Karon). Guanylhydrazone produces complete remissions in 30–50% of patients with acute myelogenous leukemia (Freireich). The toxicity of this agent limits its usefulness. Attempts to improve the therapeutic index are underway but have, to date, not proven successful. These include (1) combinations with other agents, (2) modification of route and dose schedule, and (3) study of congeners of guanylhydrazone. (See below.)

Pharmacologic and animal tumor studies indicate that the schedule of Methotrexate (MTX) administration to man should be important in achieving maximum therapeutic effect. In 21 patients with acute leukemia, 18 (86%) achieved remission with MTX administered every 4 days as compared to 30% for conventional daily administration (Freireich).

New agents currently under study in acute leukemia in the Medicine Branch include hydroxyurea, the terephthalanalides, CB–1506, nitrosourea, azauridine, and etiocholanalone.

During the past several years the Medicine Branch has increased its program in the study of patients with chronic leukemia, lymphoma, and myeloma and has decreased the number of beds allotted to patients with "solid" tumors. In order to continue our program in the solid tumor area, a 15-bed chemotherapy unit has been established at the USPHS Hospital in Baltimore under Dr. N. Tarr, their Chief of Surgery, and the Medicine Branch, NCI.

There are a number of agents of established efficacy in chronic myelogenous leukemia (CML). New agents which we have found to be effective in these patients include azauridine, hydroxyurea, and vinblastine (Carbone). One of the most important problems in the treatment of CML is that, though a number of agents are capable of inducing and maintaining remissions, survival has not been significantly prolonged. In an effort to define further this problem, an analysis of the effects of treatment with conventional agents (6-mercaptopurine, Myleran, colcemid) on the hematological, biochemical and cytogenetic abnormalities in these patients has been undertaken. The cytogenetic aspects of this problem were particularly important because a chromosomal marker, the Philadelphia chromosome, has been observed in the majority of patients with CML and does not occur in other neoplastic diseases. It has been found that 90% of patients will have effective reduction of the white blood cells and organomegaly; 40–50% of patients will in addition have return of their bone marrow to "normal"; 40% of patients will have return of the white cell alkaline phosphatase to normal; but in no patient, regardless of the extent or duration of remission, does the Ph′ chromosome disappear from the marrow. Almost invariably 100% of marrow metaphases have the chromosome prior to treatment and no reduction occurs in spite of otherwise effective treatment. If the Ph′ chromosome marks the malignant cell it is clear that the above treatment is only palliative, i.e., the majority of the leukemic cells are destroyed but some always remain. The implication of this as regards experimental therapy of this disease will be discussed (see below) (Block, Carbone).

New agents recently found to be active in Hodgkin's disease and lymphosarcoma include vincristine, vinblastine, and guanylhydrazone. The first two produce regressions in 50–80% of these patients and thus compare with the alkylating agents. Preliminary studies indicate that they are not cross-resistant clinically with each other or with the alkylating agents (Carbone). Remissions produced with guanylhydrazone are often complete but transient and occur at considerable cost in toxicity. The fact that there are now a number of effective therapeutic modalities for the lymphomas (X-ray, alkylating agents, periwinkle alkaloids, corticosteroids, guanylhydrazone, and methotrexate) indicate that a more comprehensive and disease oriented approach to therapeutics is desirable.

Most of the Medicine Branch studies in patients with "solid" tumors hahve been performed in collaboration with the Eastern Solid Tumor Group. Comparative studies of Cytoxan, uracil mustard, and nitrogen mustard have failed to show significant differences for these agents in patients with solid tumors or lymphomas. A coded study of fluorouracil, fluorodeoxyuridine, and methotrexate in patients with carcinoma of the breast and colon indicate some differences between these

agents in terms of therapeutic index. Preliminary studies of vincristine, vinblastine, guanylhydrazone, hydroxyurea, etiocholanolone, nitrosurea, and tryptophan mustard have shown limited activity for vincristine and vinblastine in cancer of the breast and suggestive activity in certain other solid tumors (Perry, Carbone).

Phenylalanine mustard affords objective benefit to 30% of patients with multiple myeloma. In a coded study performed by the Eastern Solid Tumor Group, there was no difference between a placebo and urethane in 100 patients with multiple myeloma. In this study objective improvement of any sort occurred in less than 5% of the patients. This study affords considerable information concerning the nautral history of myeloma and thus serves as a frame of reference for future studies.

In addition to the above, studies involving the following therapeutic approaches are underway:

1. Remission Induction

Since complete remissions can now be induced with a number of agents in the acute leukemias, lymphomas, and chronic leukemias, combinations of these effective agents used intensively during remission, induction and early maintenance might be curative. Preliminary studies of vincristine, prednisone, methotrexate, and 6-mercaptopurine used in combination in patients with acute leukemia, and combinations of nitrogen mustard and vincristine in patients with lymphoma have yielded promising results (Frei, Freireich, Rall, Carbone).

2. Remission Maintenance

The development of resistance of malignant cells to chemotherapeutic agents is a major problem and limits current clinical cancer chemotherapy. Approaches to control of this problem include combination chemotherapy studies involving the use of MTX and 6-mercaptopurine both concurrently and alternating at monthly intervals in maintaining acute leukemia remissions. Preliminary analysis suggests that the alternating maintenance therapy does not prolong remission. The sequestration of leukemic cells in areas not accessible to antileukemic agents (brain and meninges, and questionably, thymus) may serve as a focus for the development of the resistant cell. In a controlled study of remission maintenance the use of intrathecal aminopterin prophylactically throughout the remission has failed to prolong remissions (delay the development of resistance) (Frei). Autopsy studies of patients with meningeal leukemia indicate that, though intrathecal aminopterin markedly reduces the number of leukemic cells on the meninges, it does not eliminate them (Thomas). The nitrosourea derivatives are active against intracranial as well as subcutaneous L–1210 leukemia in mice. This drug has physiocochemical properties consistent with distribution into the central nervous system. Finally, preliminary studies in man indicate that orally administered nitrosourea is effective in treating meningeal leukemia.

Cytogenetics

The specificity of the Ph′ chromosome marker for the chronic myelogenous leukemia (CML) cell is established and 90+% of patients with CML have this change (Carbone, Whang). It is present not only in myeloid tissue but also in nucleated red blood cells and probably megakaryocytes (Whang). If the Ph′ chromosome marks the neoplastic cell then all three cellular elements of the marrow are malignant and presumably the neoplastic change occurs in a stem cell. Attempts to identify this cell are underway. Cytogenetic studies of acute leukemia have yielded varying but inconsistent patterns from patient to patient. Within the same patient, however, cytogenetic abnormalities, if present, persist and tend not to change (Whang).

Cytogenetic techniques have proven most useful in following tissue homografts. Homologous CML cells transfused into patients with acute leukemia (see below) have persisted and replicated in the recipients for as long as 60 days as evidenced by the presence of the Ph′ marker. Most important is the fact that in some of the recipients the grafts functioned in terms of maturation and delivery into the peripheral blood of granulyocytes and probably platelets and red cells. Skin graft studies have not shown tolerance to CML donor skin and overt evidence of homologous disease has not occurred (Levin, Whang, Freireich, Frei).

Marked improvement has been achieved in the prevention of thrombopenic hemorrhage by homologous platelet transfusions. Complement fixing antibodies develop and limit donor platelet effectiveness in less than 15% of patients and isoantibodies do not decrease platelet survival. Ho-

mologous CML white cells at doses in excess of 10^{11} control major infections, including pseudomonas septicemia in the majority of instances. Antibody formation limiting white blood cell survival in patients with acute leukemia has not been a major problem (Freireich). In order to expand this research and extend its clinical usefulness methods for the more effective acquisition and storage of white cells and platelets are being developed. A centrifuge is under development, which should allow for immediate return of red blood cells and plasma to the donor and continuous separation of the white cells and platelets (Judson, Freireich). This would be far more effective than the currently used batch plasmapheresis and would allow for the use of normal white cells rather than the white cells from patients with chronic leukemia.

In view of the above studies we have intensified our efforts to define more precisely the effect of cancer chemotherapeutic agents on host defense. In a controlled study of 6-mercaptopurine in man it has been found that this agent depresses circulating antibody response to primary but not to booster antigenic stimulation, that it partially depresses skin homograft rejection, that it does not decrease established delayed skin sensitivity but does suppress the induction of delayed skin sensitivity (Levin, Frei). Similar studies are being performed with relation to other cancer chemotherapeutic agents.

PHARMACOLOGY AND RELATED STUDIES

The blood-brain barrier is of major importance to pharmacology generally and to cancer chemotherapy particularly. Tumors, both primary and metastatic frequently develop within the central nervous system. Most cancer chemotherapeutic agents do not appear to pass the blood-brain barrier. Increasing knowledge of the physiocochemical properties which influence passage across the blood-brain barrier should markedly improve treatment. Finally, the problems of cell entry and passage across the blood-brain barrier have many things in common and it may well be that the central nervous system acts as a typical cell and that penetration there and penetration into other cells are fundamentally similar.

Using perfusion techniques the rate of production of spinal fluid in the dog has been determined as has the rate of removal of bulk flow. The amount of extracellular space in the brain has been the subject of considerable controversy, the major evidence being based on electron microscopy which indicated very little extracellular space. Using the above techniques and C^{14}-inulin it has been determined that the extracellular space in the brain is of the order of 10 to 15% of the brain by weight. When similar infusions are done in animals after death, the extracellular space is 2 to 6% similar to that estimated from electron microscopy studies (Rall). Many substances appeared to be removed from the cerebral spinal fluid passively. Recent evidence indicates that an active mechanism for removal of magnesium exists (Rall, Oppelt).

Studies of the pharmacology, enzymatic and biological effects of various analogs of folic acid antagonists continues. Using chlorine^{36} labelled dichloromethotrexate, it has been found in animals that absorption from the gastrointestinal tract is incomplete, that a major portion is excreted via the liver, that the compound does not enter the central nervous system, and that depending upon the species, a considerable portion of the dichloromethotrexate is hydroxylated to a much less active metabolite. The relative ability of various species to hydroxylate dichloromethotrexate explains in part the difference in toxicity between dichloromethotrexate and methotrexate which is not inactivated. The markedly superior antileukemic activity for dichloromethotrexate as opposed to methotrexate does not occur in man, and has not been explained (Davidson, Oliverio, Adamson). Tritiated methotrexate has been prepared and pharmacologic studies of this agent are under way (Oliverio). The hydroxylating enzyme for DCM has been studied in liver homogenates and has been found to be present also in the histologically mature Morris hepatoma (Adamson). The oxidative produce is 7-hydroxy-dichloromethotrexate which inhibits folic acid reductase some 100-fold less than does DCM itself (Oliverio, Misra). Seven-hydroxy aminopterin, 7-hydroxy methotrexate, 7-isoaminopterin, 7-iso folic acid, 7-methyl aminopterin and 7-methyl folic acid have been synthesized (Loo). These compounds show no inhibitory activity against L-1210 and only minimal inhibitory effect on frolic acid reductase *in vitro* (Loo, Adamson, Misra).

In collaboration with the CCNSC a number of congeners of methylglyoxal-bis-guanylhydrazone have been synthesized and studied for activity against L-1210. Almost any deviation from the parent molecule results in considerable or complete loss of antileukemic activity (Davidson, Bond). C^{14} methylglyoxal-bis-guanylhydrazone has been synthesized and its pharmacology in animals and to a limited extent in man has been studied. In rodents excretion is reasonably prompt, while in dogs, monkeys and particularly in man this compound, after a single dose, is excreted for many days (Oliverio). Methylglyoxal-bis-guanylhydrazone sensitive L-1210 leukemia concentrates C^{14} methylglyoxal-bis-guanylhydrazone 5-fold greater than does methylglyoxal-bis-guanylhydrazone-resistant L-1210 (Adamson). A chemical method for the analysis of hydroxyurea has been developed and pharmacologic studies using this method have been performed in both animals and man (Davidson). Toxicologic studies with a number of the terephthalanilides have been performed. Of interest is the fact that in monkeys most of these agents produce degenerative changes in the extraocular muscles of the eye. This has served as a useful predicting system for those which produce ophthalmoplegia in man (Rall).

The serotonin-producing mast cell tumor has been studied extensively as regards the effect of various chemotherapeutic agents. The best of these which include Cytoxan and the glutamine analogs, were selected for trial in patients with serotonin producing carcinoid tumors. In both mice bearing the mast cell tumor and in humans with the carcinoid tumor a marked increase in serotonin or urinary indoles occurs following treatment with either glutamine analogs or Cytoxan (Kelly). Whether this represents tumor destruction in man is unknown. The various plasma cell tumors in mice are being studied extensively with several chemotherapeutic agents. The antitumor effect of chemotherapeutic agents does not vary with the different forms of plasma cell tumors, many of which have qualitatively different protein abnormalities (Carbone).

Monkeys are being used increasingly for pharmacologic as well as numerous biological studies. The care and breeding of these animals is receiving major attention and considerable progress has been made during the past 18 months. Chemical carcinogens have been studied largely in rodents. A number of studies are underway concerning the ability of these chemical carcinogens to produce tumors in primates, particularly newborn monkeys (Kelly, Rall).

The use of tissue culture as a tool to support biological studies in a number of areas is increasing. The relative effects of bromodeoxyuridine on different cells in cell culture correlates well with the relative ability of nucleosides to enter these cells. Preliminary success has been achieved in the culture of human chronic myelogenous leukemic cells bearing a chromosome marker which can be identified in culture (Mohler).

BIOCHEMICAL STUDIES

A number of biochemical studies, particularly of the human leukemic cell, are underway. Several of these relate to changes induced by the administration of cancer chemotherapeutic agents and their relation to response and resistance to such agents. Hypoxanthine and guanine analogs must be converted to the nucleotide by the enzyme inosinic pyrophosphorylase before their antileukemic effect can be exerted. Deletion of this enzyme has regularly occurred in animal tumor systems with drug induced resistance. Studies in 30 patients with acute leukemia have indicated that major changes in this enzyme level in the acute leukemic cell do not occur and that resistance in the majority of patients to 6-mercaptopurine probably has another explanation (Davidson). In tissue culture studies it was shown that the variation in resistance to azaguanine may be as much as 100-fold in the presence of essentially complete inosinic pyrophosphorylase deletion. Clearly factors other than deletion of this enzyme are operative in the development of 6-mercaptopurine resistance (Davidson, Law). The initial effects of azauridine in the treatment of acute and chronic leukemia are excellent but resistance rapidly occurs precluding the development of remission in acute leukemia. Preliminary evidence would indicate that this resistance relates to a marked inductive increase in orotodylic decarboxylase or orotodine pyrophosphorylase (Bono). Studies of tritiated thymidine have indicated varying incorporation into DNA for different types of white cells which correlates poorly with biological observations concerning the proliferative capacity of these cells. Since thymidine ki-

nase rather than DNA synthesis may limit incorporation, the assay of this enzyme in various types of leukemic and normal white cells in parallel with studies of tritiated thymidine uptake is underway (Perry).

By studying the relative intra- and extra-cellular distribution of a weak non-protein bound acid it has been possible to determine intra-cellular pH. This technique plus an assay of intra-cellular acid production has been applied to normal and leukemic cells. A consistent gradient between intracellular and extra-cellular pH has been found for normal white cells which rapidly disappear following injury of these white cells. The intracellular pH is more acid than the extra-cellular pH at almost all levels of extra-cellular pH below 7.4. This pH gradient is considerably decreased in human leukemia cells and in at least some patients with chronic myelogenous leukemia returns to normal following effective treatment (Block, Rall).

Using techniques for the detection of DNA denaturation it has been observed that nitrogen mustard in very low concentration prevents denaturation by any of a number of denaturing agents. This is presumably the result of cross-linking and even concentrations of nitrogen mustard which produce very few cross links are capable of this. It has been shown that such DNA may induce genetic transformation whereas comparable DNA similarly denatured but not exposed to HN_2 loses this biological property. These techniques allow for a number of fundamental observations concerning nucleic acids and the effects of various drugs thereon (Kohn). Chromatographic analysis of normal and leukemic white blood cell RNA has revealed distinct differences in the patterns for the malignant cells which resemble those of other immature cells. There is some evidence that this difference may result at least in part from phenol-resistant ribonuclease activity rather than a difference in the original RNA (Karon).

RADIATION BRANCH

The objectives of the Radiation Branch are to introduce and to maintain high standards of clinical cancer radiotherapy and nonclinical experimental and service irradiations, to engage in supportive physics dosimetry and development programs, to study radiation-induced aberrations of structure and function, to develop biological bases for the testing of new theoretical concepts which may be introduced into clinical practice, and to introduce such new concepts into applied clinical cancer radiotherapy as have been demonstrated in biological or other models as having potential value.

The study of radiation-induced aberrations of structure and function is a continuous operation which is carried on whenever a patient is submitted to cancer radiotherapy and then followed. Importance is attached to both the anticancer and the normal tissue and organ responses to irradiation. Such studies may be general and comprehensive in relation to the specific anticancer radiotherapeutic effort which is being undertaken or the irradiation procedure may be designed around the specific study which it is intended to perform. In the former category are the single dose irradiation, the metabolic balance, and the childhood cancer studies and in the latter the renal function and the blood cell kinetics studies.

The laboratory of radiobiology of the Radiation Branch has completed a definitive study of the relationship of ionization density or linear energy transfer (LET) to the oxygen effect which provides an experimental foundation for the introduction of advanced theoretical concepts of accelerated particle irradiation into clinical radiotherapy practice. The laboratory is now adapting the DBA–P–388 model into a completely pharmacological mammalian *in vivo* quantitative tumor cell system which will permit the simultaneous experimental determination of the supplemental relationships and their mechanisms between pharmacological agents and irradiation in respect to both their antitumor effects and drug toxicity. Other studies of the DBA–P–388 systems have demonstrated that under certain conditions of host-tumor relationships the oxygen diffusion gradients and the membrane transport are physiological. Preliminary studies of what is tentatively identified as a transplantable chondrosarcoma of the Street strain mouse suggest possible experimental approaches to making more effective use of S–35 in the treatment of human chondrosarcoma.

SURGERY BRANCH

The Surgery Branch of the National Cancer Institute continues to act in a dual capacity, that of carrying out its own specific clinical and labora-

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tory investigations, and that of providing consultative surgical service to the National Institutes of Health. There were 1,068 requests for surgical consultation and 439 operative procedures were performed on these patients. This consultation service, in many instances, provided investigators with human tissue, both benign and maligant, for their research activities.

The major clinical investigative interests of the Branch are directed to the indications for and the effectiveness of an aggressive surgical approach to cancer. Patients so treated have afforded the investigator an opportunity for lifetime observations on the behavior of cancer as it is modified by therapy.

Local wound recurrence of tumor, often referred to as "wound seeding", is being studied both clinically and in the laboratory. Although we have found no experimental agent to control wound seeding, unless it be proflavine hemisulfate, it has been demonstrated that the mode of application of a potentially therapeutic agent is most important. The growth of tumor in an artificially "tumor seeded" animal wound is directly proportional to the pressure by which the tumoricidal agent is applied.

There continues to be a 26–30% incidence of wound washings positive for cancer cells following definitive surgery and a much lower but correlated incidence of cytologically recognizable cancer cells in the wound drainage during the first few postoperative days.

Patients with carcinoma of the cervix continue to show an overall 40% 5-year survival following extensive surgery for advanced, usually recurrent, disease end an operative mortality of less than 10%.

Plastic reconstruction of the vagina following its total extirpation for cancer has been carried out on 10 patients. Six of these have been followed for 6 months and have functionally satisfactory vaginas.

Both a short- and long-term evaluation indicates that the use of the ileal conduit following total pelvic exenteration is the most satisfactory method of urinary diversion. Although many of these patients have an asymptomatic course in spite of persistently positive urine cultures, the incidence of pyelonephritis is significant. Antibacterial prophylaxis has been of definite help in minimizing this complication.

An evaluation of abdominal wound closure using wire in a single layer peritoneal fascia approximation has shown that wound dehiscence can be prevented and wound herniation minimized. The development of a hernia or delayed suture abscess is directly correlated with the incidence of postoprative wound infection.

Of six postoperative hernias, five were in patients who had infection. Delayed abscesses (up to 1 year) were not seen in patients who had had wound infection primarily.

Bacteriological examination of all patients has shown that there is an increased postoperative infection rate in hemolytic staphylococcus aureus coagulase positive carriers. Ten days of postoperative antibiotic therapy lowered the infection rate from 54.3% in a nonantibiotic treated group to 14% in a treated group. When antibiotics were administered for 3 days preoperatively and 7 days postoperatively, the infection rate was 8% as compared to 17.4% in the patients who received only 7 days of postoperative therapy. A 2 year survey has shown that when extensive and prolonged surgical procedures are undertaken, antibiotics are of definite value in decreasing the number of infectious complications.

Lymphangiography has been performed on over 100 patients and its usefulness has been demonstrated. This technique has been of considerable value in demonstrating the variability of the thoracic lymphatic duct. A characteristic picture of incomplete lymph node filling by the injected dye is seen in metastatic disease from solid tumors. Large nodes with a foamy reticular pattern usually indicate lymphomatous disease. The addition of chlorophyll to the injectable radiopaque material stains the lymph nodes green and has significantly improved the completeness of lymphadenectomies.

Elective esophagostomy has been extensively used for intubation for feeding and has proven to be far superior to gastrostomy or an indwelling nasal tube.

The double blind preoperative radiation study of head and neck cancer patients has continued to be an interesting project. One thousand roentgens given to the tumor area 24 hours preoperatively probably has caused some increase in morbidity, but the followup time is too short to determine any effect the radiation may have on the end results.

Cancer cells may be present in the circulating blood of patients with cancer. The presence of circulating tumor cells cannot be correlated with survival. We have shown that present methods of recovering cancer cells are much more inefficient than had been suspected. Present efforts are directed at developing methods of tumor cell recovery which will prove to be more efficient. It is suspected that circulating megakaryocytes may, in the past, have been incorrectly designated as tumor cells.

Several patients have now been intensively studied with the cooperation of the Metabolism Service in relation to metabolic alterations incidental to preoperative bowel preparation. Initial studies indicate a rather marked loss of fluid and electrolytes and negative nitrogen balance. It is hoped that as a result of this study we may be able to ameliorate, in part, the metabolic alterations brought about by bowel sterilization.

Survival from paranasal sinus cancer, in spite of improved techniques of radiation and surgery, continues to be very poor, usually due to local recurrent disease. The Surgery Branch continues its active interest in cancer of this anatomical area and have combined a neurosurgical approach to the usual surgical approach in 23 patients. With elevation of the frontal lobe, intracranial invasion can be ruled out and the paranasal sinus area freed from the intracranial approximation. This allows *en bloc* resection of the entire area from the usual facial approach.

In wound-washing studies whereby an open wound is seeded with tumor cells, then washed with an agent suspected of having tumoricidal activity, the animal laboratory has been used to continue studies into the mechanisms of tumor growth and dissemination. Proflavine hemisulfate in a 1:500 or a 1:1000 concentration has been shown to be extremely tumoricidal when tested against several tumor-host systems. While other drugs have decreased tumor growth, this agent has been consistently most active. Large animal toxicity studies are now being completed in preparation for a clinical trial.

Attempts to alter growth of tumor by infecting a seeded wound with *E. coli*, Group A beta hemolytic streptococci, proteus vulgaris, and staphylococcus aureus revealed that only the streptococcal and coliform infections significantly decreased tumor growth.

Cortisone appeared to increase the size of pulmonary metastases, the number of large lung tumors, and the growth of the primary T–241 Lewis sarcoma in C–57 BL/6JN mice. This effect was enhanced if cortisone was administered after tumor transplantation. When the primary tumors were amputated, this effect of cortisone was not demonstrated. Amputation of the primary tumors decreased the incidence of lung metastases. The earlier the amputation, the more pronounced was its effect. Cortisone did not alter the anatomical distribution of metastases.

With the Millipore filter technique we were able to demonstrate tumor cells in the blood of offspring of pregnant mice injected with S–91 melanoma. This passage across the placental barrier had been postulated but not demonstrated.

Previous extensive laboratory investigations with S–91 melanoma and T–241 Lewis sarcoma have shown these tumors to metastasize only to the lungs. With individual housing, the life of the animal can be extended from 1 to 3 weeks. These mice will then frequently demonstrate metastases retroperitoneally to the liver and bowel.

Malignant bronchial changes have not been observed in the 5½ years during which a bronchial pouch in dogs was exposed to material thought to be carcinogenic.

Laboratory techniques are being developed and standardized which may allow us to determine if autoimmunization of patients with their own tumors produces a specific immunological response or therapeutic effect. Skin tests, tannic acid treated hemagglutination tests, fluorescent antibody techniques and cytotoxicity studies are being evaluated. Seven patients were treated with their own tumor in preliminary work. No significant response, either clinically or in the laboratory testing, was elicited. We would like to be able to detect and quantitate circulating antitumor antibodies and characterize these antibodies in terms of antigen specificity.

Additional data have been accumulated referable to serum fractions which influence cell growth. Data accumulated to date indicates that serum from normal individuals contains two fractions, one capable of inhibiting cell growth and the other capable of stimulating cell growth of the regenerating rat liver. Concentrations of these fractions potentiate these effects. Serum from patients with cancer contains a stimulating frac-

tion but does not contain an inhibiting fraction in the same concentration as does serum from normal individuals. This deficit appears to be of the magnitude of 75% or greater. The addition of the inhibiting fraction of normal serum to whole cancer serum or to the stimulating fraction of cancer serum produces inhibition of liver regeneration, indicating that the defect in cancer serum can be replaced. The periodic injection of the inhibiting substance into animals with a transplanted tumor delays the time of appearance of the tumor and slows its growth rate. Mice injected with L–1210 leukemia and treated with inhibiting substance live 10 to 12 days, while untreated controls die at 7 days.

With the acquisition of a full-time staff urologist in the Surgery Branch, a number of projects have evolved which are based on problems of current interest in the field of urology. Of considerable interest is the study of the levels of lactic acid dehydrogenase in the urine and the serum of patients in good health with disease.

Renal vascular hypertension has been under study. The diagnostic work up includes the I–131 Hippuran renogram, bilateral renal function studies performed by ureteral catheterization, as well as the usual clinical and chemical evaluations. Five patients have undergone corrective renal artery surgery for hypertension.

Studies have been performed to evaluate the effect of Angiotensin II on renal function of subjects with normal blood pressure, hypertension, and unilateral renal artery disease. The normotensive and hypertensive kidneys respond to angiotensin in different ways. Preliminary studies in the dog have shown a similar response. Sustained hypertension up to 6 months has been produced by partial ligation of segmental renal artery branches and then corrected by total artery branch ligation with relief of hypertension for up to 18 months.

INTRAMURAL RESEARCH PROGRAM

Introduction

The translation of the data of biomedical research into benefits for the sick is often incomplete and always slow. These lags—conceptual, diagnostic, therapeutic—are cumulative and even potentiating. The result is that in some diseases there is little resemblance between what is available to the average patient and to the patient with the same disease at hospitals associated with active research groups. This lag can be excused in a number of ways, but underlying all of them is failure to accept responsibility for broad application of research information. The scientist and clinical investigators who establish a new finding are best able to recognize its implications, yet are in the poorest position to reduce it to general availability. The discoverers perhaps rightly feel their responsibilities end with publication. But the responsibility deficit is not in communication, for rapid communication of new findings already exists. The responsibility is that for action—to take new findings and to see that they reach the right patients through the complex social, economic, traditional, political and emotional milieu of our free society.

During the past 2 years the physicians of the National Cancer Institute have been reviewing those research findings which should be applied broadly, and the means by which this might be done. The many new findings related to the acute leukemias led to a decision to form an Acute Leukemia Task Force. The membership* includes scientists and physicians who are making the new observations, clinicians with responsibilities for large numbers of leukemic patients and scientists engaged in drug development. A discussion with officials of a large industrial corporation which had used the task force mechanism effectively led to the conclusions that a task force should limit itself to one or at the most two objectives: set up criteria for their accomplishments; be dissolved when these end points were reached. The Acute Leukemia Task Force chose two objectives; (1) the extension of platelet and granulocyte replacement to many patients with acute leukemia; and (2) the establishment of accurate predictive animal systems for selecting antileukemic drugs.

The repair of platelet and granulocyte deficit in the depleted leukemic patient is part of routine care at National Cancer Institute. A number of patients who would have died from hemorrhage or infection are thereby saved and can often attain

*Membership: Dr. J. H. Burchenal, Dr. S. Farber, Dr. E. Frei, Dr. E. Freireich, Dr. J. Leiter, Dr. J. Louis, Dr. E. K. Marshall, Dr. T. McGinn, Dr. M. L. Murphy, Dr. H. E. Skipper, Dr. G. Taylor, Dr. P. Waalkes, Dr. R. Whittington, Dr. S. L. Rivers, Mr. W. Lourie, Dr. M. Sloan.

additional drug induced remissions. If these techniques were available throughout the country, the average survival time of patients with acute leukemia would be lengthened, perhaps doubled. The major problem in extending this technique is that of platelet and granulocyte supply. In 1962 6,000 units of blood were processed for platelets, as compared to 4,000 units required for the heart-lung apparatus and 4,000 for all other transfusions at National Institutes of Health. Additional limitations are placed on supply since platelets must be used within a few hours of collection. The Task Force has sponsored discussions among many interested parties and taken action to increase the supply of donors, improve harvesting by plasmapheresis, develop a continuous flow centrifuge, and to study freezing and storage. It is even more difficult to obtain adequate numbers of granulocytes and only chronic melocytic donors can supply the 100 billion cells needed per transfusion. To solve the supply problem, continuous plasmapheresis of normal donors will probably be needed using a continuous flow centrifuge. Throughout all of these problems runs the need to expand the research and services of blood banks to meet the present and future clinical needs.

The Task Force has made considerable progress with its second objective—the establishment of better predictive systems for selection of antileukemia drugs. Six drugs can induce complete remission in the acute leukemias—6-mercaptopurine and methyl gas in acute myelocytic leukemia; 6-mercaptopurine, prednisone, methotrexate, vincristine and cyclo-phosphamide in acute lymphocytic leukemia. The L–1210 mouse leukemia successfully discriminates for four drugs but misses vincristine and prednisone. The Task Force with the cooperation of Cancer Chemotherapy National Service Center and other units of the Task Force has supported an examination of the six agents and a number of clinically negative compounds in a wide variety of animal leukemias and lymphomas. In this recent work P1534 leukemia seems to discriminate for vincristine. The goal is a battery of tumors sensitive to the six drugs and insensitive to clinically inactive agents. Ten additional compounds have passed the L1210 screen and are in preclinical or clinical trial. Some have definite clinical activity, but their introduction into broader clinical trial will depend on studies of degrees of effectiveness and toxicity. The Task Force has also had under study the problems of combination chemotherapy. If one considers the possibly analogous situation of tuberculosis chemotherapy, it may be recalled that pulmonary tuberculosis could not be cured by either PAS or streptomycin. Cures were achieved when the drugs were given together. It is conceivable that a combination of two or more of the antileukemic drugs could bring about prolonged or permanent remission. The Task Force has developed studies of combinations of the six drugs in the animal leukemias, in toxicity models and in patients. The ability to predict the toxicity or usefulness of drug combinations from animal systems would be of great value.

It had been planned to consider the viral etiology of the leukemias in the deliberations of the Acute Leukemia Task Force. When its goal was sharply focused, virus problems were removed from its scope. Research in the viral etiology of cancer had resulted in so many new findings it became apparent there was an opportunity to pursue more vigorously the viral etiology of some clinical cancers. It was decided to establish a second group—the Virus Task Force.* It has recently been formed and has as its central objective the rapid examination of the virus etiology of the leukemias.

During the past year there have been several important observations on canced viruses by National Cancer Institute scientists. The murine models continue to be studied profitably, and there is gradually emerging a reconstruction of the morphologic events occurring between inoculation and appearance of leukemia. Of importance is the morphology of the murine virus in electron micrography. Using new negative staining techniques, the Moloney agent has been shown to have a tail and a hexagonal head not unlike the bacteriophages. It is apparently unlike the bacteriophages in having a longer tail, lacking a complex injection apparatus, attacking mammalian cells and being composed of RNA. Similar morphology has been found for the Rauscher and Friend agents. This finding permits specific identification of these three agents. It has a further significance in that it will permit examination of mechanisms by which the virus affects the mam-

*Members: Dr. W. R. Bryan, Dr. J. Grace, Dr. R. Huebner, Dr. F. Horsfall, Dr. P. Kotin, Dr. J. Melnick, Dr. R. Miller, Dr. R. Stevenson.

malian cell. This is well understood for the bacteriophages and bacteria, and similar studies can be done for the murine leukemia agents. A large effort is underway to uncover a viral agent in the acute leukemias and in stomach cancer. There are definite positive findings, but at the time of writing no clear cut proof of a clinical oncogenic agent exists. The importance of the use of research data in the control disease must not be allowed to obscure the realization that such data can come only from an organization with scientific excellence. The quality of the research during 25 years of National Cancer Institute is evidence enough that this has been achieved. More important is the resulting current staff of dedicated scientists and physicians who individually contribute so many new observations and collectively have such a broad and deep understanding of cancer. Both qualities are apparent in the accompanying summaries of the work of the past year by the Laboratory and Branch Chiefs. Since it is not possible to review all the important new contributions, mention will be made of four areas of significance for the future.

1. The need has been apparent for a tissue culture medium which is chemically defined and free of serum and protein hydrolysates. Quantitative studies in cell nutrition cannot be done while serum or protein contribute unknown amounts of vitamins and amino acids. Neither can valid studies on virus growth in tissue culture be performed in the presence of added serum because of the possible presence of antibodies or viruses.

Scientists of the Tissue Culture Section of the Laboratory of Biology are now able to carry 14 cell strains in chemically defined media. With three strains of mouse fibroblasts, for the first time cells have been as hardy as those grown in the serum medium.

2. Study of the relations of nuclei acids and proteins within the cell will not be precise until methods exist for the separation, purification and characterization of the various macromolecules. In the Biochemistry Laboratory emphasis has been placed on the perfection of techniques analogous to those developed in the column separation of proteins. Considerable success has been achieved in purifying ribosomes and ribonucleoproteins, oligonucleotides and lecithin and choline containing nucleotides. While advances are being made in purification of these macromolecules, much remains to be done. A somewhat different approach has been undertaken in the joint Oak Ridge-National Cancer Institute effort to develop high speed zonal ultracentrifuges.

3. A knowledge of the interdependence of tumor and host as they make joint demands on nutritional resources would help to understand and perhaps indicate a way to control tumor growth. One of the scientists in the Biochemistry Laboratory has continued study of this problem in a system wherein a tumor inserted under a kidney or ovarian capsule of the rate develops a single arterial supply and venous drainage. He has shown that collagen of such a tumor is produced by cells of the host, not of the tumor. It has also been found that the interstitial fluid of this tumor has a constant composition in spite of tumor necrosis. It differs from composition of serum in that protein is 40% less, and contains no fibrinogen. Glucose is absent from the interstitial fluid unless there is marked hyperglycemia, but the lactic acid content is twice that of serum. Cholesterol and lipid phosphorous are much lower than in serum. These discrepancies give a hint of marked differences in the metabolic patterns of this tumor and normal tissues.

4. The successes achieved by the physical scientists in the use of computers have not been matched in biology. Perhaps this is because physical systems can validly be studied in each of its parts, while living systems must always be studied whole. A more attractive possibility is that the physical scientist can move his problem directly to the computer while the biologist needs a middle man who understands biology and mathematics. Developments in the National Cancer Institute give some hope that the second explanation is correct. For 2 years the Energy Metabolism Section of the Laboratory of Physiology has had a 1620 computer. It is in use by a number of National Cancer Institute scientists with the help of members of this section. The following research studies have been profitably programmed:

(*a*) Analysis of arrangement of mononucleotides within polynucleotides derived from RNA.

(*b*) Effect of irradiation on production of sheep antihemolysins.

(*c*) Retrieval and correlation of pathological diagnoses in patients.

(*d*) Evaluation of dose-response phenomena in toxicity and antitumor effects of drugs.

(*e*) Determination of the pattern of urinary excretion of nucleic acid metabolites by leukemia patients.

(*f*) Another scientist has in collaboration with Dr. Mones Berman of National Institute of Allergy and Infectious Diseases and the Rand Corporation programmed a Monte Carlo model for the effect of irradiation on the survival and growth of populations of cells.

(*g*) Working with the Princeton computer group another scientist has programmed models for carcinogenesis due to ultraviolet light, X-rays and viruses. The success of these programs leads one to hope that the biomedical scientist will one day gain from the computer the same advantages enjoyed by the physical scientist.

In considering the productivity of scientists and physicians of the National Cancer Institute, the question must be posed—"How is present excellence to be maintained in the future?" It is clear to all that this depends upon the recruitment of scientists and physicians of the highest quality and the creation of an atmosphere which will ensure their retention and unlimited scientific development. The problems are known to all and need not be listed in their entirety. While salary levels and isolation from the academic life are important, the greatest problem is the growing conviction that the National Institutes of Health is of such size it can no longer respond to scientific need with the speed and flexibility essential to excellence. The intramural program of the National Cancer Institute is a fragment of a much larger National Institutes of Health complex, many of whose functions are not related to the needs of intramural research. If a new start were being made a National Cancer Institute, complete within itself, might be considered desirable. Yet much would be lost by such splendid efficiency—the contacts with many fine minds in the same and other disciplines, the great hospital which has been created with the seven institutes, and the attractiveness of the National Institutes of Health to scientific visitors from the entire world. On the whole, the advantages of the larger complex to the National Cancer Institute outweigh the disadvantages. This does not relieve any of us from the duty of imaginative administration to create the ideal climate for the flowering of biomedical research. The quickest way to control disease is to make it easy for the best scientists and physicians to work at it.

LABORATORY OF BIOCHEMISTRY

Cytochemistry Section

Drs. Woods and Burk have found that reduced diphosphopyridine nucleotide (DPNH) produced very marked inhibition of anaerobic glycolysis of intact ascites cells and certain other tissues. Addition of excess oxidized diphosphopyridine nucleotide (DPN), pyruvate or methylene blue, converting the added DPNH to DPN via reductases, could completely abolish this inhibition. The DPN/DPNH ratio is thus believed to be important in tumor cell metabolism, and chemotherapy and radiotherapy also. The ratio probably dominates the glycolytic activity at the triosephosphate-dehydrogenase step inside the cell. Many chemotherapeutically employed quinones may exert their effect via this DPN/DPNH ratio.

Drs. Burk, Woods and Giger (NIH Postdoctorate Fellow) showed that methylglyoxal-bisguanylhydrazone (MeGAG) was a potent inhibitor of respiration in L–1210 mouse leukemic and other ascites tumor cells; much more so in MeGAG-sensitive than in MeGAG-insensitive lines. The inhibition was found to be localized at two DPN-dependent loci, namely malic and pyruvic dehydrogenases, but at no other DPN- or TPN-mediated enzyme systems. Anaerobic glycolysis was not effected by MeGAG but aerobic glycolysis was increased, as a result of the respiratory inhibition, due to a Pasteur effect. 6 aminonicotamide (6-AN) both *in vivo* and *in vitro* enhanced the action of MeGAG on tumor cells and decreased its toxicity for the host. Detailed chemotherapeutic studies with 6-AN and MeGAG combinations are thus strongly indicated, particularly with mice.

Dr. Woods and Mrs. Kolbye (guest worker) in collaboration with Dr. M. Landy are studying the possibility that cellular glycolytic responses of endotoxin represent a delay hypersensitivity reaction. This has led to observations that BCG-sensitized spleen and macrophages showed abnormally high aerobic glycolysis with altered response to injected endotoxin. Endotoxins appeared to increase nonspecific (phagocytic) and specific (antibody) resistance to infection. Low nonstressing injections of endotoxin could cause marked lower-

ing of glucose levels in normal and alloxan-diabetic mice.

Important differences between extravascular and intravascular leukocytes have been found by Dr. Evans (NIH Postdoctoral Fellow) in collaboration with Dr. Mueller (NIMH). They were able to induce myelopoiesis in guinea pigs by the intraperitoneal injection of sterile casein. Marrow, exudate, and blood myeloid leukocytes all phagocytized particles such as polystyrene but only the first two took up fatty acid (albumin-bound palmitate). With marrow this uptake of fatty acid produced little respiratory stimulation, but with myeloid exudate cells there was a marked respiratory stimulation that was cyanide-insensitive. With blood myeloid cells (polymorphonuclear leukocytes (there was also marked, cyanide-insensitive respiration stimulation when polystyrene particles were taken up. The glucose uptake of normal marrow cells was only one-third that of immature (casein-stimulated) marrow myeloid cells or of exudate myeloid cells. It appears that mature myeloid marrow cells have less glucose uptake and higher palmitate uptake than do the immature myeloid marrow cells. The respiration of exudate, and mature and immature myeloid cells was virtually the same.

Further studies on the effects of vincaleukoblastine sulfate (VLB) have provided evidence that the alkaloid inhibits the Pasteur effect. This effect may well be involved in the causation of metaphase arrest by the drug. Visible light can markedly increase the effects of added dyes on tumor cell metabolism, greatly increasing respiration but totally eliminating aerobic glycolysis (Hunter and Burk).

Nucleic Acids Section

The enzymes in normal and cancer tissues responsible for the synthesis of the deoxynucleoside triphosphates needed for DNA synthesis are being studied by Dr. Kielley. She has found that the low thymidylate kinase activity of normal mouse liver as compared to hepatoma was apparently an artifact resulting from the fact that the enzyme was bound to particles in the liver. If the liver was frozen and thawed several times, the activity of liver extracts became the same as that of hepatoma extract. She has also observed that normal liver could form deoxycytidine triphosphate much more efficiently than could the hepatoma. These findings have important implications with regard to the currently held concept that thymidylic kinase is a limiting factor in DNA synthesis. The nature of the thymidylate kinase bond to the particles of normal liver is being studied.

Dr. Rotherham has continued her studies of the deoxyribonucleosides of the tissues and urine of normal and tumor rats to examine the changes that occur in relation to the cancer process. Male rats have been found to excrete about five times as much deoxyribosidic material in the urine as do female rats. Castration of the males removed this difference. The deoxyribonucleosides present in the urine of male and female rats were the same with deoxycytidine accounting for approximately 67% and 5-methyldeoxycytidine for 23%. No evidence could be obtained for the occurrence of the latter nucleoside in blood or tissues.

Experiments by Dr. Ram Behki (Fellow of the Jane Coffin Childs Memorial Fund) and Dr. Schneider have shed new light on the intracellular locus of DNA synthesis. While the biochemical literature had indicated that DNA synthesis occurred in the soluble fraction of tissues, present experiments, however, show that the endogenous deoxyribosidic compounds of normal liver, regenerating liver and hepatoma were present in the nucleus provided that the nucleus was isolated in such a way as to prevent redistribution, i.e., the use of lyophilized tissues and of dry organic solvents of graded densities for the isolation of the nuclei. When aqueous solutions were used, these compounds were recovered in large part in the soluble fraction and considerable degradation of nucleotides occurred. A study of DNA polymerase led to similar findings, i.e., the enzyme was associated with nuclei when these were isolated by the nonaqueous method. It appears, therefore, that the precursors of DNA as well as the DNA polymerase are normally found in the nucleus but are lost when nuclei are isolated in aqueous media.

Studies by Dr. Schneider on the enzymatic synthesis of deoxycytidine diphosphate choline and its utilization for lecithin formation were continued in an effort to discover the reason for the occurrence of this nucleotide in the Novikoff hepatoma. The activity of the enzyme responsible for the synthesis of dCDP-choline in extracts of both normal liver and the Novikoff hepatoma was found to increase several-fold when tissue preparations were aged at 0° for several days, incubated at 37°

for three hours, dialyzed or passed through Dowex-50-Na^+ columns. The enzymatic synthesis of lecithin was stimulated 5-fold by adding an emulsion of diglyceride to the reaction mixture. This stimulation occurred in both liver and hepatoma although the ability of the latter to form lecithin was considerably less than that of the liver. However, CDP-choline was much more active in lecithin formation than was dCDP-choline in both liver and hepatoma and in the presence or absence of added diglyceride. These experiments provide further support for the previous conclusion that the occurrence of dCDP-choline in the hepatoma is related to the limited ability of this tissue to form phospholipids but leave open the possibility that this compound may be used in DNA synthesis.

As part of a continuing study of structure and sequence of nucleic acids, work on the nucleotide distribution of ribonucleic acids, on the development of enzymatic methods for the cleavage of nucleic acids at specific points, on the development of fractionation methods, and on the preparation and characterization of oligonucleotide sequences has been continued by Drs. Rushizky and Sober with the collaboration of Dr. E. Bertos (NSF Postdoctorate Fellow) and Dr. Edith Townsend (Visiting Fellow). They have been able to show with Dr. C. A. Knight of the Virus Laboratory, University of California, that the RNA obtained from various strains of tobacco mosaic virus could be differentiated on the basis of the nucleotide products produced after hydrolysis by pancreatic RNase and RNase T_1. Nucleases from Takadiastase and from *B. subtilis* have been purified and characterized with respect to their hydrolytic specificity. RNase T_1, obtained from Takadiastase, showed strict specificity for the hydrolysis of the $5'$–PO_4 linkage distal to the guanylic acid residue leaving a terminal $3'$–PO_4 guanylic acid. However, RNase T_2, also from Takadiastase, did not show a strict hydrolytic specificity but rather only a preference for adenylic residues. Complete digestion by this enzyme resulted in complete reduction of the nucleotide chain to mononucleotides. This enzyme has become extremely useful for the determination of nucleotide base composition. The RNase from B. *subtilis* has been purified extensively. It has a low molecular weight but only shows partial purine specificity, favoring guanylic residues over adenylic residues but ultimately reducing the whole nucleic acid chain to the di- and mononucleotide stage. In the course of the above studies, most of the di-, and tri-, and tetranucleotide sequences of RNA have been prepared and characterized by a combination of chromatographic and electrophoretic methods. They are being used as model and standard substances for measurements of a number of physicochemical parameters and enzyme specificity. Studies of reactivation of the immune response of immunologically suppressed animals by DNA and its degradation products have been initiated with Dr. M. Feldman, Laboratory of Experimental Biology, Weizmann Institute of Science, Rehovoth, Israel.

Nutrition and Carcinogenesis Section

The development by Dr. Morris of a large number of transplantable rat hepatomas showing much similarity to normal rat liver has led to the concept of a "minimal deviation tumor." The great interest in these biochemically interesting tumors has led to a large number of cooperative investigations of their enzymatic patterns and their metabolism. Much detailed information of individual systems has been obtained but, in general, these tumors exhibit enzymatic distribution patterns which are more like those of normal liver than the rapidly growing hepatoma. One of the most important observations to date is that no two of the minimal deviation tumors are alike. The enzyme patterns themselves and responses to physiologic stimuli show striking individual differences.

Further studies of experimental gastric cancer in the rat have revealed for the first time that this cancer can be induced by N,N′-2,7-fluorenylene-bisacetamide (2,7-FAA) injected into the animal at a site distant from the site of the cancer. Studies are underway on inhibitors and promoters of 2-fluorenylacetamide carcinogenesis. Although these are long term experiments, and all details have not yet been completed, it appears that bladder tumors can be produced in Fischer strain rats without the addition of tryptophan. Jaundiced rats ingesting the carcinogen developed as many liver tumors as nonjaundiced animals of the same genetic background.

Biochemical studies in experimental cancer by Drs. Dyer and Morris have shown that the metabolism of 2–FAA by the Rhesus monkey resembles that of the guinea pig, a species resistant to induc-

tion of cancer by this agent. However, the monkey unlike the guinea pig does not excrete N-OH-2-FAA glucuronide (a potent carcinogenic metabolite in the rat) after 2-FAA ingestion. 2-Fluorenyldiacetamide, 2-fluorenylbenzoylamide and 2-fluorenylphthalamic acid may owe their carcinogenic activity to the production of 2-FAA *in vivo*. Differences in carcinogenic activity may be due to the rate of absorption and to the amount of unmetabolized material excreted by the large intestine. 2,7-Fluorenylenebisacetamide, a potent carcinogen for the rat and the dog, however, does not yield 2-FAA. Hydroxylated metabolites are excreted after feeding 2,7-FAA to both species. The dog, unlike the rat, excretes a large amount of unstable free amino metabolites of administered 2-FAA and of 2,7-FAA. Tryptophan pyrrolase cannot be detected in some minimal deviation hepatomas but is present in others in low concentrations compared with liver. In tumor lines where activity is detectable, that activity is increased by intraperitoneally-administered L-tryptophan.

In attempts to study the effect of natural and purified diets on tumor induction and growth, Mr. Otey and Dr. Birnbaum have initiated studies in rats in which dimethylbenzanthracene is fed by stomach tube. Preliminary experiments have indicated that tumor induction was relatively slow on both diets with a slight variation in tumor incidence tentatively ascribed to the difference in food intake. In attempts to obtain a 100% incidence of tumors all locally available strains were tested and it was confirmed that the Sprague-Dawley strain was the most susceptible. Since in the early experiments there appeared to be some relationship between the age of weanling and the development of mammary tumors, current experiments are studying the effect of adding progesterone and anhydroxyprogesterone to the synthetic liquid diets.

Preliminary results indicate an earlier appearance of the first palpable tumor.

It has been shown by Dr. S. Birnbaum and Mr. Otey in their studies on the nature of renal aminopeptidase, that, like many other intracellular peptidases, the renal enzyme was activated by but not necessarily dependent on metal ions. Further work has been discontinued due to Dr. Birnbaum's transfer to the Division of Research Grants.

Protein Chemistry Section

A comprehensive investigation of the "Bence-Jones" proteins produced by mouse plasma cell tumors has been undertaken by Drs. E. L. Kuff, M. Potter, R. McIntyre, and W. Dreyer (NIAMD). This has revealed that the Bence-Jones protein elaborated by tumor RPC 20 has a molecular weight of 24,000, that it contains one reactive sulfhydryl group per molecule, and that it is different from every other murine Bence-Jones protein thus far studied in its immunological properties and in the peptide "fingerprint" produced by tryptic hydrolysis. It forms a disulfide dimer and also a hybrid molecule with serum albumin. It appears in the urine partly in the form of dimer and partly in combination with oligopeptides linked apparently through the sulphhydryl group, since the latter is no longer free. The murine Bence-Jones proteins can be divided into two classes on the basis of ultracentrifugal analysis: one with sedimentation rates near 2.8S and the other with rates between 3.3 and 3.9S, with both classes heterogeneous by starch gel electrophoresis. The situation is thus analogous to that observed in human multiple myeloma.

Dr. W. Hymer (NIH Post Doctorate Fellow) and Dr. E. L. Kuff are developing methods for the isolation of nuclei from plasma cell tumors and have found that these exceptionally fragile components of the cell are stabilized by treatment with Triton X–100, a nonionic detergent. Similarly, very clean nuclei have been obtained from normal liver and kidney by dispersing other particulate cell components with this detergent. The liver nuclei retained their DPN-synthesizing capacity after treatment. The use of Triton X–100 has also made possible the isolation of tubular casts from the kidneys of tumor-bearing animals, and their composition is now under study.

Drs. E. A. Peterson and E. L. Kuff, in collaboration with Dr. R. W. Hendler (NHI), have found that ribosomes isolated by the column method from normal rat liver labeled *in vivo* with radioactive amino acids were markedly less radioactive than ribosomes isolated by conventional ultracentrifugal procedures. However, the microsomal lipoprotein fraction washed off the column by Triton X–100, a nonionic detergent, was extremely radioactive. The results suggest that the

radioactivity associated with liver ribosomes isolated by the ultracentrifugal method may be due to a small amount of active, lipid-containing substance contaminating them, as has been recently found to be true of *E. coli* ribosomes. This active substance may have emerged from the column in the highly radioactive Triton X–100 eluate, which is known to contain newly synthesized protein.

Isolation and fractionation of the ribosomes of mouse plasma cell tumor RPC 20 by the column procedure previously described (1960) yielded a pattern similar to that obtained from rat liver, but the two major peaks were eluted at significantly lower salt concentrations than the corresponding liver ribosomes. Chromatography of microsomal RNA on ECTHAM-cellulose has been complicated by the large size of the molecules, their high density of negative charge, and their lack of a fixed configuration. It was necessary to use relatively high initial salt concentrations to minimize chromatographic anomalies that arise from the extended configurations assumed by RNA molecules at low salt concentrations because of repulsion between neighboring charges. It is of interest that the major portion of the RNA emerged from the column in the same region as the intact ribosomes when chromatographic conditions were the same. This suggests that the protein of the ribosome is recessed within an RNA structure that dominates the surface of the particles.

Dr. R. W. Hartley, Jr., in continuing his studies on the heterogeneity of crystalline bovine plasma albumin has found it possible to separate gram quantities of the components having a high content of sulfhydryl groups from those having a low one by scaling up the sectioned column procedure previously reported. Chromatography on Sephadex G–200 was found to fractionate albumin purely on the basis of molecular weight, and separation of monomer and dimer from each other and from higher polymers was accomplished in a single pass. Equilibrium ultracentrifugation of the dimer fraction provided the first unequivocal demonstration that this commonly observed albium component (S_{20} of 6) does have a molecular weight just twice that of albium monomer.

Dr. Hartley has collaborated with Drs. G. W. Rushizky and H. A. Sober in the purification and characterization of an extracellular ribonuclease from *Bacillus subtilis*. Phenol extraction followed by a series of precipitation and chromatographic procedures yielded a product pure enough for molecular weight determinations which have indicated a value in the 10,000–12,000 range. This was supported by its behavior in chromatography on Sephadex G–75. The relatively small size, stability, reported absence of disulfide bridges, and easily measured activity of this enzyme recommend it for a study of the relation between enzyme structure and function.

Mrs. Mary Wyckoff and Dr. Peterson made a detailed study of the effects of temperature (0 to 60°) on the ionization of the acidic and basic groups of several cellulosic ion exchanges and found that both types became progressively weaker as the temperature increased. Since similar effects were observed when simple soluble compounds containing the same groups were titrated in free solution, the phenomenon appeared to be a result of the effect of temperature on the dielectric constant of water rather than on the structure of the adsorbent, itself. Because of an inflection in the temperature vs. ionization curves of the amines, the ionization of these (including "tris" buffer and DEAE-cellulose) was essentially the same at 5° and 25°, accounting for the nearly identical patterns obtained when serum proteins were chromatographed at these two temperatures. Albumin, however, was significantly displaced in the direction of tighter binding at the higher temperature, apparently because of some unusual property of its own.

Difficulties previously interfering with the isolation and characterization of the metal proteins of plasma have been overcome. Drs. S. R. Himmelhoch (Research Associate), H. A. Sober and E. A. Peterson, in collaboration with Dr. Bert Vallee of Harvard University have perfected procedures for the routine removal of contaminating trace metals from buffers, adsorbents, and equipment, and preliminary experiments have shown several metals (Ca, Mg, Ba, Zn, Cr, and Fe) to be present in specific fractions of dialyzed plasma. Larger scale experiments are expected to permit the detection of additional trace metals. It is believed that this work will ultimately aid in the interpretation of metal fluctuations in the plasma proteins in disease by revealing the changes

in individual metalloprotein components instead of net changes in the total amount of a given metal.

Drs. A. Yaron (Visiting Fellow) and H. A. Sober have undertaken the preparation and examination of a variety of single and mixed amino acid polymers of known chain length, composition, and sequence for use as model substances in studies of the relationships of the structure of proteins to their biological activity and their chromatographic behavior. Homologous poly-alpha-amino acid mixtures having different average chain lengths (prepared by Drs. A. Berger, E. Katchalski, and M. Sela in the Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel) have been chromatographed on CM-cellulose and DEAE-cellulose to resolve the individual components. Resolution has been studied as a function of temperature, pH, buffer, and load. Adequate resolution of the lower homologs of polylysine was obtained with a one gram load, and a scaling up of several fold is contemplated. Eluting systems have been devised that do not interfere with examination of the effluent at 220 microcuries, and techniques have been developed for the recovery of the polyamino acid fractions from the effluent by precipitation.

Dr. T. T. Otani has tested the N-chloroacetyl derivatives of a number of Beta hydroxyamino acids for growth inhibitory effects in three microbial systems as a preliminary screening for possible antitumor activity, N-Chloroacetyl-Beta-hydroxyleucine and N-chloroacetyl-Beta-hydroxynorleucine were the best inhibitors of microbial growth. When these two compounds were tested in collaboration with Mr. John Venditti for antitumor activity in leukemic mice (L–1210) significant decrease in tumor nodule size was noted, although there was no increase in survival time. One of the 10 mice receiving N-chloroacetyl-Beta-hydroxynorleucine at a level of 500 mg/kg/day showed complete regression of the tumor nodule and 4 of 10 mice showed significant decrease in tumor size after 1 dose of the compound. While no significant loss of weight was observed in the animals before death, there were more deaths among the treated animals than among the controls on the third day of treatment. The possibility that this was the result of an acidotic condition deriving from the action of tissue acylases on the chloroacetyl derivatives will be taken into account in future tests.

Tumor-Host Relations Section

Increased efforts are being made to investigate the relationships between the tumor cells and the various tissues of the host. This has required the development of new *in vivo* and *in vitro* techniques and has resulted in an increased amount of collaboration and consultation with colleagues in the Laboratories of Immunology, Virology and Biology.

In studies which are exploiting the "tissue isolated" tumor technique of Dr. Gullino the development of connective tissue during the growth of the tumor has been investigated. It has been found that although the host fibroblast produced the collagen in the tumor, the concentration of collagen was controlled by the tumor type. In addition, it has been demonstrated that the collagen formed in the tumor was predominantly of the adult type. By the development of a small diffusion chamber which can be implanted in the tumor and connected to the exterior of the animal through a very fine capillary, studies of the interstitial tissue of the tumor during tumor growth have become possible. The interstitial fluid was characterized by a constant composition despite the presence of necrosis, a protein concentration of less than 40% that of plasma, an amino acid level equal to or higher than that of serum, the absence of fibrinogen, a negligible content of free glucose, a lactic acid concentration of 2- to 3-fold higher than serum, and a cholesterol and lipid phosphorus content much lower than that of aortic serum.

Dr. Shelton, with the aid of intraperitoneally implanted diffusion chambers, has studied the interaction between normal and tumor cells. She has found that small numbers of lymphocytic tumor cells markedly inhibited the collagen formation of norma peritoneal cells, but that hepatoma cells had a much less striking effect on connective tissue fibroblasts. She has been able to show replication of the Moloney leukemia virus, after 38 days but not at 9 days, when grown together in the chamber with new-born BALB/c mouse thymus. In collaboration with Dr. Virginia Evans, she has demonstrated that fibroblasts were transformed into malignant cells in the millipore diffusion chamber. This finding provides another example of the malignant transformation in

an essentially *in vivo* environment quite different from that of tissue culture.

Dr. Rechcigl's studies on the multiple factors effecting catalase level of tissues has done much to clarify some of the relationships between the catalase content of liver in a tumor-bearing host with the growth of a neoplasm. Nutritional studies have indicated that amino acid deficiency may be one of the mechanisms by which the catalase reduction in liver of the tumor-bearing rat is mediated. It was shown that the stress of a protein-free diet produced a characteristic lowering of liver catalase in both normal and tumor-bearing animals. However, in hepatoma 5123, a minimal deviation tumor, the catalase level was not altered under these same protein-free conditions. Sex, age, and location of tumor did not affect the tumor catalase level. In some tumors, namely, the transplanted hepatoma 5123, the ethionine-induced hepatoma of rats and the spontaneous hepatomas of C3H mice, high catalase activity was found in the tumor tissue even though a definite depression in catalase content was observed in the liver and kidneys of these tumor-bearing animals. Transplantation of a single, primary ethionine-induced hepatoma into OM/N rats gave rise to two lines of hepatomas, one possessing extremely high catalase activity, sometimes exceeding that of the normal liver, and the second with a low catalase activity. No other significant chemical or enzymatic differences have been obtained in these 2 hepatoma lines which have been carried through 20 transplant generations to date.

In preliminary studies with Dr. Heston it was found that most strains of the C57 B1 mice had half the normal liver catalase concentration except for the C57 B1 substrains, C57 B1/He and C57 B1/An, which had values similar to that found for the liver of other mouse strains. These studies indicate the genetic control of the catalase content of liver. In collaboration with Dr. Wollman it was demonstrated that the removal of the pituitary gland does not prevent or overcome the characteristic catalase depression in the tissues of the tumor-bearing host, previously reported by Utsugi. The catalase depression was present in the tumor-bearing rats, whether hypothosectomy was performed before or after tumor implantation. Preliminary studies with Dr. Warren Evans (NIH Postdoctorate Fellow) have shown that when the catalase of leukocytes was inhibited with aminotriazole the respiration and glucose uptake was not altered.

Development by Dr. Greenfield of methods for the *in vitro* maintenance of hepatic cells in functional state progresses nicely. A high oxygen requirement of liver cell suspensions has been established even at 0° C. in polyvinylpyrrolidone-sucrose mixtures. When maintained in growth media under 95% 0_2—5% CO_2, the cells reaggregated at the miniscus. Initial studies using 95% 0_2—2% $C0_2$ at 2 atmospheres appears promising since under these conditions the cells formed uniform round aggregates on the bottom of the flask. Cells in the aggregates maintained respiration and gave other evidences of viability over a period of 48 hours.

Additional effects of the Lactic Dehydrogenase Agent have been demonstrated. Infection by this viral agent has been found to produce an elevation in both normal and tumor-bearing mice of a number of plasma enzymes besides lactic dehydrogenase, namely: glutamic-oxalic transaminase, isocitric dehydrogenase, malic dehydrogenase, and phosphohexose-isomerase. As has been reported previously for lactic dehydrogenase, the elevation of these enzymes is much more striking in the tumor-bearing animal than in the normal animal. The plasma aldolase levels were, however, not affected by the virus in either the normal or tumor-bearing animal.

Studies of immune phenomena in mouse histocompatibility systems have been initiated by Drs. Mishell (Research Associate) and Greenfield. Since analysis of histocompatibility loci has been handicapped by the lack of suitable *in vitro* tests, development of methods for detecting *in vitro* antibody reactions which reflect histocompatibility loci are underway. Studies of the mouse leukocyte agglutination system are being extended to determine which histocompatibility loci determine these antigens. In early results it has been found that the BALB/c mice have been found to produce satisfactory leukocyte agglutinins when hyperimmunized with DBA/2 normal tissues.

LABORATORY OF BIOLOGY

The Laboratory of Biology continues to maintain a broad biological approach to the cancer problem. Emphasis is on etiology. This year the Tissue Culture Section was added to the programs of the Carcinogenesis Section and the Gen-

eral Biology Section. With the single cloning techniques and the chemically defined media in which cells can now be grown directly from the animal, and with the inbred mouse to which the tissue can be returned for testing for malignancy, this section is in a good position to study malignant transformation.

Carcinogenesis

Dr. Deringer has continued her studies of the multipotential carcinogenic action of urethan using hairless strain Hr mice as test animals for skin carcinogenesis and strain DBA/2eB mice as test animals for induction of leukemia. Urethan painted on the skin of the hairless mice significantly increased the occurrence of epidermoid carcinomas and decreased the latent period of both epidermoid carcinomas and papillomas. Urethan painted on the skin of DBA/2eB mice increased the occurrence of reticulum cell neoplasms and they arose earlier. Most of these were lymphocytic neoplasms, whereas most of those in the untreated controls were reticulum cell neoplasms of types A and B. The painted Hr mice also developed lung tumors and both the Hr and DBA/2eB mice developed multiple hepatomas and hemangioendotheliomas of the liver. Hemangioendotheliomas of the Hr mice were transplatable.

Using a genetically more resistant strain C3H/Lw, Dr. Law found that the urethan administered to neonatal mice was ineffective in inducing leukemia and even inhibited leukemia in C3Hf/Lw mice carrying the Moloney leukemia virus. Multiple hepatomas were found in the treated mice.

Dr. Law has found that thymectomy within the first 24 hours following birth and at 1 month prevents lymphocytic neoplasma in his three high leukemic C3H sublines. However, in those animals in which thymic remnants remain granulocytic leukemias were found relatively late in life. Splenectomy at birth and at 1 month has been ineffective in influencing occurrence of leukemia in the three high leukemic C3H sublines which raises the question of the significance of those histopathogenic changes described in the spleen preceding the appearance of leukemia. Isologous thymic grafts introduced under the kidney capsule following standard, fractionated, X-irradiation did not develop into lymphosarcomas nor induce leukemia in C57BL/Ka recipient mice, but such such grafts given subcutaneously became leukemic.

Dr. Potter has continued the study of induction of plasma cell tumors with mineral oil (Bayol F). Such tumors can be induced in BALB/cAn mice and in F_1 hybrids resulting from outcrossing to the closely related strain A, but not in a number of other strains including A or in other F_1 hybrids. Plasma cell tumors have also been induced in BALB/c mice with Drakeol-6VR which has been used clinically because it was thought not to be carcinogenic. Subcutaneous sarcomas have also been induced with incomplete Freund's adjuvants and to a lesser degree with Bayol F.

In studying the mammary tumor inducing effects of added pituitary glands, Heston and coworkers were able to induce mammary tumors in genetically resistance C57BL virgin females with the implantation of five extra pituitary glands under the capsule of the kidney. None were induced with one extra pituitary gland. This illustrates the relative importance of hormonal factors and the mammary tumor virus. These C57BL females did not have the virus and it is difficult to induce mammary tumors in them with the virus, especially when they are kept as virgins.

Immunology

Because of Miller's report of a wasting illness of mice thymectomized at birth, Dr. McIntire has been interested in this procedure to study the role of the thymus in leukemogenesis and in immunologic responsiveness. His technique of removing the thymus within the first 24 hours of birth resulted in a low mortality (10–20%) compared with (75–100%) reported by others, but later at autopsy there was no evidence of residual thymic tissue. Many of the animals became weak with weight loss and ruffled hair and some had diarrhea, but they had normal or only slightly decreased lymphocytic tissue. Only 1/20 thymectomized mice had a delayed rejection of homologous skin which did not differ at the H–2 locus. Natural antibody titers to gram negative bacilli of these mice were no different from sham thymectomized litter mates. Failure to confirm the reports of Miller suggests that there may be other factors in the illness and death following neonatal thymectomy, the most likely being viral infection.

Miss Uphoff has continued her program on immunologic responses of irradiated mice and protection with bone marrow transplantation. One observation bearing directly on the cancer prob-

lem was the high incidence of leukemia in AKR mice that had received 700r X-irradiation and protected with bone marrow from low-leukemic strains C3Hf, CBA, ST, and the high-leukemic strain C58. Transplantation proved most of these neoplasms to be of donor tissue origin. Similar results were obtained from F_1 hybrids (ARK x a low-leukemic strain). Low-leukemic strains protected with AKR marrow had a low leukemia incidence and all of these neoplasms were of AKR tissue origin.

Virology

Work in this area is concerned with the biology of tumor viruses in experimental animals.

Two mouse leukemia inducing viruses have been discovered by Law and coworkers during the past year. P–LLV was isolated from a 5 month old C3Hf/Bi mouse. BALB/c, C3Hf/Bi, and C3Hf/Lw mice inoculated during the neonatal period with the filtered agent developed lymphocytic neoplasms at 3 months, but DBA/2 and C57BL/Ka mice did not. Passage of P–LLV through the mother's milk was observed (it may later be shown that this is the same as the Moloney virus). S–180LV was isolated from Sarcoma 180. This virus induces leukemia, principally granulyocytic in C3H mice and lymphocytic in BALB/c. (This apparently is not the same as any other known leukemia virus.)

In his continuation study of the natural transfer of mouse lekemia viruses, Law has found that they are transferred readily through the milk but scarcely or not at all prenatally from the mother or from the father. The extrachromosonal pattern for congential transmission of these viruses stands in sharp contrast to the chromosomal pattern of transfer potentialities to develop leukemia in the high leukemia strains such as AKR, C58, F, and C3Hf/Fg and raises the important question of whether viruses are involved in the etiology of the disease in high leukemia lines.

Dr. Andervont has shown that the mammary tumor virus can disappear from certain sublines of strain RIII mice. These lines are remaining low tumor lines. New evidence this year indicates that this disappearance is not limited to the RIII agent in RIII mice but that the potent C3H agent introduced into RIII can also disappear from certain lines.

Further studies by Dr. Andervont on transmission of the mammary tumor agent by the male indicate that the foetus can be infected directly *in utero*. The agent does not pass through the wall of the uterus to infect the mother.

Dr. Barrett has been studying the long latent period of the mammary tumor virus. By injecting agent-free but susceptible mice of different ages, (newborn, 1 month, and 10 months) he showed that the latent period of the tumors was about the same in all groups. They became less susceptible with advancing age. These results suggest that the "latency" represents a maturation of the host-virus relationship and does not represent aging in the host tissue.

To test the concept held by some investigators that the low mammary tumor incidence in C3HfB mice is due not to their being free of the agent but to their having obtained a duplicating inhibitor from the foster strain C57BL, Heston and Vlahakis have started a strain C3HfC by fostering C3H on BALB/c. BALB/c lacks the agent but is susceptible to it and theoretically then would not have the inhibitor. According to this concept the C3HfC, free of inhibitor, should have a high tumor incidence. This has not been observed. We still believe that these agent-free lines have a low incidence of tumors because they are agent-free.

Doctors Sanford and Law have been studying the effects of polyoma virus on cell transformations *in vitro*. All but one strain tested have shown the malignant change and in certain cases the virus treated cells showed this change earlier than the untreated controls. Antigenic alteration has been obtained. Experiments are still in progress to determine whether the malignant and antigenic changes are associated or are independent phenomena.

Cell Physiology and Nutrition

The Tissue Culture Section made an important contribution this year in getting freshly explanted mammalian tissue cells to grow directly in protein-free chemically defined culture media. Three strains of mouse cells started in this manner have been grown continuously. This is the first time cells have been grown in chemically defined media without first undergoing a period of adaptation in media to which serum had been added. It will represent a great advancement for the study of cell

nutrition, for attacking the problem of the malignant change, and for virus work using tissue culture. It ranks in importance with the single cell cloning and the chemically defined media. This contribution can be contributed directly to the work of Dr. Evans and Mr. Bryant, but of course would not have been possible without the work of the whole section in methodology and cell nutrition.

Further advance has been made in growing a variety of cells in chemically defined media after the period of adaptation. Fourteen established cell strains from man, mouse, monkey, and hamster are now growing in chemically defined media. This gives rise to the hope that any cell strain that can be grown in undefined media can ultimately be adapted to chemically defined media.

In their studies on cell nutrition, Dr. Sanford and associates have shown that serum protein when added to otherwise chemically defined media prevented several vitamin and amino acid deficiencies. It appears that the serum protein contributes appreciable amounts of vitamins, amino acids, and other components of the cells. These results raise serious doubts that any accurate quantitative studies on the nutrition of cells can be carried out on a media containing serum protein supplement.

In a protein free media biotin was identified for the first time as a vitamin required for survival of a mammalian cell strain in culture, and vitamin B_{12} was shown to be necessary for maximal proliferation of certain but not all cell types. With the strain of mouse fibroblasts the requirement for biotin could be demonstrated only when the media lacked the purine nucleic acid derivatives and 10 of the 18 vitamins. Vitamin B_{12} was demonstrated to prolong the survival of cells when the media lacked folic acid. Results of studies of effects of 16 other vitamins have led to formulation of simpler media than had formerly been practicable.

In chemically defined protein free media, galactose was found to be utilized in place of glucose. The study is being pursued in order to correlate the activity of enzymes for galactose utilization in 929-L cells with growth responses to galactose.

Evans and Parker have been continuing their studies of the malignant transformation of cells in culture. Cultures of kidney tissue from 3-day-old mouse becomes malignant when cultured no more than 145 days *in vitro*. This indicates that early malignant transformation is not restricted to embryonic tissue in culture. It must now be determined whether cells explanted directly into chemically defined media become malignant and how soon. Tissues grown in diffusion chambers for 18 months did not produce tumors but did, after 23 months in the chambers. In collaboration with Dr. Yosida the chromosome picture of these cells is being followed through this malignant transformation. He has found that cells remain predominantly diploid up to 7 days in culture after which there is an increase in polyploid cells.

Bryant and coworkers have been studying a fragile strain of monkey kidney cells in culture. Mechanical or flow stresses were much less on these cells in suspension cultures in smaller flasks. The cells grew in 125 ml flasks but in 500 ml flasks they grew at a much slower rate or not at all. They grew better in stationary cultures than in the suspension cultures. They also have studied binding of methylcellulose molecules to these cells. This protects the cells not only in suspension culture, but also in the large stationary cultures, possibly when they are scraped from the floor of the flask. Glucose utilization and lactic acid production of these monkey kidney cells in suspension cultures was relatively high. Even in nonproliferating cultures the cells were metabolizing actively. In proliferating cultures the rate of glucose utilization was not directly correlated with growth rate, but in none of the cultures was the amount of glucose remaining in the used fluid so low as to be a limiting factor. These cells were also used in work with Dr. Montes de Oca on the damaging effect of X-irradiation. It was found that serum added to the culture immediately before or directly after irradiation lessened the damaging effect.

In the study of preservation of large stocks of cultured cells in both serum-free and serum-containing media, both types have now been successfully preserved by freezing. In collaborative work with Dr. Deringer, Dr. Evans is attempting to freeze fertilize mouse ova for future implantation.

Genetics

Studies of effects of specific genes on occurrence of tumors are continuing. Heston and Vlahakis have now shown that the lethal yellow (A^y) gene of the mouse increased the incidence and average number of both epidermoid carcinomas and papillomas induced in the skin by painting with meth-

ylcholanthrene. It also decreased the latent period of the papillomas. Deringer has shown that the A^y gene increases the occurrence or reduces the latent period of reticular neoplasms. Formerly this gene had been shown to increase the occurrence of pulmonary tumors and hepatomas and greatly decrease the average tumor age of mammary tumors. Results of transplantation of ovaries indicate that the effect of the gene on mammary tumors is not manifest through the ovary. Studies are underway to see if this action of the gene is through the hypophysis or localized in the mammary gland itself.

The effect of the genetic difference between strains A and C3H both of which have a relatively high incidence of mammary tumors in breeding females with the virus is revealed when the virus is absent. Whereas the tumor incidence in virus-free breeding C3Hf females is about 30 percent, in a new line of virus-free Af mice not one mammary tumor has yet appeared.

Biochemical Genetics

Interest has increased in biochemical genetics of the mouse. In the Laboratory of Biology it is represented by such work as that of Dr. Potter and collaborators on proteins associated with plasma cell tumors and mouse urinary proteins; the work of Heston and Rechcigl on catalase; and the nutritional differences in cells in culture discussed in the preceding section.

Dr. Potter has transplanted over 100 plasma cell neoplasms in the mouse and has characterized the associated protein abnormalities. Seventeen different Bence-Jones proteins associated with these tumors have been characterized. Each Bence Jones protein has a characteristic peptide that is a stable heritable characteristic of the neoplasm. Effort is directed toward determination of number of different chains (i.e., gene product units) and whether the different peptide maps are a function of different disulfide linkages within one molecule. Resolution of the question should provide chemical information on antibody formation since the peptides of Bence Jones proteins are similar to those in antibody molecules.

In collaboration with Dr. Finlayson, Potter has been studying mouse urinary proteins (MUP). They have shown that inbred strains differ in their urinary proteins and these are genetically controlled.

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In the study that Heston, in collaboration with Dr. Rechcigl, is carrying out on catalase activity of the liver and kidney of mice, a survey of various strains has been made. All strains are approximately the same in respect to level of kidney catalase and all strains show a sex difference with the level in males being higher than in females. But a distinct strain difference has been noted in level of liver catalase, strains derived from the original Lathrop stock (C57BL, C57BR, C57L, and C58) having about half as much as other strains. However, the C57BL/An substrain and the C57BL/He derived from C57BL/An have a high level. Genetic analysis of the difference between C57BL/He (high) and C57BL/6 (low) indicates that the difference is due to a single gene with low level dominant over high. This means that what is actually controlled by the gene must be something like an inhibitor holding down the level of catalase activity in the liver. The original Lathrop stock must have had the dominant mutation for low and a reverse mutation to the recessive gene for high must have occurred in the progenitors of the C57BL/An substrain.

Pathogenesis

Dr. McIntire has continued his studies on the pathogenesis and pathophysiology of plasma cell tumors in mice and man, and has made a detailed study of the myeloma kidney in mice associated with these tumors. The characteristic lesion is the clear, homogeneous, eosinophilic hyaline cast within the lumina of the renal tubules. There is a consistency of pattern for a single tumor that would indicate that factors such as infection, obstruction, and blood supply are not so important as the specific characteristics of the Bence Jones protein in the production of the myeloma kidney. The only component in significant concentrations in the casts are the Bence Jones proteins, which by themselves are sufficient to cause the casts. Correlation studies of the characteristics of the plasma cell neoplasms and their associated renal damage indicate that those tumors associated with only a small amount of renal damage have a greater amount of cytoplasm. Bence Jones proteins that produce the least renal damage are those that do not precipitate when heated to 56° C. Some of the Bence Jones proteins are in the form of glycoproteins and this, too, seems to decrease their ability to cause renal damage.

Drug Resistance

In continuing studies on azaserine-sensitive and -resistant lines of plasma cell neoplasm 70429, Dr. Anderson has studied the effects of azaserine, DON, and the structurally similar duazomycin at four steps (glutamine-requiring reactions) in the purine synthetic pathway. Three resistant tumor lines showed marked cross resistance to duazomycin as well as to DON. These results characterized duazomycin as probably a glutamine antagonist. It exerted inhibition on all these glutamine catalyzed reactions much like DON and possibly less like azaserine. In exploring effect of azaserine and DON on sensitive and resistant lines at lower levels of the inhibitors a difference between the sensitive and resistant lines was observed, the reactions being inhibited by lower concentrations of the antagonists in the sensitive cells than in the resistant cells.

In studying biochemical differences in the metabolism of sensitive and of fluorouracil- and fluorouridine-resistant lines of the mast cell neoplasm P815, resistance has been found to be associated with a marked decrease in the activity of uridine kinase which is probably the major mechanism involved in the resistance.

LABORATORY OF CHEMICAL PHARMACOLOGY

From the standpoint of public health the major objectives in cancer continue to be: (1) better methods of treatment; (2) widely applicable methods of earlier diagnosis; and (3) preventive measures.

Progress toward the achievement of these goals requires more knowledge and understanding of the many phenomena involved. The facets of the malignant diseases are so numerous as to justify the many different approaches that have been employed by others. The various programs that have been carried out over many years by the Laboratory Chief and his associates have had as a basis the underlying concept that study of chemical agents and of chemical processes would be useful for chemotherapy, for prevention of carcinogenesis, and for development of laboratory methods of early diagnosis. In continuation during the past year of long-range investigations, there was planned convergence of these basic science studies to illuminate selected aspects of the immunology, molecular biology, virology, and chemotherapy of cancer.

The methods and materials employed ran a wide gamut, cutting across the conventional scientific disciplines. They included the methods of immunology, bacteriology, and virology (both bacteriophages, and viruses that induce tumors in mammalian cells); mammalian cytology in tissue cultures and in tumor ascites; biochemistry (endotoxic polysaccharides, intermediary metabolism as affected by anti-cancer agents, and enzymology); physical chemistry (density gradient centrifugation, streaming birefringence, amperometric titration, nuclear magnetic resonance and electron paramagnetic resonance spectroscopy); radioactive tracer techniques; methods of inhibition analysis of anti-cancer agents in tumor-bearing animals; etc.

During the past year, research on cancer-causing virsuses and on cancer immunology was extended. All the organizational units of the Laboratory contributed to one aspect or another of these subjects. Highlights of the work are integrated in the following pages without separation on the basis of administrative Sections.

Endotoxins

Progress was made in several directions: overcoming the tolerance, induced by a single dose of endotoxic polysaccharide, which prevented tumors from responding to a second dose; success in the passive transfer of immunity to endotoxin; chemical and physicochemical characterization of endotoxic polysaccharide preparations; preparation of biologically potent endotoxin, containing a minimal amount of bound lipid, in a homogeneous state; demonstration of the presence in the mammalian host of enzymes capable of inactivating endotoxin; demonstration of a basic protein from liver which inactivated, reversibly, endotoxic polysaccharide; and synthesis of additional polysaccharides with functional groups.

In the investigation (O'Malley, Shear) of the nature of the tolerance induced by *S. marcescens* polysaccharide; and of measures designed to overcome it so that tumors would repeatedly respond to successive doses, the first two reports appeared in the December 1962 number of the JNCI. The third paper in this series, describing the surmounting of tolerance by appropriate spacing and size

of the second and later doses, has been completed for publication. It reports the repeated repression of the growth of mouse Sarcoma 37 by each of four successive doses. The high mortality accompanying this procedure, especially after the first dose, continues under investigation. Deaths from a first dose several times the LD_{100} have been completely prevented by administration of saline. These results are now being prepared for publication as well as the progress made in the fractionation of blood serum, from mice treated with *S marcescens* polysaccharide, to isolate the potent tumor-damaging component.

The immunity engendered by endotoxins is generally held to be ineffectual. Most of the published work on endotoxic immunity has been carried out in the mouse; the criterion employed has been the number of MLD's neutralized by antisera. However, normal mice are highly refractory to the lethal action of endotoxins. The prevailing pessimistic view was re-evaluated (Landy) in mice rendered sensitive to the lethal action of endotoxin by two different methods: implantation of Sarcoma 37 (6 days prior to use); or, vaccination with BCG (ten days before test). These treatments greatly enhanced the sensitivity of mice to the lethal action of endotoxin so that the highly protective effect of rabbit antiserum to *Salmonella enteriditis* was now demonstrable. Passive transfer of 1 ml. of antiserum (0.3 mg. antibody nitrogen), a half hour prior to challenge with massive doses of *S. enteriditis* endotoxin, neutralized as much as 100 LD_{50} and 1,000 tumor-damaging doses in mice bearing Sarcoma 37; in BCG-vaccinated mice this quantity of antiserum protected against more than 1,000 LD_{50}. Failure of previous efforts to appreciate this powerful protective effect, of passive transfer of antiserum to endotoxin, was thus found to be a consequence of the unsuitable experimental conditions which had masked the effectiveness.

Physico-chemical characterization (Oroszlan, Mora) of the tumor-necrotizing polysaccharide from *Serratia marcescens* was carried out by ultracentrifugation and zone electrophoresis. Two components were identified. The one with the higher molecular weight ($S'=8.24$) and which migrated more slowly in the electrophoretic field was identified as the component possessing tumor-damaging activity. Further purification of this latter component was accomplished by centrifugation in a CsCl density gradient. It was found to be dissociable with sodium dodecyl sulfate into smaller units with a sedimentation constant of $S'=0.9$. Depolymerization into these subunits, of about the size of haptene, was accompanied by loss of tumor-damaging activity. Upon removal of the dissociating agent, reaggregation took place with reappearance of the biological activity. These findings indicated that a macromolecular complex of critical size, held together by other than covalent bonds, is required for the polysaccharide to elicit characteristic endotoxic activity.

Collaborative work (Landy, Ribi) with endotoxic materials from other gram-negative organisms has further clarified the relationship of the chemical composition of endotoxic polysaccharides to their biologic activities. The method (Ribi) of isolation from *Salmonella enteriditis* of an endotoxin with minimal amounts of lipid and of nitrogen was extended to other bacterial species to assess the generality of the important findings. Viable bacilli (*E. coli*, *Bordet. pertussis*, *Serr. marcescens*) were extracted with water saturated with diethyl ether. The crude endotoxin was deproteinized with phenol-water. Then the bulk of bound fatty acids and esters was complexed with $LiAlH_4$ and removed. Polysaccharides were obtained containing only 0.3–0.5 percent nitrogen and 2–3 percent fatty acid esters. These products displayed undiminished potency in evoking the host reactions characteristic of bacterial endotoxin.

The widely-heralded importance of "lipid A" as the component in endotoxins which is responsible for their characteristic activities thus has not obtained any support in these careful studies. To the contrary, evidence continues to accumulate that it is the polysaccharide moiety which is responsible for the potency of these complexes. In Ribi's preparations a second component was found which was immunochemically similar to the haptenic polysaccharide obtained on acid hydrolysis of endotoxin. The content of this material in the endotoxin was reduced, by centrifugation, to less than one percent as estimated from gel-diffusion data.

Biologically potent endotoxin has thus been obtained in an essentially homogeneous state, as indicated from chemical, ultracentrifugal, immunodiffusion, and quantitative precipitation analyses. As was indicated by the work in another part of

the Laboratory (Oroszlan, Mora) discussed earlier, it appears increasingly likely that the biological effects require a macromolecular structure composed of polysaccharide units similar to, if not identical with, haptene.

The discovery of host factors which inactivate endotoxin was carried further (Landy, Waravdekar, Shear). Enzyme preparations from two sources—blood plasma and cell-free homogenates from liver—were incubated *in vitro* with highly purified endotoxin; the products were examined with immuno-diffusion techniques. With plasma, the reaction product acted like the haptene polysaccharide obtained on acid hydrolysis. With the enzyme from liver, no lines of precipitation were discernible in the agar gel diffusion test; this indicated that the endotoxin had been degraded in a fashion which did not yield haptene.

By another procedure a fraction was obtained, also from rabbit liver, which inactivated endotoxin by a quite different mechanism, viz., by a complexing which was reversible (Oroszlan, Mora). Material was leached out of intact cells of rabbit liver which rendered *S. marcescens* polysaccharide incapable of damaging Sarcoma 37. This factor from liver was in the basic protein fraction. Macromolecular interaction between these cationic proteins and the anionic endotoxic polysaccharide was demonstrated by zone electrophoresis and by metachromasia. Polyglucose sulfate, a synthetic polysaccharide with more strongly anionic properties than the bacterial one, blocked the inactivation. After inactivation of endotoxin by the basic protein fraction, the original tumor-damaging potency was restored by treatment with polyglucose sulfate. Other experiments, with cationic derivatives of synthetic polyglucose, confirmed the conclusion that the inactivation was a consequence of complex formation, and that the complex was dissociable.

Synthetic polysaccharides (Mora), and derivatives containing groups which confer basic or acidic properties, have already been useful in a variety of investigations. Additional derivatives have been prepared (Wood) containing various functional groups.

Cancer Immunology

In addition to basic science investigations in immunology, attention was directed during the year to several selected aspects of immunology which bear directly on the cancer problem.

The question of antigenic differences between malignant and normal tissues was investigated further (Leise, Landy). The report of McKenna and Blakemore on the isolation of a distinctive antigen from extracts of HeLa cells could not be confirmed; the results of complement-fixation, hemagglutination, and gel diffusion tests indicated that the antigen they reported was not present in the HeLa strain used here.

Our scientists found that the intact HeLa cell evokes antibodies even after a short course of immunization (3 injections). Bacterial endotoxin as adjuvant speeded up antibody production. These antibodies were demonstrable only with complement-fixation. On the other hand, extracts of HeLa cells gave rise to antibodies which could be demonstrated by hemagglutination and gel diffusion as well as by complement-fixation. Here, too, endotoxin was an effective adjuvant in obtaining a quick response. Fewer antibodies were obtained in the rabbit with intact HeLa cells than with cell extracts.

In previous work, it had been reported (Messineo) that rabbit antisera to deoxyribonucleoprotein (DNP) from normal human leucocytes did not cross-react with DNP from leukemic cells. This line of work was continued. DNP produced, with rabbit antisera directed against it, only one line of precipitation in agar gel diffusion tests, suggesting that the DNP preparations were homogeneous. More highly concentrated solutions of DNP were prepared, as high as 20 mg. per ml. (the approximate limit of solubility). Antisera were also prepared with considerably larger amounts of DNP than had been used previously. The antisera of higher titer, examined in gel diffusion against the more concentrated solutions of DNP, still showed but one line of precipitation, indicating the presence of but a single antigenic component.

Rabbit antisera were prepared against homogenates of leukemic leucocytes (human) and were examined in precipitation tests against antigenic material from normal and leukemic leucocytes. The results indicated that the aforementioned immunological reactions of DNP were not attributable to extra-nuclear material.

The so-called natural antibodies have been recog-

nized for many years but their origin and properties are still but little understood. The assay for natural antibody against gram-negative bacteria was greatly simplified (Landy, Weidanz). With this improved method, further information was obtained (Landy, Weidanz, Henthorne, Silverstein) on the presence of natural antibodies in several distinctive situations. These included the foetus and neonate, especially in those mammalian species where the likelihood of maternal transfer of antibody is minimal, and in animals maintained in a germ-free environment. Among the sera examined were those from foetal lambs, newborn swine, calves, and sheep. Among the findings was presumptive evidence that in the sheep, at least, the foetus is capable of synthesizing natural antibody *de novo*.

Cytolytic and cytotoxic antibodies are attracting the attention of an increasing number of investigators. A new electronic method of estimating such antibodies was devised (Hirata) which is both simple and sensitive. Cells damaged by the immunologic system of antibody and complement are digestible by trypsin; undamaged cells are not digested. An electronic particle counter then enumerates viable cells but not those digested by trypsin.

This method was employed in a comparison of the potencies of antisera to Sarcoma 37. Ascites tumor cells were exposed to various dilutions of the antisera, plus complement, after which they were treated with trypsin. The surviving cells were then enumerated with the electronic particle counter. The dilutions which lysed 50 percent of the cells represented the relative potencies of the antisera. This method is capable of application to many cellular problems. The particle counter was also found (Hirata) useful in objective estimation of hemagglutination.

Where the electronic method is not available, an alternative method of cytolytic antibodies can be employed. This was developed (Hirata) on the basis that: (1) specific antibody plus complement render cells non-viable, and hence susceptible to depolymerization of their DNA by DNAase; (2) native DNA takes up methyl green whereas depolymerized DNA does not. With Sarcoma 37 ascites cells as a model system, this method was found to be as precise as the electronic one.

The highly important subject of the mechanism of antibody synthesis is being advanced by the use of experimental cell-free systems *in vitro*. Such a program was initiated (Sussdorf) at the molecular (sub-cellular) level during the past year. The system currently employed has yielded results which indicate that it is biosynthetically active. This line of investigation has reached the stage where rapid data accumulation should permit interpretations.

Tumor-Producing Viruses

In preparation for investigation, on the molecular level, of the mechanisms by which viruses transform normal mammalian cells into malignant ones, experience in techniques of virology was acquired (Mora and coworkers) in previous years with bacteriophages and bacteria. Inactivation of T_2 coli phage with polyanions and polycations was reported previously.

The orientation of the DNA in the intact phage head has been examined (Rizvi, Gellert of NIAMD)) by streaming birefringence. Treatment with polyglucose sulfuric acid disrupted the organized DNA molecule. The mechanism was similar to that of cadmium cyanide, i.e., the phage lost its ability to kill and lyse bacteria but retained full ability to attach. It was concluded that the proximal tail structure was damaged.

In work with oncogenic viruses, it is of course helpful to purify further the virus preparations. Starch agar gel electrophoresis has been developed (Oroszlan, O'Connor) for purification of the various strains of polyoma virus. The large (hemagglutinating) and the small (nonhemagglutinating) plaque-forming strains were investigated. In the case of the large plaque-forming strain, complete recovery of hemagglutinin and infectivity was obtained in a sharply separated zone. The hemagglutinin of the small plaque strain is known to be masked by an inhibitor substance. Separation of the hemagglutinin from the inhibitor was achieved with starch agar gel electrophoresis after heat treatment of the virus.

A new medium for the separation of viruses by density centrifugation has been found (Oroszlan, O'Connor). The polyglucoses prepared here (Wood) gave very stable density gradients which provided resolution of polyoma virus into two components: intact virus, and empty capsules. The resolution was superior to that obtained with the methods in the literature. Polyglucose, moreover, is inert to virus and non-toxic to mammalian

cells; the dialysis prior to assay in tissue culture, which is necessary when CsCl gradients are employed, is rendered unnecessary.

In view of the importance of quantitation in assays of oncogenic viruses, attention has been devoted to elaborating a standardized technique. Several beneficial modifications have been introduced (O'Connor) in the plaque assay of polyoma virus in mouse-embryo monolayers. Titers were obtained in excellent agreement with those in Dulbecco's laboratory on the same stocks. Work has also been started (O'Connor) on the propagation of the Rauscher virus in tissue culture with the aim of developing a quantitative *in vitro* assay.

In the course of systematic investigations on chemotherapy of animal tumors, unexpected findings (Goldin and co-workers) indicated that immunologic phenomena were involved in some of those situations. The mice used were pure strains (DBA, BALB/c) and F_1 crosses of them; the tumors employed were L1210 and leukemias induced in these mice with either Moloney virus or Rauscher virus. Experiments were carried out with skin grafts and with normal tissue implants, with and without X-irradiation of the tissues and of the hosts. Evidence was obtained (Glynn, Bianco, Humphreys) that antigenic differences exist in certain of these situations, and that the antibodies which form against the implanted tumors can enhance the therapeutic effect of the antitumor drug administered. Analogous findings were made with carcinoma 755 in C57B1 mice treated with 6–MP; this was shown by blocking of the immune response by pretreatment with Melphelan. Abrogation of the homograft response was previously obtained with folic acid antagonists; this permitted L1210 to grow in BALB/c mice. This line of work was extended to other tumors (resistant sublines of L1210, Ca 755), to other strains of mice, and with other antitumor agents (6–MP, 6–TG, BW 322 and 323, Cytoxan, Melphelan). Suppression of the immune response has continued to be obtained, in varying degree, in these various situations.

Chemotherapy

The various lines of investigation in chemotherapy of animal tumors continued to be prosecuted vigorously and extended (Goldin and coworkers) in a number of directions. The influence of antibodies, contributing to the effect of chemotherapeutic agents, has already been mentioned.

The scope of this large and varied program is indicated by the following:

Tumors Studied: Leukemia 1210 and many sublines, resistant to one or another anti-tumor agent, developed in this Laboratory. Adenocarcinoma 755. Spontaneous mammary tumors. Leukemia P1534. Plasma cell and Mast Cell tumors. Tumors induced by Moloney Virus and by Rauscher Virus.

Mice Employed: Various pure strains, and their F_1 hybrids.

Chemical Agents Examined: Many folic acid and purine antagonists, alkylating agents, plant products, and newly introduced synthetic compounds.

Topics Investigated: Role of antitumor antibodies in chemically induced regressions; chemical repression of the immune response; central nervous system leukemia; relative efficacy of different dosage schedules of drug administration; combination therapy; drug resistant variants of sensitive transpalted tumors; virus-induced leukemias, and transplantable lines derived from them; antiviral chemotherapy; mechanisms of drug action and of resistance to therapy (Dihydrofolic reductase; Carbamylphosphate synthetase; Lactic dehydrogenases); evaluation of efficacy of anti-tumor drugs; selection of compounds for clinical study.

Team Work and Collaborative Work: The members of the staff of the Biochemical Pharmacology Section operate as a harmonious team, working together in group attack on the various facets of the chemotherapy program. (This precluded a simple way of indicating the individuals working on each of the many lines of work.) Intimate liaison is maintained with the clinicians studying chemotherapy in the cancer patient in our Clinical Center. Active collaboration is conducted with leading scientists in outside institutions. The Drug Development and Evaluation Program (Contract No. PH–43–62–182) is conducted with the Project Officer (Goldin) and Assistant Project Officer (Venditti) dovetailing the project operation with the program within the Biochemical Pharmacology Section.

These activities have resulted in a steady increase in our knowledge and understanding of ex-

perimental chemotherapy in many directions. For example, a new class of drugs—the terephthanilides—has been found to produce longer survival of leukemic mice than amethopterin. Quantitative comparisons have been made, in mice with advanced leukemia, of six clinically active drugs; these included Vincristine and Methyl-GAG. These data were of value for the new Leukemia Task Force which sponsored acquisition of information on the correlation of animal data with clinical results. Surgical removal of spontaneous mammary tumors in C3H mice was followed by recurrence in 80% of the cases; with surgery plus Cytoxan therapy, recurrences were reduced to 10%. An important extension of the work consisted of inclusion of chemotherapy against virus-induced tumors and transplantable whole-cell tumors derived from them, and chemotherapy against the viruses themselves. Four compounds were tested against intracerebrally-inoculated L1210; hydroxyurea and methylglyoxal-bis-guanylhydrazone increased median survival time over the controls by 80 and 70%, respectively. Fourteen pairs of compounds were evaluated for ability to provide therapeutic synergism in the treatment of advanced leukemia; at their optimal combination dosage levels, five of the pairs were more effective than the optimal daily dose of either drug alone. In the treatment of advanced mouse plasma cell tumor LPC–1, four drugs were found to increase survival time as compared with untreated controls.

A battery of six transplantable virus-induced lines of leukemia is being developed for large-scale assay of antitumor agents. This is required because such tumors exhibit a wide range of sensitivity to chemotherapeutic agents. The six clinically active drugs mentioned above were rated, also, with regard to their effectiveness in mice bearing virus-induced transplanted tumors; Cytoxan was the most effective in those experiments. A large number (52) of chemical agents is being screened in an antiviral assay system. These experiments are still in progress but, already, the technique is being modified by starting with still younger mice in an effort to shorten the latent period of the virus induced leukemia. Various other assay methods for investigations on virus tumors are in course of development. With primary leukemia induced by the Rauscher virus, 4 of 11 drugs examined produced an increase in survival of more than 100%. Chemicals were tested for antiviral activity both *in vitro* and *in vivo*. Alkylating agents exhibited antiviral activity in the former situation; of seven compounds tested in the latter system, Vincristine was found the most effective.

Biochemical studies of the mechanisms of drug action, and of resistance to chemotherapy, were continued. Attention was focused on dihydrofolic reductase activity as a measure of resistance. Metabolite-antimetabolite relationships were investigated by searching for metabolites capable of reversing the anti-leukemic potency of selected antimetabolites. Other avenues explored involved: the elevation of carbamylphosphate synthetase activity in livers of mice receiving antileukemic levels of hydroxyurea; lactic dehydrogenases in normal and malignant tissues; the effects of antitumor agents on amino acid transport into Sarcoma 37 ascitic cells; the association of chromosome alteration with increased levels of dihydrofolic reductase in amethopterin-resistant variants of L1210; the distribution of C^{14}-labeled Methyl-GAG in sensitive and in Methyl-GAG-resistant L1210; biochemical indices of chemotherapeutic effectiveness; and, correlation of therapy against a plasma cell tumor with disappearance of the abnormal protein associated with advanced stages of this disease.

LABORATORY OF PATHOLOGY

The work in the Laboratory 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. It is, therefore, convenient to divide this summary into a number of sections.

1. 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. The 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 histologic sections in a given experiment; he may serve as a

consultant to review selected material with no responsibility for the entire experiment or 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.

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

It would be tedious to consider all the collaborative work now in progress in the Laboratory of Pathology, especially since it is often covered in reports from other laboratories. However, the following are noteworthy: Fluorescent antibody studies by Dr. Malmgren: (a) plasma cell neoplasms in man and animals carried out with Dr. Fahey and Dr. Soloman; (b) relationship of autoimmune diseases to sclerosis of blood vessels with Drs. Elmore and Sokoloff, and (c) location of SV 40 virus in cells in tissue culture with Dr. Rabson, Dr. O'Conor, and Dr. Kirschstein.

Dr. Malmgren has used cytodiagnosis techniques in collaborative studies of metastasis of animal tumors with Dr. Madden, Dr. Retick, and Dr. Sabesin. It was found that after an intravenous injection of a large number of tumor cells, most of the cells had disappeared from the circulation in 1 minute and all were gone in 10 ten minutes. Homologous and isologous tumor cells were removed from the circulation at the same rate and the metastatic potential of the tumor did not influence the rate of removal from the blood stream. Cytologic techniques were also used in collaboration with Drs. Retick, Sabesin, Ketcham and Mrs. Hume to study the transplacental transmission of melanoma in animals. It was observed that in a few instances melanoma cells were found in the blood of new born mice whose mothers had received an intravenous injection of S–91 melanoma cells.

Dr. Ross MacCardle in collaboration with Dr. Frederick Bartter has described hyperplasia of the juxtaglomerular apparatus that is peculiar to a condition of hyperaldosteronism and hypokalemia with normal blood pressure. Dr. MacCardle has also collaborated with Dr. Van Scott in showing aborative mitosis during the resting phase of the hair growth cycle.

Dr. Clyde Dawe has collaborated with Dr. Helen Curth and grown a human tumor in tissue culture. This tumor was associated with acanthosis nigricans in man, but growth in the hamster cheek pouch failed to induce skin changes.

Dr. H. L. Stewart and Dr. K. C. Snell continue collaborative work with Dr. Harold Morris on tumors induced in rats by several compounds of the fluorenamine class; one of these N,N′-2-7-fluoroenylenebisacetamide, is remarkable because of the great variety of tumors induced.

Dr. T. B. Dunn has collaborated with Dr. Andervont on a study of tumors in wild house mice; a great variety were found in mice over two years of age. Dr. Dunn also collaborates with members of the Laboratory of Viral Oncology on the induction of tumors in mice by viruses.

Dr. Elizabeth Chu is collaborating with Dr. Richard J. Wurtman (Laboratory of Clinical Science, NIMH) in the investigation of the relationship between the pineal gland and the estrous cycle of rats. Injection of an extract of the pineal gland alters the cycle, and disturbs the hormonal balance. Such conditions are always of interest in cancer research.

2. Accumulation of Data Relating to Laboratory Animals

This is a continuing activity in the Laboratory of Pathology. Precise knowledge regarding the normal anatomy of the laboratory animals is often lacking, particularly as regards variations in inbred strains and the alterations appearing when animals reach the age when cancer can be expected. The investigator using laboratory animals must know his basic material for he relies upon it much as a chemist relies upon the substrates in a reaction. Dr. Stewart and Dr. Snell are accumulating data on aged rats from five inbred strains. Dr. Snell is also collecting data on the Mastomys, a species of rodent recently introduced to the Laboratory. Dr. O'Gara is acquiring information on spontaneous diseases of monkeys, especially the newborn. Dr. Banfield, Dr. Dunham, Dr. Herrold, Dr. Chus, and Dr. Swarm are collecting data

on the hamster, a species now popular in cancer research, and about which our knowledge is still limited. Dr. Dunn continues to collect information on the endocrine system of the mouse. Dr. Swarm has under observation a breed of rats with an inborn error in the metabolism of bilirubin. Basic data are required before many of these animals can be used with greatest profit.

3. The Natural History of Cancer in Man and Applications to Research With Animals

Pathologists, even when doing animal research, remain aware that cancer is a leading cause of death in man. They are acquainted with cancer in man and rely upon this knowledge for important information and leads for animal research. Several studies are now in progress on cancer in various ethnic groups in different geographic areas. Animal studies have also been devised, and are being carried out in the Laboratory of Pathology to correlate with observations in man. The incidence of cervical cancer in women is being studied by Dr. Dunham and others, and Dr. Elizabeth Chu is inducing carcinoma of the cervix in the Syrian hamster and noting factors which will alter the effect of carcinogens. Dr. Katherine Harrold is reviewing lung cancer in veterans of World War I, and has devised a method for inducing lung cancers in hamsters. Dr. Roger O'Gara has studied esophageal cancer in natives of the Transkei, and obtained an iron pot used by victims of this disease, and mice have been exposed to metal from this pot as a test for a possible carcinogen. Dr. Lucia Dunham has duplicated the quid used by betal nut chewers who often develop cancer, and inserted it into the cheek pouch of hamsters, but no cancer has yet resulted. Dr. Katherine Harrold is carrying out studies on the hamster cheek pouch with known carcinogens. A cooperative study on gastric cancer in Japan has been started with Mr. Haenszel of the National Cancer Institute and Dr. Mitsuo Segi of Japan. Urinary bladder cancer is especially frequent in New Orleans, and plans have been made to obtain adsorbates of the drinking water to test on laboratory animals. Because bladder cancer in Egypt appears directly related to schistosoma hematobium infection, Dr. Louis Thomas has infected a large number of hamsters with this organism. No bladder cancers have been found.

An important study on the natural history of cancer is being carried out by Dr. Mearl Stanton and collaborators. This is the "cancer eye" appearing in some cattle herds. This form of cancer is of economic importance and may also furnish important information on the natural occurrence of cancer. Many lesions do not progress to cancer and during incipient stages those that will progress cannot be distinguished from the others. The lesions begin on nonpigmented areas of the lid and the possibility of an insect-borne virus is considered.

4. Carcinogenesis

Carcinogenesis continues to be a fruitful field for study by the pathologist since pathogenesis is always of major interest. Types of carcinogenesis now under study may be divided into (*a*) Viral, (*b*) Chemical, (*c*) Endocrine, and (*d*) Others.

(*a*) *Viral carcinogenesis studies with polyoma virus*

Dr. Dawe has worked for several years in collaboration with Dr. Law, Dr. Rowe, and Dr. Rabson. Recent findings are that the transformations produced by the virus in organ cultures are similar to those produced *in vivo* and transfer from tissue cultures back to the mouse produce typical polyoma type tumors; tissue cultures made from old animals react to the virus as readily as cells from young animals; total body irradiation makes adults susceptible to induction of tumors; specific antipolyoma antibody if used before the 5th day of a culture infected with polyoma will prevent tumor formation; and epithelium does not show neoplastic poliferation in the absence of mesenchyme, in fact a combination of both elements is required. It is expected that time lapse photography will help to elucidate this perplexing problem of epithelial-mesenchymal association. Discovery of this association raises important problems relating to embryogenesis and tumorigenesis. Dr. Mearl Stanton with Dr. H. Otsuke, a Visiting Scientist, has continued studies on polyoma-induced hamster tumors. Dr. Alan Rabson has produced a variant of polyoma with reduced oncogenic potency by growing it on a milk medium. Dr. Robert Friedman has found a reduced interferon production in this strain. He has also been studying the role of interferon in recovery from viral infections and the possible therapeutic use of agents that stimulate interferon production.

Dr. Malmgren and Dr. Rabson found that infection with polyoma virus did not affect chemical carcinogenesis.

Studies have also been conducted with SV 40 virus, which produces tumors in hamsters. Dr. Dawe observed transformation of tumor kidney cells in tissue culture by this agent. Ependymomas in Mastomys were produced by Dr. Rabson and coworkers by the SV 40 virus, and morphologic changes were produced in human thyroid cells in culture. Dr. O'Conor is now studying the polyoma and SV 40 virus with the electron microscope. Dr. Dunn has continued collaborative work on the leukemia-inducing viruses of Dr. Moloney and Dr. Rauscher. An experiment with the Rauscher virus in BALB/c mice failed to induce leukemia, probably because the animals died early from hemorrhages in the spleen. An experiment now in progress with a hybrid mouse should be more successful since reaction in spleen is less severe. Dr. Kamel has examined over 1,000 BALB/c mice injected with material from human cancer. Material from one gastric carcinoma caused an increase in renal tumors that appears to be significant.

(*b*) *Chemical carcinogenesis*

Some of these studies have already been referred to in this summary, because they were attempts to duplicate conditions that might appear in man. Dr. Mearl Stanton has produced lung cancer in rats by a combination of an infarct-producing chemical and methylchloanthrene. Other potential carcinogens including viruses are now being investigated, especially those implicated in human lung cancer. This experimental device offers many possibilities and represents a combination of factors which may simulate conditions in human carcinogenesis. Dr. Roger O'Gara and collaborators are investigating two interesting and important facets of chemical carcinogenesis. (1) Induction of neoplasms by minute doses of a carcinogen given to newborn animals; the great susceptibility of the newborn animal indicates that special precautions should be taken to avoid exposure in the young human being. (2) Attempts at induction of cancer in newborn monkeys have not yet succeeded. However, much needed experience and information is being acquired on care of the young primate, and diagnosis of its diseases. Dr. Ambadas Mulay has continued his studies on the induction of hepatomas in rats and substances that affect the growth of these tumors. Dr. Dunn has found a high percentage of cancers of the vagina in mice given a single dose of diethylstilbestrol at birth, and kept 12–26 months. Three granular cell myoblastomas were also found and one has been successfully transplanted. Dr. O'Gara has attempted to induce epithelial thymic tumors in mice by stilbestrol and radiation, but although lymphosarcomas are induced true thymomas have not yet appeared. Early results indicate that mice thymectomized when newborn have developed more tumors from carcinogen injection that sham operated controls but the results are not final. This work is being done in collaboration with Dr. Malmgren. Dr. Richard Swarm is studying thorium induced neoplasia in man and in experimental animals.

5. Transplantable Tumors

The first studies on transplantable tumors in animals were made by pathologists and this interest has continued. The interpretation of the effects of certain tumors on the host has given important information regarding the function of normal organs, especially the endocrine organs. Work on transplantable adrenal cortical carcinomas is being continued by Dr. Mulay. Dr. Mulay has found an absence of glucocorticoid secretion by one of these tumors. Dr. Swarm has found that the uptake of S^{35} in a chondrosarcoma is reduced when compared with normal cartilage of the host. Dr. Swarm is also investigating the transplantation behavior of this tumor in comparison with normal cartilage. Dr. Banfield, with Mrs. Brinddley, has continued to study a contagious reticulum cell sarcoma in the hamster. There is some indication that contagion is not always by cellular transfer because some animal contacts have developed tumor 17–19 months later. Dr. MacCardle has made further studies on freshly-induced and transplantable plasma cell tumors. The same type of cell, the plasma cell, produces different types of globulin at different stages of maturation. Dr. El Bolkainy, a Visiting Scientist, has studied a hormonally active adrenal cortical tumor in the mouse. He has developed methods for hypophysectomy and observed variation in the tumor when grown in hormonally altered hosts. This work will be continued when he goes to the University of Michigan for postgraduate work.

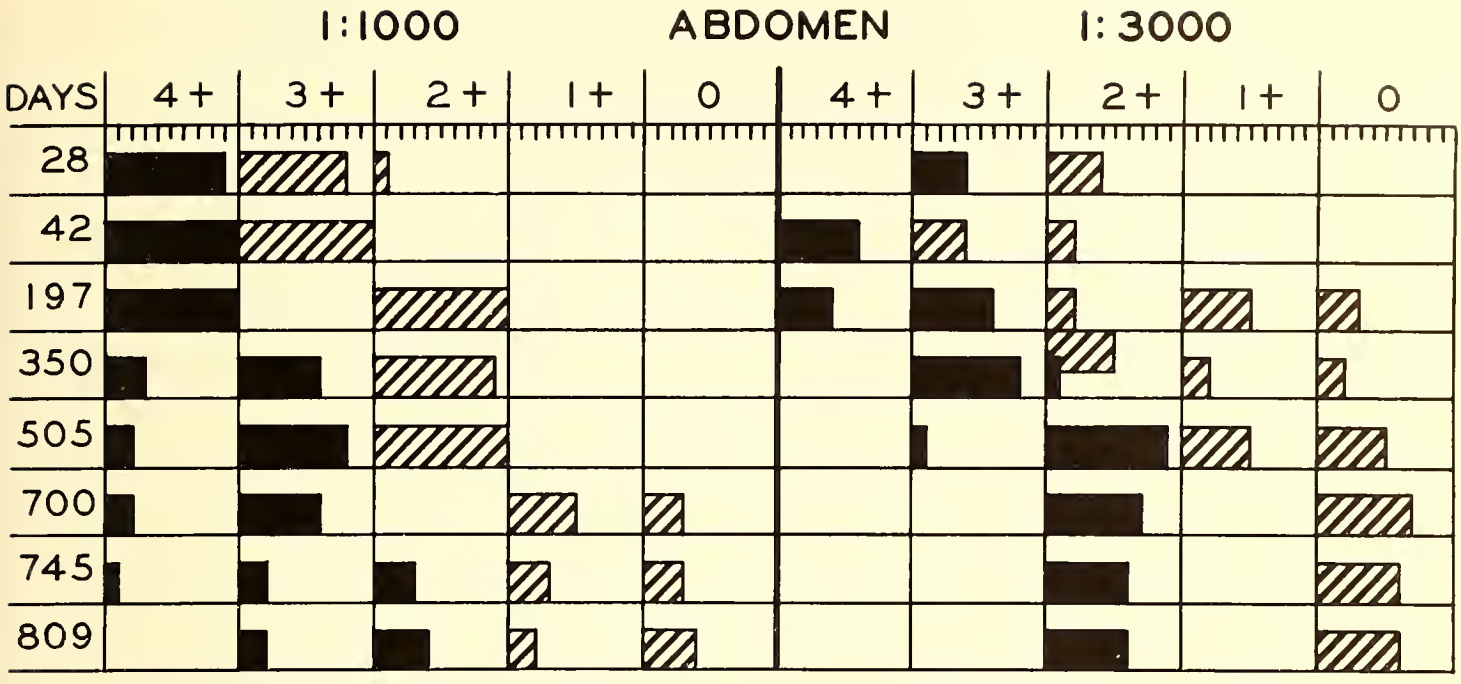

Chart 1a

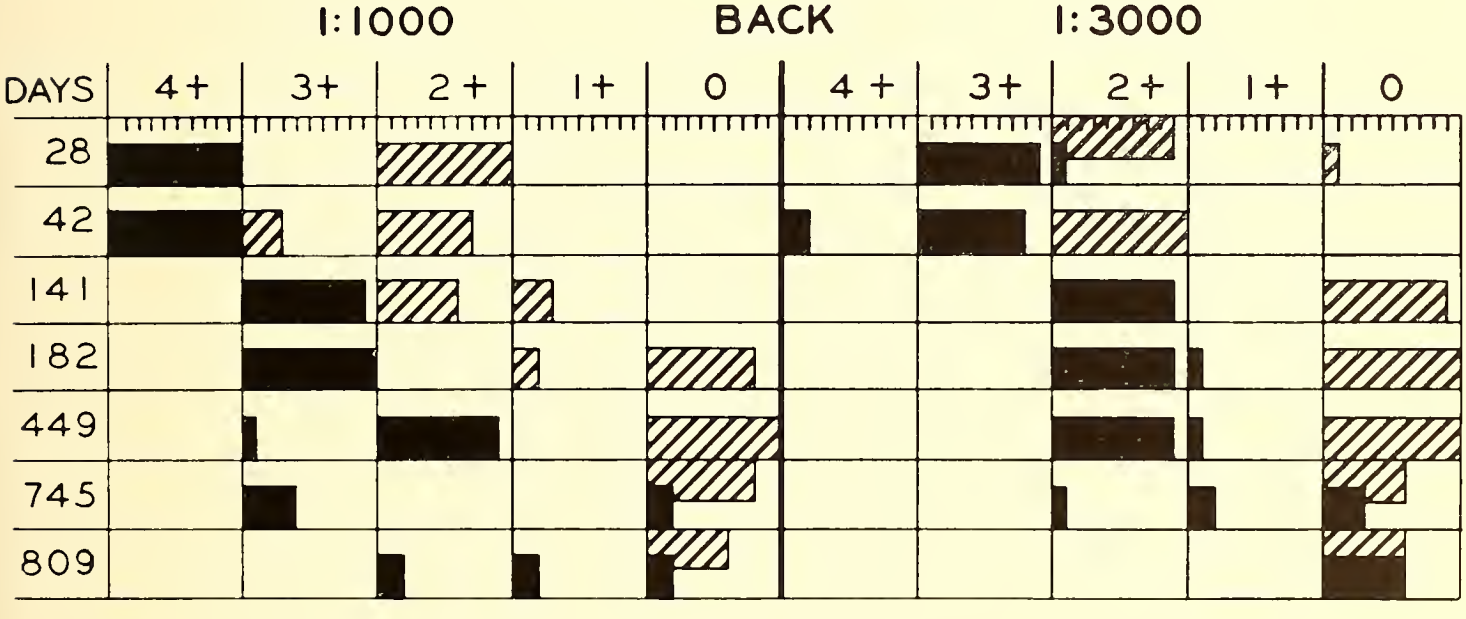

Chart 1b

Age variations in the acetic acid-extractable collagen from hamster skin

One-tenth of a gram of skin, kept in a single piece as far as possible, was extracted with 5 cc. of dilute acetic acid (1 : 1000 or 1 : 3000) for 24 hours at about 9° C. About 1.5 cc. of the extract was then transferred to a 10X75-mm. test tube and about 0.3 gm. of sodium chloride crystals added. If soluble collagen was present, it precipitated in characteristic white fibrils, which were lighter than the extracting medium and tended to float to the top of the tube. The precipitate was graded visually and estimated at 0 to 4+. A few strands of precipitate floating to the top of the tube were graded 1+; a veil over the salt subsequently floating to the top, 2+, and a heavy precipitate sticking to the salt, 3+. A concentration of collagen in solution great enough to form gel around the salt and causing all the granules to clump was read as 4+.

A portion of the initial extract was diluted 1 : 8 and the precipiate measured again to obtain a finer measure of the amount of collagen extracted.

Animal age in days is in the left hand column. The results for extraction using 1 : 1000 dilution of acetic acid are on the left of the chart and those for 1 : 3000 dilution on the right. The bars represent the number of animals of each age whose skin extract gave the precipitate designated by the plus reading at the top of each column. Each mark below the top line represents one animal. The solid bars represent the undiluted extracts and the hatched bars the extracts diluted 1 : 8. Chart 1–a is for abdominal skin and 1–b for back skin.

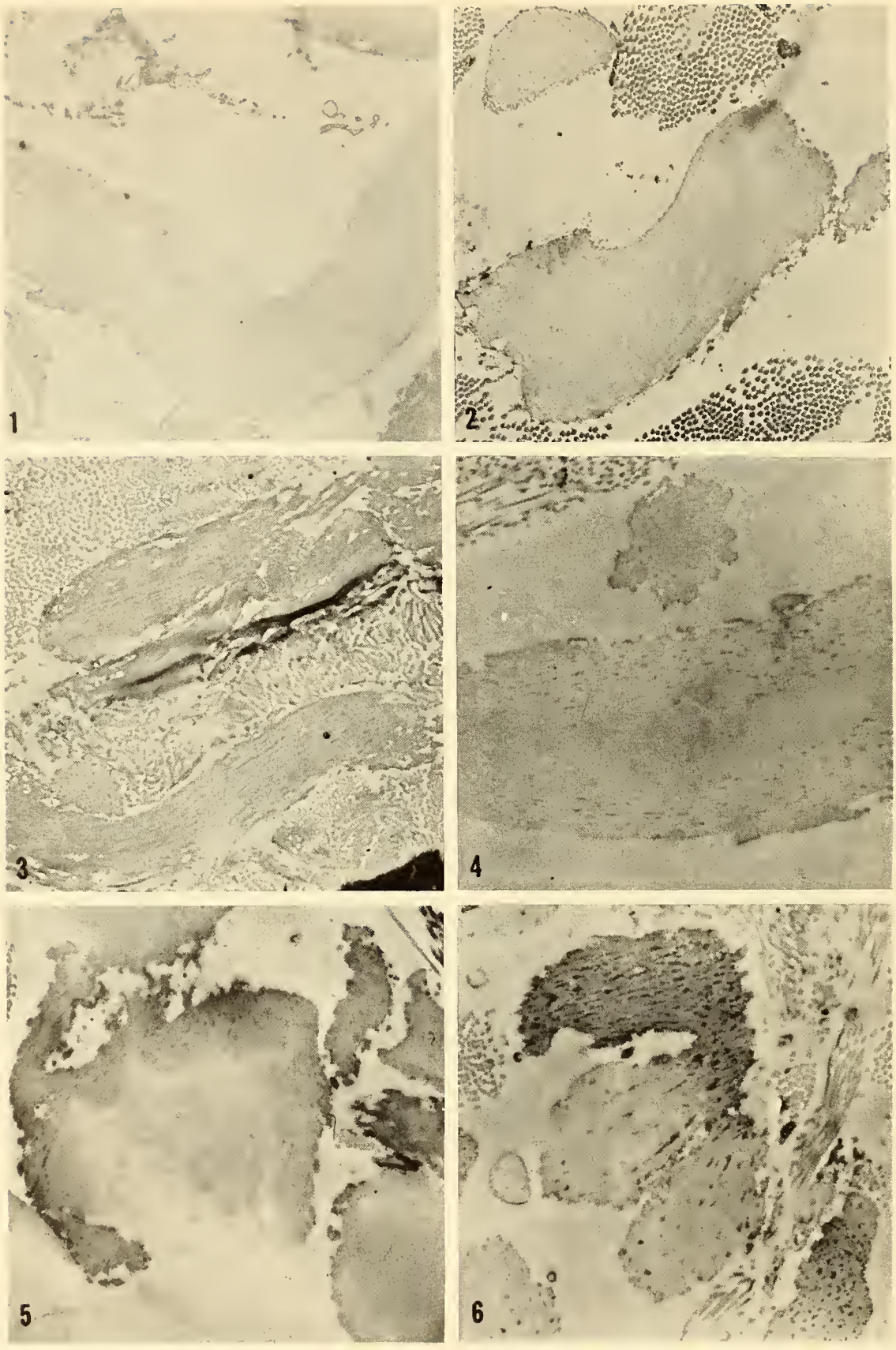

FIGURE 1.—Normal elastic fibers. Papillary layer abdomen, 6-year-old W.M., Acute leukemia. Autopsy. × 7200.

FIGURE 2.—Normal elastic fibers. Skin, abdomen, 21-year-old W.M., Tetrology of Fallot. Autopsy. × 7200.

FIGURE 3.—Normal elastic fibers. Note prominence of fibrils and density of matrix. Papillary layer buttock, 61-year-old W.M., mycosis fungoides. Clinic. × 7200.

FIGURE 4.—Normal elastic fibers. Note peripheral arrangement of fibrils. Reticular layer buttock, 75-year-old W.F., mycosis fungoides. Clinic. × 7200.

FIGURE 5.—Probably normal elastic fibers which appear exploded. This may be an artifact. Papillary layer buttock, 56-year-old W.M., psoriasis. Clinic. × 7200.

FIGURE 6.—Elastotic degeneration. Note the large size of the fibrils in the otherwise normal-appearing portions of the elastic fiber. There is a transition within the same fiber to more advanced degeneration with an increase in density of the matrix which begins to appear very finely granular.

6. Miscellaneous

(a) Dr. Louis B. Thomas has studied the intracerebral localization of leukemic L1210 cells in mice treated by several agents. A remarkable correlation with children treated for acute leukemia has been found. As a result of this work, important information on the spread of leukemic cells and a better understanding of the anatomy of the brain of the mouse has also been obtained.

(b) Dr. Richard Swarm continues his work with hyperbilirubinemic rats. It is desirable that some degree of inbreeding be achieved so that they will accept tumor transfers.

(c) The aging process in collagenous and elastic connective tissue has been studied by Dr. Banfield. Much fundamental information has been acquired on these important body components. During an electron microscopic investigation of senile elastosis observations on age changes in the elastic fibers were made, best brought out in the accompanying figures 1–4.

The observations on senile elastosis can be summarized as follows:

The elastotic degenerated material in senile elastosis was found to be derived from elastic fibers. There was no definite evidence that a change in collagen could also result in elastotic degenerated material. Observations on normal elastic fibers incidental to the problem of senile elastosis indicated that the possibility of a normal collagen-elastin transformation should not be dismissed.

The acetic acid solubility of the skin collagen in aging hamsters has been followed from 28–809 days and is summarized in the accompanying chart. Here only the data for the transition points have been charted. Organ weights and body weights were also recorded and regression lines are being plotted. With one exception, there is no obvious correlation of age changes in the organs or pathology of the organs with the collagen solubility. The exception, a slight increase in solubility at the maturation of the testis after 28 days.

(d) Dr. Louis Thomas is a member of the Committee on Nomenclature and Classification of Disease of the College of American Pathologists. Classification of neoplasms is a particularly difficult field.

(e) Dr. Ross MacCardle is engaged in writing a monograph on the cytological characteristics of a variety of neoplasms. He is also writing the Introductory Chapter for the new revision of Cytology and Cell Physiology, by G. H. Bourne, to be published by the Academic Press.

LABORATORY OF PHYSIOLOGY

Excellent progress has been made by Dr. Pratt in developing mathematical models and statistical techniques for evaluating cancer chemotherapy screening data, particularly in the analysis of structure-activity relationships of alkylating agents in a spectrum of rat tumors. A method was formulated for creating a file of pathological diagnoses and vital statistics in a form available for selective retrieval. By employing an IBM computer 1620, programs have been prepared and tested in which it was possible to demonstrate an ability to retrieve data either on a single or multiple selection basis. The program started last year by Dr. Pratt in collaboration with Drs. Rushizky and Sober of the Laboratory of Biochemistry to develop chemical, mathematical and computer programming techniques to describe the arrangement of mononucleotides within polynucleotides from yeast RNA has made considerable progress. Mononucleotides of adenine, guanine, cytosine and uracil from yeast RNA, prepared by alkaline hydrolysis or enzymatic methods, show mathematically similar identity to their counterparts from other sources, including tobacco mosaic virus RNA, micrococcal RNA or polyadenylic acid. Using the four above-mentioned nucleotides (Ap, Cp, Gp and Up) in their library, they have been able to mathematically describe, identify and obtain base ratios for di-, tri-, and tetranucleotides of known chemical identity (e.g., ApCp, CpApGp, GpGpUp, UpGp, ApApApAp, etc). Of particular interest are the hyperchromic and hypochromic (shifting of spectral maxima) effects observed after hydrolysis which appear to be highly characteristic expressions of near neighbors within the polynucleotide. As a result of this technique it may be possible to tackle problems of chemical structure and sequence that have been too large or too laborious to undertake previously. Dr. Pratt in conjunction with Drs. F. K. Millar, Seoras D. Morrison and Peter Baillie have been investigating

the total metabolism of the tumor-bearing animal with a view of determining the mechanism by which the presence of a tumor affects the normal patterns of energy and material exchange. They hope to study the problem of caloric imbalance in the tumor-bearing animal. This involves an understanding of the relationships between calories, nitrogen, electrolytes and water as these relationships change with progressive tumor growth. Some of these relationships are being studied in animals made hyperphagic or aphagic by hypothalamic lesions; a study of temporal patterns of food intake in normal intact rats, in rats after gastric vagotomy, in rats after lesions in the hypothalamus; temporal patterns of gastric motility and its relationship to food intake. The problem of ulcer occurrence in tumor-bearing rats is being studied with respect to time of appearance and tumor development and methods which can be used to block their occurrence.

Characterization of nucleic acids and nucleoproteins of normal and malignant tissues in terms of physical, chemical and biological properties as well as the development of new procedures for the isolation of nucleic acids continue to be explored. Dr. Shack has obtained experimental conditions whereby complete separation of native and denatured DNA in the density gradient electrophoresis apparatus is possible. Denaturation experiments in which DNA is partially denatured can be resolved into one fraction which is completely native and the other completely denatured. These data provide the first definitive proof that denaturation of DNA is an all or none phenomenon. This strongly suggests that the DNA macromolecule exists either in the native or denatured configuration. No evidence was found for intermediate hybrid species which possess a partly native and a partly denatured configuration. Native DNA derived from partially denatured material has a higher content of guanine and cytosine, and a lower adenine and thymine than the original total DNA, while the denatured fractions show a lower guanine and cytosine and a higher adenine and guanine than the original DNA. The extent of these deviations depends on the extent of partial denaturation.

Dr. Wollman is continuing his program dealing with properties of transplantable thyroid tumors, the iodide-concentrating mechanisms of the thyroid gland, and the intracellular properties of the thyroid gland. He has found that in the large family of transplanted thyroid tumors derived from feeding rats a goitrogen that transplanted independent tumors (those which will grow in rats not fed a goitrogen) arose from very small regions in the parent donor tumor. Some of the functional independent tumors can clear radioiodide from the blood at rates which are independent of pituitary production of thyroid-stimulating hormone. The rate at which these functional tumors clear blood radioiodide indicate a high rate of blood flow comparable to rates in the liver and brain. Kinetic studies on the distribution of radioidide among the iodinated amino acids of the thyroid gland in the rat and guinea pig indicate that I^{131}-labeled thyroxine in the gland is turned over in the gland much more rapidly than it is secreted into the blood. Studies are underway on the properties of intracellular colloid droplets in the thyroid gland to determine whether they are newly formed colloid or colloid which has been resorbed from the lumen of the follicle. The purpose of this study is to obtain a better understanding of the mechanism of hormone secretion and, thus, radioisotope release in the thyroid gland.

Dr. Elkind is pursuing his studies on the mechanisms of survival and recovery of X-irradiated mammalian cells in tissue culture. Employing tracer counting and radioautographic techniques he is also examining the effect of X-irradiation on DNA, RNA and protein synthesis. He has observed that after irradiation the synthesis of DNA, RNA and protein continues during the division delay period; the rate of DNA synthesis after irradiation undergoes fluctuations, probably as a result of the combined effects of suppression of DNA synthesis in cells undergoing division (S) at the time of exposure and the progression of the G_1 cells (those about to undergo mitosis) into cells being synthesized. After a single exposure there does not seem to be a temporal relationship between the fluctuations in the rate of DNA synthesis and the fluctuations in survival previously observed. He is also developing models for the radiological response of cells and tissues. His objective is to construct models for a better understanding of radiation effects in tissues. In collaboration with Dr. R. Bellman of the Rand Corporation and Dr. M. Berman, NIAMD, a Monte Carlo model has been programmed for the effect

of irradiation on the survival and growth of a population of cells.

Dr. Willie Smith has been engaged in studying irradiation injury as well as spontaneous and induced recovery. She has set as her objectives (1) to characterize and quantitate various aspects of irradiation injury and recovery, (2) to correlate natural factors such as age, weaning, intestinal flora on injury and recovery, (3) investigate the processes involved in the initiation of recovery by treatment with substances such as endotoxin and various mitotic inhibitors, and (4) to study the effect of mitotic inhibitors on the response of tumor cells to radiation. Experimental data indicate that there is a definite relationship between radiation dose and granulocyte or lymphocyte count three or four days after irradiation. Higher dose ranges result in lower counts and an increase in the time interval between exposure and recovery. Young mice (4 weeks) required a longer time for recovery although the counts are the same. Colchicine-treated mice require less time for recovery although the counts are the same as the controls. Velban (a mitotic inhibitor) promotes early granulocyte and lymphocyte recovery and increase in per cent survival in irradiated mice.

Dr. Draper has been studying the status of antibody-forming mechanisms after multiple exposures to radiation, as well as investigating the action of the antigen and non-specific stimuli during the immune response in irradiated animals. Rabbits were irradiated and then immunized with various doses of washed sheep erythrocytes. Serum samples obtained during the response were assayed for hemolysin content. The radiation-induced depression of hemolysin formation can be overcome to a large extent by a second antigen injection one day after a second dose of irradiation. The results indicate that the depression of antibody formation by a given dose of high energy (2.0–2.5 Mev) X-irradiation is less severe than that of lower energy irradiation. The modification of depressive effects of irradiation is dependent upon the dose of each of the two antigen injections and upon the time interval between the two radiation exposures and antigen injection. Relatively large amounts of antibody are produced in rabbits given a total of 1,200 r in two exposures one week apart, provided antigen is injected one day after each exposure. Dr. Draper is also studying the effect of daily, low level gamma irradiation on the ability of rabbits to produce antisheep hemolysins. Thus far he has demonstrated that the hemolysin response following accumulated doses from 100 to 1,900 r is not markedly depressed. The spleen appears to be more radiosensitive to radiation under these conditions than nonsplenic sites of hemolysin synthesis.

The chemical effect of ionizing radiation on aqueous solutions of organic compounds, particularly acetone, is being studied by Dr. P. Reisz employing Co^{60} gamma rays. In the present study of aqueous acetone solutions, it has been possible to distinguish between the chemical effect of the solvated election and the hydrogen atom and to measure the relative rate constant of these two species of acetone. The initial yield of hydrogen atoms and solvated elections have been shown to be a function of pH. Isopropanol has been identified as a new product in addition to those reported last year. The mechanism of action is currently being investigated and one mechanism consistent with the current experimental results has been proposed.

Dr. C. Maxwell is furthering his studies on the effect of ionizing radiation on amino acids in aqueous solution. He is actively pursuing the mechanism of the chemical reactions induced by ionizing radiation with the ultimate goal of compiling information on simple systems of biological interest with the intention of applying these observations to more applied complex systems which are not amenable by direct investigation.

Studies of urinary excretion patterns of nucleic acid congeners in human leukemia are being continued by Dr. J. C. Reid. Because of the complex nature of the urinary profile, mathematical as well as chemical techniques had to be employed. Chemical procedures comprise an integrated chromatographic system developed by Dr. Reid in which a urinary specimen can be fractionated into a large group of mixed fractions. The mathematical techniques, which are primarily those of linear algebra, represent an approach to the further resolution of mixed peaks by means of computational analysis of their ultraviolet absorption spectra. Data processing machinery is used to handle the large volume of data generated. Thus far an integrated system for data reduction, error monitoring, retrieval, etc., has been created and is working satisfactorily. In addition, a system of programs has been written which analyzes unknown mix-

tures containing as many as eleven components in which one of the components can be computed with errors as low as a few tenths of one percent. This type of mathematical and machine approach makes it possible to detect the presence of meaningful information in such datum which may be the ultimate interpretation.

Studies on protein synthesis in normal and tumor cells and the effect of antagonists are being continued by Dr. M. Rabinovitz. His objectives are to study in both normal and tumor cells the pathway taken by amino acids in the formation of protein through an analysis of biochemical lesions introduced within the pathway by specific inhibitors of protein synthesis and, where applicable, to extend the information obtained to the tumor-bearing animal with the view of selective inhibition of protein synthesis in cancer cells. He has found that hemoglobin synthesis can be inhibited by α-amino-β-chlorobutyric acid at the earliest stages of incubation. Synthesis appears to continue for a period of 5 minutes at half the normal rate and then stops abruptly. The accumulation of labeled protein on the ribosomes, which is characteristic of this inhibition, is the result of continued labeling of ribosomal protein beyond the time when normal ribosomes would have reached a steady state of amino acid incorporation and protein release. The initial rate of labeling of ribosomal protein is identical in normal cells and in those treated with the antagonist. The maintenance of labeled ribosomal protein accumulated in the presence of the antagonist requires the functioning of energy yielding processes of the cells. The use of amino acid antagonists in cell-free systems which incorporate amino acids into protein offers both a means of studying the partial reactions of the system and can also serve as a basis of comparison of the cellular and acellular processes. Preliminary studies have indicated that the incorporation of labeled amino acids into protein of isolated Ehrlich ascites ribosomes is insensitive to inhibitors which interfere with the process by intact cells. The results suggest that the acelluar system may represent a small insensitive component of the total cellular activity.

With isolated rabbit reticulocytes, a striking similarity between the celluar and acellular processes was observed.

Drs. J. White, F. K. Millar and Mrs. J. N. Toal have continued their studies of the growth stimulating factors in tumor tissue and sodium balance in tumor-bearing rats with emphasis on the participation of the adrenal gland. A diet containing dried tumor tissue (10%) as a source of protein produces a 30% better growth (increased over-all weight) in normal animals than does a 10% casein diet. Changes in adrenals have been observed in tumor-bearing rats. The zona glomerulosa was 40–50% wider in the adrenals of tumor-bearing rats than in the adrenals of normal rats. This observation was in line with previously reported increase in aldosterone secretion by adrenals of the tumor bearers. Studies have been initiated on the effect of sublethal does of a X-irradiation on the incidence of cirrhosis in rats ingesting a low protein diet. Sprague-Dawley rats ingesting a 6% casein diet and exposed to 450 r all develop cirrhosis of the liver in 145 days. Control non-irradiated rats also develop cirrhosis but not as severe although no irradiation had been administered. Osborne-Mendel rats similarly treated develop cirrhosis only in the irradiated group. Supplementing the diet with choline or methionine prevents the appearance of cirrhosis of all animals previously affected.

LABORATORY OF VIRAL ONCOLOGY

The research of the Laboratory of Viral Oncology continues to be primarily in the area of viruses in relation to cancer, although other basic problems such as the ultrastructure of normal and nonviral cancer cells, cancer immunology, and the induction of plasma cell tumors with chemically inert substances are also being pursued.

Dr. Moloney has continued investigations on the strain of mouse leukemia virus which he first isolated from the transplantable tumor of mice, S 37. Dr. Moloney has confirmed the work of Sachs in which it was shown that the transplantability of tumors induced with the Moloney strain of mouse leukemia virus is inhibited in isologous mice by preinoculation of the leukemia virus into recipient hosts. This phenomenon was first observed for the polyoma mouse-virus system, independently, by Habel and co-workers and by Klein. The phenomenon is thought to indicate that a new cellular antigen is induced by the virus in the membranes of the virus-induced tumor cells, and that the host is able to react against the transplanted cells containing this foreign antigen

through immunological factors brought into play by the prior inoculation of virus. However, much work remains to be done to establish this concept as the actual mechanism involved. A possible application of this new information is the development of a relatively short-term biological assay of the virus. Using the rejection of transplanted tumor cells as the biological indicator, and prior injection of the candidate virus suspension, quantitative differences in potency up to 1,000-fold have been determined by Dr. Moloney in assays completed within less than 30 days. This is in comparison with a period of 4 months or longer required for comparable bio-assays involving leukemia development as the biological indicator. Another potential practical application of the phenomenon resides in the possibility that it is highly specific for virus strains and may therefore be used for detecting antigenic differences between leukemia virus strains. Little progress has been made thus far toward this important objective because of the failure of conventional serological methods to yield satisfactory results with the murine leukemia viruses.

In other studies initiated during the past year Dr. Moloney has achieved preliminary success in isolating infectious nuclei acid from extracts of murine leukemic tissues induced by his strain of virus. The method employed was a modification of the phenol method of Gierer and Schram which others had successfully applied to several viruses. The incidence of leukemia in mice which received the nucleic acid fraction was 11.8 percent within 6 months, which is well above the natural incidence (less than 1 percent) at this age in the strain of mouse employed (BALB/c). The biological activity of the nucleic acid fraction was destroyed by RNase but not by DNase. The activity of the intact virus, on the other hand, was not altered by RNase (or by DNase). The leukemia induced by nucleic acid has been confirmed by Dr. Dunn as being identical to that induced by the intact virus, and complete viral particles have been observed by Dr. Dalton in the leukemic cells and megakaryocytes of mice with the nucleic acid induced disease. These results have now been repeated in three out of five tries, with essentially the same incidence of tumors.

In collaborative studies, Drs. Moloney and Dunn have found that inoculation of the Moloney strain of leukemia virus 48 hours after thymectomy induces recticulum cell sarcomas (type B) in recipient BALB/c mice, rather than lymphocytic neoplasms as in intact hosts. Also, the reticulum cell tumors appear after a much longer latent interval than the lymphocytic neoplasms. Splenectomized hosts may develop chloroleukemia, lymphocytic leukemia, or reticulum cell sarcomas (type B) as a result of inoculation with Moloney's virus strain.

Collaborative studies by Drs. Moloney, Dalton, and Dunn are being conducted on the kinetics of virus development and the early pathogenesis of the disease in mice and rats inoculated with Moloney's strain of virus. Virus particles can be detected by electron microscopy in megakaryocytes of the bone marrow as early as 7 days after inoculation of the virus, long before any histological evidence of disease can be detected. Relatively large quantities of virus can be detected after 14 days. As previously reported, large quantities of virus can also be demonstrated in the blood within a few weeks after virus inoculation and before the appearance of overt disease. This finding has made possible the production of virus suspensions of a much higher degree of purity than can be achieved when tissue extracts are used as the source of virus. Blood contains relatively little extraneous particulate material in the virus size range and a high degree of purification can be obtained by differential centrifugation alone.

Dr. Dalton, in collaboration with Dr. Moloney and Dr. Haguenau (of the Institute of Cancer Research, Villejuif, France) has recently initiated studies on the morphology of the Moloney strain virus utilizing the method of negative staining by salts of phosphotungstic acid. Earlier results utilizing thin sectioning techniques had indicated that some Moloney virus particles possessed tails, giving the particles a "tadpolelike" appearance. The negative staining of intact virus particles on monolayer film preparations revealed a high percentage of particles with tails. In many instances an hexagonal geometric structure of the body of the virus particle could be made out. Although "tails" and other pleomorphic formations of other animal viruses have been described as artifacts under certain conditions (e.g., drying in the presence of strong salts), observance of the tails of Moloney's virus in preparations that were fixed before drying, in thin sections, and the absence of other pleomorphic forms indicate that the tail

in this instance is real and that it may represent a stage in maturation. This characteristic morphology in negatively stained material has made possible rapid analysis of extracts from blood, tissues and tissue culture for the presence of virus and has thus greatly opened up studies on kinetics of virus reproduction.

In further studies on the Moloney strain leukemia virus in tissue culture, Dr. Manaker has demonstrated that it can be propagated in cultures prepared from mouse lung and kidney, in addition to mouse spleen preparations previously reported. Whether the virus was propagated in cells of the reticulo-endothelial system present in the different organs tested was not determined.

Other studies by Dr. Manaker indicate that this murine leukemia virus does not propagate in cultures prepared from human or monkey bone marrow, whereas it can be readily propagated in mouse bone marrow cultures. This suggests that man and other primates may not be susceptible to this murine leukemia virus. Mouse tissue cultures held at 40° C. continued to release Moloney virus without detectable difference from cultures held at the usual temperature of 37° C. Efforts by Dr. Manaker to infect tissue cultures with nucleic acid fractions of virus supplied by Dr. Moloney were unsuccessful. Tests on mouse tissue cultures originally infected with Moloney strain virus 1½ years ago, and maintained since that time by Dr. Manaker, showed that virus was still being released.

Dr. Fink has continued her efforts to adapt conventional serological methods to the Moloney leukemia virus. Slight complement fixation at low titer was achieved but the sensitivity of the system will have to be increased before complement fixation becomes a useful tool. All efforts to demonstrate specific fluorescence in leukemic cells using fluoroscein conjugated antibodies in both mouse and rabbit antisera have failed thus far. However, in view of the importance of this tool to studies on human leukemia, intensive efforts to devise adequate methods of immunofluorescence for this mouse model system are being continued. Exhaustive tests involving numerous controlled variables have failed to demonstrate a hemagglutinin associated with the Moloney virus which might permit the establishment of a hemagglutination test.

Dr. Rauscher has continued investigations on his strain of mouse leukemia virus, the isolation of which was reported last year. This virus induces a dual type of disease the early phase of which is represented by marked erythrocytopoiesis and tremendous enlargement of the spleen; the second phase is represented by the development of lymphocytic leukemia. The early enlargement of the spleen (at about 15 days with the strongest does) permitted the development of practical bioassay procedures based upon time to palpable spleen, or weight of spleen at sacrifice. Using these assay procedures the virus was found to remain stable and show no loss in potency when stored nine months at 70° C. Also, it has been possible to devise quantitative neutralization tests based upon bioassay procedures for studying immunological relationships of this virus with other murine leukemia virus strains or isolates.

Kinetic studies on the growth of virus in intact mice were also conducted by Dr. Rauscher, using quantitative bioassay procedures. Plasma or spleen removed from mice within 1 day after infection contained little or no virus, and virus could not be recovered through the third to fourth day after inoculation. Beginning on the fifth day virus was found to be present in both spleen and plasma and to increase with time at a logarithmic rate until a maximum was reached which persisted until the development of overt disease. No significant difference was found in the time to appearance of virus in spleen and plasma, but the quantity of virus reached higher levels in the plasma in some instances.

Of significance also in indicating that this virus may differ entirely from other isolates are the pathogenesis studies of Dr. Dunn carried out in collaboration with Dr. Rauscher. Unlike the murine leukemias induced by the Gross and Moloney viruses, the thymus appears not to be the primary site of leukemogenesis or of viral synthesis.

Dr. Fink has initiated serological investigations on the Rauscher strain virus and has succeeded in producing relatively potent antiserum in rabbits. Also, she has developed vaccines with formalin-inactivated and heat-inactivated virus, combined with Freund's adjuvant, which effectively protected mice against challenge with strong doeses of virus. Vaccinated mice also showed high levels of neutralizing antibodies, as determined by Dr. Rauscher in bioassay studies. The knowledge that a strong immunity can be developed to another

murine leukemia virus (Dr. Friend was also successful in vaccinating against her agent) increases the hope that if human leukemias are caused by viruses, some of them may be effectively controlled by classical immunization procedures. While immunological studies on the Rauscher strain virus are incomplete, preliminary findings indicate that there is no strong antigenic similarity between this strain and the Friend, Moloney, and Schoolman-Schwartz isolates.

Dr. Zeigel, in collaboration with Dr. Rauscher, has studied the morphology of the Rauscher strain agent under the electron microscope in both tissue and blood using both the thin section and the negative staining technique. Morphologically, the virus is indistinguishable from the Moloney strain agent, including the existence of a tail and an hexagonal body as revealed by negative staining. Up to 80% of the virus particules were found to have "tails."

Studies by Dr. Manaker on the Rauscher strain virus showed that it, like the Moloney strain, could not be propagated in tissue cultures of human or monkey bone marrow, although it could be grown in cultures of mouse bone marrow. Nor could this virus be grown in mouse liver cell cultures.

Dr. Manaker has continued to study the isolate of mouse leukemia virus which he reported last year. Serial blind passages of extracts prepared from mouse spleens and thymus glands were made 14 days after neonatal virus inoculation, and several weeks before leukemia could be expected to develop. Sixteen successive passages were carried out. Five further passages were then made at 10-day intervals. Some mice of each passage group were retained for observation of leukemia development. Leukemia has been observed through all passages, indicating that early virus proliferation occurs long before leukemia may be expected to develop. Other experiments were designed to determine how soon appreciable quantities of virus appeared in mice following the initial virus inoculation. It was found that new virus was present within 10 days in mice that had been injected when newborn. When older, immunologically competent mice were inoculated, new virus was observed at 7, 21 and 28 days after inoculation, but not at 14 days. The failure of attempts to recover virus at 14 days in the immunologically competent animals is thought to be due to the production of inhibitors (e.g., antibody), the subsequent reappearance of active virus being due to saturation of available inhibitor with the increasing production of virus. Further investigations on this phenomenon are in progress. Other studies by Dr. Manaker on the effects of cortisone, and the enhancement of very low (subclinical) doses of virus are in progress, but the results are incomplete. These studies are directed toward the establishment of methods which might enhance the chances of recovering viral etiological agents from human leukemia, if such exist.

Previous reports have described the finding of Dr. Zeigel of viral type particles budding from the membranes of acinar cells of the pancreas in apparently normal chicks and chick embryos. Similar particles were observed in other tissues of the same birds, but not in the process of budding. Birds which failed to show budding in the pancreas also failed to show virus in other tissues. These findings suggested that the particles might represent lymphomatosis virus which is known to be endemic in most flocks of chickens and which is passed through the egg in some instances. Also, they suggested that the pancreas might be the most important primary site of viral replication during subclinical infection.

These studies have been followed up by Dr. Zeigel in collaboration with Dr. Rauscher of this laboratory and Dr. Burmester of the Regional Poultry Research Laboratory (USDA), East Lansing, Michigan. The following evidence indicates that the particles observed by Dr. Zeigel are very probably the virus which causes lymphomatosis in chickens. Hens from several colonies were isolated and trap-nested for identifying eggs with dams. As previously shown by Burmester and by Rubin, dams which shed lymphomatosis virus have a high percentage of infected eggs (and embryos) as demonstrated by biological tests. Contrariwise, hens which do not shed virus and which show no antibody to the virus yield eggs free from biologically demonstrable lymphomatosis virus. The results of Dr. Zeigel and associates to date show varying percentages of embryo pancreases from infected hens which have budding virus particles, whereas embryos from hens which do not shed virus have failed thus far to show electron microscopic evidence of particles in the pan-

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creas. Parallel biological studies on the same preparations examined by Dr. Zeigel are being conducted by Dr. Rauscher.

The procurement of human cancerous tissues and the methods of approach used in attempts to demonstrate viral etiological agents in association with them were described in detail in the report of last year. An initial series of 10 experiments involving as many different human tumor specimens was described and preliminary evidence of the induction of tumors in mice injected neonatally with the microsome fraction of one of the human tumors was reported. These experiments have now been completed and all mice have come to autopsy. Only one of the 10 experiments (HT–8), namely that on which the preliminary report was made last year, showed significant differences between test and control groups in the studies involving newborn mice. The specimen employed in this experiment was gastric carcinoma tissue from an elderly man.

The most striking finding was the occurrence in all 3 experimental groups of carcinoma of the kidney a tumor type that has not been observed spontaneously in the strain of mouse employed (BALB/c) and only very rarely among all strains of mice. The polyoma virus was ruled out by monitoring tests on all mothers that supplied newborn litters and by tests on mice developing tumors. It is emphasized that carcinomas of the kidney have not been observed as a result of the polyoma virus, although this agent does induce sarcomas of the kidney. In addition to the kidney tumors, cysts of the liver were observed in most of the mice bearing this lesion and four of them also had carcinomas of the acinar cells of the pancreas, another lesion not observed spontaneously. The kidney tumors appeared first and most frequently in mice that had received microsome material plus Freund's adjuvant (group *c*), but the group that received microsome suspension in buffer (group *b*) was only slightly behind. These two groups showed highly significant differences from control groups in time of appearance, frequency, and type of tumors. The group which received crude extract of the gastric carcinoma developed only one kidney tumor, late, but two tumors of types that occurred also in control groups appeared slightly earlier. These results leave no doubt that some factor associated with the human cancerous material induced a significant tumor response in mice injected neonatally.

On a basis of these findings a larger study of human cancerous materials using newborn mice has been initiated. The latter study has not been under way sufficiently long for significant results.

The finding by electron microscopy of virus particles in the blood and bone marrow of leukemic mice, and the development of a practical approach to the study of the human disease based on the animal model system by Doctors Dalton and Moloney was reported last year. The observance of virus-like particles in 11 of 14 human leukemia specimens was also reported. An additional electron microscope procured for speeding up the human studies has now been in operation for several months and Mrs. Mitchell has been trained in its operation. Dr. George Porter has also joined Doctors Dalton and Moloney in the human study and is taking responsibility for the broader survey. A total of 47 human leukemic cases has now been examined, 20 of which were found to contain particles with characteristics similar to the particles associated with murine leukemia. The processing of specimens was carried out by Dr. Moloney who also has injected particle postive materials into newborn monkeys and mice. The oldest monkeys have been under observation for only 6 to 8 months, and no neoplastic reactions have yet been obtained. Among the various test groups of BALB/c mice that were injected neonatally with human leukemic materials, 8 out of 100 in one group have developed hemangioendotheliomas at the sites of inoculation, 2 out of 50 in another have developed thymic lymphomas, and 1 in a third group of 25 has developed a reticulum cell sarcoma, within periods of 7 to 12 months following inoculation. No tumors have yet occurred among comparable numbers of control mice. Although still preliminary in nature, these findings appear to be comparable to the findings with the solid tumor (gastric carcinoma) described in the foregoing section, and they further justify the continued investigation of newborn mice as a potentially valuable indicator of tumor-inducing (or enhancing) factors in human neoplasms.

Following his success in isolating infectious nucleic acid from murine leukemic tissues, Dr. Moloney is now applying the same techniques to human leukemic tissues. The nucleic acid frac-

tions have been injected into newborn mice, and 1 newborn monkey. Drs. Manaker and Stewart are also testing the nucleic acid fractions in tissue cultures of human cell lines.

Dr. Sarah Stewart is now spending most of her effort and facilities in intensive efforts to propagate virus from human leukemias in tissue culture. Primary cultures of human fetal tissues such as thymus, spleen, liver and bone marrow, as well as calf embryo spleen are used as substrate. Leukemic materials used for inoculation include bone marrow, cells from the buffy coat, extracts and concentrate prepared from tissues obtained at autopsy, and "microsome fractions" separated from blood by differential ultracentrifugation (supplied by Dr. Moloney). Explants of human leukemic bone marrow and buffy coat cells are also made and fluids from them are used for animal inoculations and electron microscopic study. Twenty such specimens from human leukemic patients (or autopsy specimens) have been successfully established in tissue culture. Several have shown "suggestive" viruslike particles under the electron microscope, and one, involving buffy coat, has shown definite cytoplasmic particles similar to murine leukemia viruses. The electron microscopic studies are being made by Dr. David Ferreira (a visiting scientist in the Cellular Biology Section) and Miss Valentine.

In two instances embryonic calf spleen cultures that had been inoculated with bone marrow or blood pellet material from leukemia cases positive for virus-like particles (determined by Drs. Dalton and Moloney) showed a marked increase in rate of proliferation as compared with the controls. The fluids of the "infected" cultures became milky in appearance because of the massive cellular proliferation. Uninoculated cultures did not exhibit this effect. The same two "infected" cultures also showed a marked increase in motility in comparison with control cultures, as determined by time-lapse cinemophotography. The latter studies were in collaboration with Mr. Davenport, of the Pfizer Company in Maywood, New Jersey. If the phenomena of increased proliferation and increased motility are found by further study to be caused by a propagatable viral entity, it is possible that they may be used in the future as diagnostic criteria. Such criteria are badly needed for tumor viruses of the classical type since most of them do not produce CPE or other morphological changes that can be recognized in *in vitro* systems.

Encouraging preliminary biological results have also been obtained by Dr. Stewart with the same two "infected" bovine spleen cultures which showed increased proliferation and motility. Four out of 35 hamsters inoculated neonatally with fluids from one of the cultures, and 5 out of 33 with fluids from the other have developed reticulum cell sarcomas within 4½ to 11 months. One such tumor was observed after 9 months, among 75 hamsters inoculated with fluids from control cultures. Although reticulum cell sarcomas are known to occur spontaneously among older hamsters with low frequency, the higher frequencies of such tumors, and their appearance as early as 4½ and 5 months in animals of the test groups suggest enhancement of the neoplasia as a result of the experimental processes described. This finding is comparable to the enhancement of tumor development in mice by human materials as described in preceding sections.

A total of 19 monkeys have been injected when newborn with fluids from tissue cultures of human leukemia explants or human cell lines that had been inoculated with leukemic material. Six have died and 13 remain. The oldest have been under observation for about 7 months. No neoplasms have been observed to date.

Dr. Manaker is also carrying out extensive investigations on human leukemia using cultures of human and monkey tissues as substrates. Particular emphasis is being placed upon the use of bone marrow from normal adults. Ribs removed at operation for heart surgery, at the Clinical Center, represent the primary source of human marrow. Fetal bone marrow and other tissues are also used when available. Viable human leukemic bone marrow obtained by aspiration at the Clinical Center has been the chief leukemic material investigated. Marrow from 45 leukemic cases has been put into tissue culture, both as primary explants and by seeding onto other human and monkey tissue cultures. The cultures are incubated for 3 weeks and then carried through one or more additional passages. Fluids from the cultures are stored at low temperature and used as facilities permit for animal inoculation and electron microscopic examination for virus particles. Only a few preparations have been examined thus far by electron microscopy and none have shown virus particles. Biological tests in newborn mice have

not been in progress for sufficient time to yield significant results.

In collaboration with Dr. John Fahey (Metabolism Service, NCI), Dr. Dalton has made electron microscopic studies on bone marrow aspirates from a series of cases of multiple myeloma and macroglobulinemia. Among the multiple myeloma cases at least two each of those producing gamma globulins, B2A globulin and Bence-Jones protein were included. Six cases of macroglobulinemia have been studied. Characteristic cells with details of ultrastructure indicating their origin from plasma cells were found in all cases, but the degree of differentiation was least in the cells from patients producing Bence-Jones proteins. The finding of characteristic cells in all six cases of macroglobulinemia is of interest because it had not been possible with the light microscope to make this identification.

Dr. Merwin has continued her studies on the induction of plasma cell tumors in BALB/c mice with diffusion chambers. She has found conditions which yield incidences of plasma tumors as high as 45 percent in mice with empty chambers, as compared with the previous highest incidence of 16 percent. The chief factor in increasing the incidence appeared to be the size of the chamber. The techniques for producing a relatively high percentage of plasma cell tumors now makes possible further practical systematic studies on the etiology of this tumor. The time of residence of the plexiglas was also of importance since among 51 mice in which the material was removed at periods up to 6 months, only 2 (4%) developed plasma cell tumors. Nine months or longer was required for inducing the higher incidences of plasma cell tumors. Work is being continued on the effects of different types of viable tumor and other tissues within intact chambers placed in the peritoneal cavity. As reported last year, a particular transplantable sarcoma of BALB/c mice maintained by Dr. Merwin caused an increase in plasma cell tumors over the frequency of such tumors caused by blank chambers alone. Another transplantable mammary gland carcinoma, probably containing Bittner agent induced a high percent of sarcomas in the peritoneal cavity, and when Sarcoma 37 was included within chambers, leukemia and reticulum cell sarcomas resulted. Dr. Merwin is continuing these studies with particular interest in determining whether the chamber technique may be used to detect small amounts of viruses associated with mouse neoplasms which might be below the threshold of detection by other methods now available. If successful this technique will represent a major advance in technology of work with tumor viruses.

Dr. Zeigel has launched an important study of the fine structure of normal and abnormal monkey tissues. Little electron microscopic work has been done on this type of laboratory animal thus far, and relatively little is known even regarding the normal structure of different cell types. With the growing interest and use of non-human primates in tumor-virus and other cancer research, baseline studies of the type being pursued by Dr. Zeigel are essential. Studies on normal cells of other laboratory animals are also being carried out by Dr. Zeigel. An investigation on the normal chick pancreas has revealed occasional ciliary processes associated with the apical surfaces of acinar cells, centroacinar cells and duct cells. It was not established whether every cell, or every acinar possessed at least one cilium. The cilia are similar in their fine structural character, however, to those cilia described in loci suggesting a sensory rather than a motile function.

NATIONAL HEART INSTITUTE

INTRODUCTION

The Heart Institute has undergone a number of organizational rearrangements within the last year. Most of these changes were formally instituted in the final few months of the year. The reports of the heads of laboratories which follow and constitute the body of this report reflect the new organizational pattern although most of the last year's work had been accomplished before these changes had been effected.

The modifications in the Institute's organizational structure were necessitated largely by Dr. C. B. Anfinsen's acceptance of a position elsewhere. Dr. Anfinsen had been head of the Laboratory of Cellular Physiology and Metabolism since the organization of the intramural program of the National Heart Institute in 1950. His departure is a great loss to his associates, his program and the National Institutes of Health. Nevertheless, the Heart Institute can consider itself fortunate in the relatively minor dislocation which has ensued. The administrative and scientific autonomy which the sections of Dr. Anfinsen's laboratory had enjoyed has made easy their assumption of laboratory status under excellent scientific leadership. Dr. Anfinsen's own section under Dr. Kielley has become a part of the Laboratory of Biochemistry. Other changes have been (1) the abolition of the Laboratory of the Chemistry of Natural Products and the establishment of part of it under Dr. Fales as the Section on Chemistry, Laboratory of Metabolism; (2) the organization of the Section on Molecular Disease under Dr. Fredrickson in the Laboratory of Metabolism; and (3) the establishment of the Section on Biochemical Genetics under Dr. Nirenberg in the Laboratory of Clinical Biochemistry. The Heart Institute is fortunate to have been able to offer the space and facilities to Dr. Nirenberg for the expansion of his program.

The reports which follow are those of the Laboratory Chiefs. We believe that the quality of the work they describe will speak for itself.

LABORATORY OF BIOCHEMISTRY

Section on Enzymes

The activities of the Section on Enzymes continue to be concerned with detailed studies of diverse metabolic processes. The specific enzymatic systems under investigations are regarded as model systems and have been selected because they offer unique opportunities to delineate some basic biochemical mechanisms of more general biological significance. Attention has been focused on the characterization of individual reactions and metabolites involved in (1) the biosynthesis of fatty acids, (2) the dissimilation of heterocyclic compounds, (3) the activation and utilization of one carbon compounds, (4) the activation of molecular hydrogen, (5) the dissimilation of amino acids, (6) the metabolism of ethylene glycol, (7) transsulfuration, (8) permeation and intracellular concentration of purines, (9) thiol-alkyl transfer reactions, and (10) the intracellular regulation of fatty acid biosynthesis.

Metabolism of Heterocyclic Compounds

Nicotinic Acid Dissimilation. Last year we reported the isolation from soil of an anaerobic bacterium which is capable of fermenting nicotinic acid with the formation of stoichiometric amounts each of acetate, propionate, CO_2, and NH_3.

Insight into the mechanism of nicotinic acid dissimilation by this organism has been obtained by studying in parallel experiments the decomposition by cell suspensions of substrates specifically labeled in individual carbon atoms. The isotope from nicotinic acid-2-C^{14} is found equally distributed in the methyl groups of acetate and of propionate; the isotope from nicotinic acid-5-C^{14} is found equally distributed between the carboxyl group of acetate and the alpha group of propionate; the isotope from either nicotinic acid-6-C^{14} or from nicotinic acid-7-C^{14} is found equally in the CO_2 and in the carboxyl group of propionate. These results are consistent with the conclusion

that nicotonic acid is either converted (1) to a symmetrical six carbon compound, or (2) that it is converted to two three carbon derivatives which are in isotopic equilibrium with each other. The latter conclusion is supported by the observation that pyruvate and lactate are readily fermented by cell-suspensions of the organism to mixtures of propionate, acetate and CO_2. Moreover, during the decomposition of nicotinic acid-7-C^{14} in the presence of a large pool of pyruvate, the pyruvate becomes labeled with C^{14}. Under the latter conditions several other intermediates accumulate in significant concentrations. Two of these have been isolated in highly purified crystalline form and have been positively identified as α-methylene glutarc acid and 6-oxo-1,4,5,6-tetrahydronicotinic acid. In addition, 6-hydroxy nicotinic acid was established as an earlier intermediate. These results are consistent with the working hypothesis that nicotinic acid degration involves the following reaction sequence: nicotinic acid→6-hydroxy-nicotinic acid→α-methylene glutaric acid→α-keto, γ-hydroxy, γ-methyl glutaric acid→2 pyruvate→ $+CO_2+$propionate$+$acetate.

It was further observed that this organism contains exceptionally high concentrations of vitamin B_{12} coenzyme. Preliminary experiments with cell free extracts suggest that this coenzyme may be implicated in the over-all nicotinic acid fermentation.

Riboflavin Degradation. In an effort to learn more about the biological dissimilation of heterocyclic compounds, studies on the bacterial degradation of riboflavin have continued. As reported earlier, a pseudomonad isolated by the soil enrichment procedure has been shown to degrade riboflavin to urea, oxamide and 3,4 dimethyl-6-carboxy-α-pyrone with the intermediary formation of 1-ribityl-2,3-diketo-1,2,3,4-tetrahydro-6, 4-dimethyl-quinoxaline (compound I) and 3,4-dimethyl-2, 3-quinoxalinediol (compound II). Ribose has now been tentatively identified as the other product formed in the conversion of compound I to compound II. This conversion requires the presence of molecular oxygen.

Fatty Acid Synthesis. As noted in last year's annual report, the first step in fatty acid synthesis involves a condensation of acetyl CoA with malonyl CoA to produce an acetoacetyl-enzyme complex and CO_2. Previous studies in this laboratory using cell-free extracts of *Clostridium kluyveri* as a source of enzymes showed that this reaction involves the participation of two enzyme fractions, one of which is readily inactivated by heat (fraction A) whereas the other is relatively stable to denaturation by heat (enzyme II).

Since much higher concentrations of these enzymes are present in *Escherichia coli*, extracts of this organism have been selected for additional studies. Further purification of the two enzyme fractions involved in the condensation reaction has been made. The heat labile fraction A has now been separated into at least two sub-fractions both of which are needed in addition to the heat stable enzyme II for catalysis of the condensation reaction. One of these fractions catalyzes thioltransacetylation reactions between acetyl CoA and free CoA or suitable analogues such as pantetheine. The other labile fraction designated as enzyme I contains an essential sulfhydryl group which is protected from inhibition by N-ethylmaleimide by prior incubation of the enzyme with any of several fatty acyl CoA substrates.

The heat stable enzyme II has been purified nearly 200-fold from extracts of *E. coli*. From sedimentation analysis in a sucrose density gradient it was shown to be a small protein with a molecular weight of about 10,000. The role of enzyme II as the receptor protein involved in the acetoacetylenzyme complex formation is indicated by the observation that incubation of C^{14}-malonyl CoA and acetyl CoA with fraction A and enzyme II leads to the formation of a C^{14}-labeled acetoacetylprotein complex which upon subsequent purification by salt fractionation and DEAE-cellulose chromatography remains associated with enzyme II activity. Incubation of the isolated C^{14}-labeled acetoacetylenzyme II complex with fraction A, malonyl CoA and TPNH results in the formation of C^{14}-labeled long chain fatty acids which are labeled in the third or fourth carbon atoms from the methyl terminal end of the molecules. This result supports the conclusion that the acetoacetyl-enzyme II complex is a bonafide intermediate in the synthesis of long chain fatty acids.

The Regulation of Fatty Acid Biosynthesis

Previous studies in this and other laboratories have shown that the stimulation by citric acid of fatty acid synthesis in animal enzyme preparations is due to a specific action on the activity of acetyl CoA carboxylase which catalyzes the synthesis of malonyl CoA. In continuing studies with purified preparations of acetyl CoA carboxylase derived from rat adipose tissue, it has now been established that the activation of this enzyme by citrate is associated with changes in the sedimentation behavior of the enzyme. Ultracentrifugation in a sucrose density gradient reveals that the untreated (inactive) enzyme has a sedimentation constant of 18.8 S whereas active enzyme obtained by prior incubation with citrate has a sedimentation constant of 43 S. Both the activation and increase in sedimentation constants are reversed when the citrate concentration is decreased by dilution. These results suggest that citrate induced activation of the acetyl CoA carboxylase involves the aggregation of two or more inactive monomeric units. In view of the relative specificity of citrate for aggregation and because of the freely reversible nature of the activation, this appears to provide an effective control mechanism for regulation of malonyl CoA formation and indirectly of fatty acid biosyntheses as well.

The exact mechanism by which citrate exerts its effect on aggregation is not known. The possibility that it serves merely as a chelating agent appears to be ruled out by the fact that other strong chelating compounds are ineffective. Moreover, the possibility that a metabolic derivative of citrate is the actual activating agent is made unlikely by the observation that fluorocitrate also activates the enzyme.

One Carbon Metabolism

Formate Activation. The incidental observation that some crystalline preparations of tetrahydrofolic acid formylase obtained from *Clostridium cylindrosporum* catalyze the formation of formyl hydroxymate in the presence of ATP, formate and hydroxylamine was mentioned in last year's annual report. This observation has provoked a more intensive study to determine if the formokinase activity is a manifestation of the tetrahydrofolate formylase enzyme or if it is due to a contamination of the latter enzyme with another formate activating system. The latter possibility is correct and has led to the discovery that cell-free extracts of *C. cylindrosporum* contain two distinct formokinases which are separable by protein fractionation procedures. Both enzymes catalyze the reaction:

$$\text{ATP} + \text{formate} \rightarrow \text{formyl-P} + \text{ADP} \qquad (1)$$

The reaction catalyzed by one of these enzymes, formokinase I, is apparently nonreversible; this enzyme is further characterized by having high substrate specificity; it catalyzes the activation of formate only. The other enzyme, formokinase II, catalyzes the activation of both acetate and formate in freely reversible reactions. In the presence of coenzyme A and a transacylase present in the bacterial extracts, the formyl-phosphate formed by either formokinase serves as a formyl donor for the synthesis of formyl CoA.

$$\text{formyl-phosphate} + \text{CoA} \rightleftharpoons \text{formyl-CoA} + \text{orthophosphate} \qquad (2)$$

From equilibrium measurements of this reaction the ΔF was calculated to be -1200 calories.

The coupling of reaction 1 and 2 provides a new mechanism for the enzymatic synthesis of formyl CoA. Previous studies in this laboratory have established that formyl CoA is produced also by thioalkyl transfer of the CoA moiety from acetyl CoA to formate. The presence in cell free extracts of enzymes capable of forming formyl CoA and formyl phosphate calls attention to the potential role of these substances as activated one carbon metabolites in intermediary metabolism.

The Oxidation of Methylamine. In search of a biological material particularly well suited for investigations of fundamental mechanisms involved in the metabolism of one carbon compounds, several aerobic organisms have been isolated from the soil that are capable of utilizing methylamine as the sole source of carbon and nitrogen for growth. One of these organisms tentatively identified as belonging to the genus *Pseudomonas* was selected for more intensive study. Although several sugars and other simple compounds such as lactate, glycerol and pyruvate support growth, methylamine is the only single carbon compound of several substances tested that will serve as a carbon source

for growth. Nevertheless, other one-carbon compounds including methanol, formaldehyde and formate are actively oxidized by washed resting cell suspensions of organisms which had been grown on methylamine. Formate has been identified as an intermediate in the oxidation of methylamine to CO_2 and NH_3 by resting cell suspensions. Further insight into the mechanism of methylamine oxidation is being sought in studies with cell free extracts of the organism.

Carbon Dioxide Activation. The net synthesis of acetate through the condensation and reduction of two molecules of carbon dioxide is catalyzed by numerous heterotropic microorganisms. Since this synthesis constitutes a major metabolic activity of *Clostridium thermoaceticum*, this organism has been selected as a source of enzymes for studies on mechanisms of carbon dioxide activation and assimilation. Previous studies in this laboratory have shown that the fixation of $C^{14}O_2$ into acetate by cell-free extracts of *C. theromaceticum* is stimulated by additions of pyruvate, ATP, CoA, DPN and an electron donor system such as isopropanol and alcohol dehydrogenase. The acetate formed in the presence of $C^{14}O_2$ is labeled mainly in the carboxyl group but variable amounts of isotope are found also in the methyl carbon atom. In addition to its incorporation into acetate, $C^{14}O_2$ is readily incorporated into the carboxyl group of pyruvate, formate and succinate. The possibility that serine is an intermediate in the synthesis of acetate is made unlikely by the fact that large pools of glycine and serine do not influence the rate of $C^{14}O_2$ incorporation into acetate. Participation of vitamin B_{12} coenzyme in the overall reaction is suggested by the observation that intrinsic factor inhibits the incorporation of $C^{14}O_2$ into acetate. An intermediary role of succinate is suggested by the fact that C^{14}-labeled succinate is readily converted to C^{14}-labeled acetate and this conversion is inhibited by intrinsic factor also.

Methane Fermentation. Another approach to the general problem of one carbon metabolism is made possible as a result of the isolation of the anaerobic bacterium *Methanosarcina barkerii* in pure culture. This organism derives all of its energy and carbon for growth through the fermentation of methanol or acetate to mixtures of methane and carbon dioxide. Originally isolated by Schnellen in 1947, the organism was subsequently lost. Because of its unusual metabolism it has long been recognized as an interesting medium for the study of one carbon metabolism, but all efforts to re-isolate the organism in pure culture in this and other laboratories, have until now been unsuccessful. During the course of its isolation it was discovered that pyruvate also serves as an excellent substrate for growth of the organism. Conditions for the rapid growth of the organism in large scale cultures are now under investigation in order to provide sufficient material for more detailed studies at the enzyme level.

Metabolism of Amino Acids

Lysine degradation. Studies on the anaerobic decomposition of Lysine by cell-free extracts of two strains of clostridia (*Clostridium stricklandii* and *Clostridium* (Ecuador strain)) have been continued. Previous studies in this laboratory have shown that lysine degradation by both organisms leads to the formation of one mole each of acetate and butyrate and to two moles of NH_3. The subsequent finding that orthophosphate and ADP are needed for lysine degradation by cell-free extracts has led to the discovery that lysine decomposition is obligately coupled with the esterification of one mole of orthophosphate to form ATP. Correct formulation of the overall reaction is therefore:

$$\text{lysine} + \text{Pi} + \text{ADP} \rightarrow \text{ATP} + \text{acetate} + \text{butyrate} + 2\ NH_3$$

In addition to DPN, CoA, and 6,8-dimercaptooctanoate (lipoic acid) previously implicated, it has been established that vitamin B_{12}-Coenzyme, Mg^{++}, Fe^{++}, pyruvate and acetyl CoA are required also as co-factors in the over-all enzyme system.

The requirement for vitamin B_{12} coenzyme, suggested in earlier experiments by the partial stimulation of charcoal-treated extracts by addition of pure dimethylbenzimidazole cobamide, was more firmly established by the discovery that intrinsic factor inhibits lysine degradation by cell-free extracts of both organisms; the inhibition is reversed by the addition of cobamide coenzyme equivalent to the cobamide binding capacity of the intrinsic factor protein.

The catalytic level of acetyl CoA required for the overall reaction can be supplied directly or

generated from added acetyl phosphate or added carbamyl phosphate and a catalytic amount of acetate together with free CoA.

So far no intermediates in lysine degradation have been detected. Some possible intermediates including monoamino derivatives and hydroxy analogues of lysine are not attacked by cell-free extracts; they are therefore, not probably intermediates. On the other hand a highly specific deacylase catalyzing the hydrolysis of α-N-acetyl lysine is present in extracts of both organisms, suggesting the possibility that an α-N-acyl derivative may be an intermediate.

The separation of at least one of the enzymes involved in the over-all reaction appears possible from the discovery that some enzyme preparations of the Ecuador Clostridium exhibit an almost complete dependency on the addition of a very small amount of a colored protein fraction derived from *C. Sticklandii.* This protein, presumably of rather low molecular weight, can be separated from the bulk of other protein by chromatography on Sephadex–C–100. The possible identity of this enzyme with the recently described iron containing protein "ferredoxin" is under investigation.

Continued study of this complicated enzyme system promises to yield information on several fundamental problems such as reductive deamination, anerobic phosphorylation, electron transport and vitamin B_{12} coenzyme action.

Cystathionine Metabolism. In the past year attention has been focused on continuing studies of the mechanism of pyridoxal-P potentiation of enzymatic elimination and replacement reactions. Formally, transsulfuration (the transfer of sulfur between cysteine and homocysteine) offers, in *Neurospora*, a complete spectrum of these possible reactions, being mediated by four reactions consisting of β- and γ-replacement, and β- and γ-elimination.

The synthesis of systathionine by β- and γ-replacement by *Neurospora* enzymes has been investigated. Synthesis by β-replacement (from serine and homocysteine) has been demonstrated for the first time in a microorganism. This reaction, characteristic of liver, appears to be absent in *E. coli.* Its presence in me–2 mutants indicates that it is not catalyzed by the same enzyme performing β-elimination from cystathionine, as had been proposed in the past. It does, however, appear to be catalyzed by rather highly purified preparations of the enzyme catalyzing γ-elimination from cystathionine.

During the past year, a preliminary report by English workers has described the enzymatic synthesis of cystathionine by γ-replacement in an *E. coli* mutant. With very sensative methods for detecting cystathionine synthesis from cysteine and homoserine, a search has been made for this reaction in cell-free extracts of *Neurospora* without success. It is not yet certain that a different pathway is involved in *Neurospora*, although 3 genetic loci are implicated in the process, in contrast to 2 in *E. coli.* Neither has it been possible to confirm the presence of the reaction in extracts of yeast or wild-type *E. coli.* The search is now being continued with *Salmonella*, in which the genetic results appear to more nearly duplicate those in *E. coli.* The situation with regard to the two-systathionine cleavage reactions in *Neurospora* is somewhat changed since last year's report. Wild-type *Neurospora* appears to contain 2 separable cleavage enzymes, catalyzing predominately β- or γ-elimination from cystathionine. The former is absent from me–2 mutants, but the latter is not absent from me–7. A definite but elusive question remains as to the true separate identities of these enzymes since a number of conditions appear to bring about β–γ enzyme transformations.

In last year's report we described studies which identified the mechanism of a β-elimination (from L-cystine) catalyzed by a cystathionine γ-cleavage enzyme purified from *Neurospora.* The same enzyme has now been used to gain an unexpected insight into the mechanism of γ-eliminations (from cystathionine or homoserine) catalyzed by the same enzyme. N-ethylmaleimide has been found to trap a highly unstable precursor of α-ketobutyrate in these reactions, though it does not interact with any comparable intermediate in β-elimination (from lanthionine) catalyzed by the same enzyme. The product of the reaction has not yet been identified, but present results suggest that it is an acylvinylglycine, N-(N-ethylmaleyl)-vinylglycine. The immediate significance of these results lies in the fact that intermediary formation of free vinylglycine would establish a unique reaction course for the γ-elimination reactions, and would pinpoint the point of bifurcation in the reactions by which homoserine yields α-ketobutyrate, and P-homoserine yields threonine.

This bifurcation point would lie in alternate protonation of an α or a γ carbon, and would be quite analogous in this respect to the divergence leading to either racemization or transamination.

Thioalkyl Transfer

Thiolester substitutions mediated through the enzymatic transfer of thioalkyl groups from thiolesters to free carboxylic acids are key reactions in diverse metabolic pathways. Such thiolester interchange reactions, especially those involving the transfer of the coenzyme A-moiety from one acyl CoA derivative to form another, constitute important biochemical mechanisms for the transfer of chemical energy from one intermediate metabolite to another.

In an effort to establish intimate details of the enzymic mechanism underlying this fundamental reaction, studies have been initiated to examine the enzyme "CoA-transferase" that catalyzes the reversible transfer of the CoA-moiety of acetyl CoA to butyrate to form butyryl CoA and acetate. *Clostridium aminobutyricum* was found to contain relatively high concentrations of the "CoA-transferase." Optimum conditions for preparing cell-free extracts of this organism have been determined; conditions for the measurement of "CoA-transferase" activity have been defined and procedures for the partial purification of the enzyme have been developed.

When enzyme preparations of sufficient purity have been obtained, the mechanism of the CoA-transfer reaction will be examined with the aid of isotopically labeled compounds.

Permeation and Intracellular Concentration of Purines

A new study in this laboratory is directed toward elucidation of mechanisms underlying the transport and intracellular accumulation of purines. Biochemical studies of cellular transport systems have been hampered by the difficulty of identifying the components of the transport system which are presumably located in the cytoplasmic mebrane. For this reason, the transport system of *Bacillus subtilus* has been selected for study since the cytoplasmic membrane of this organism is readily isolated from the non-membranous parts of the cell. It is hoped that such membrane preparations can be used to form an artificial phase boundary across which the transport, or flux, of purines can be studied apart from the non-membranous parts of the cell.

Preliminary to the more exhaustive studies planned, methods have been developed for the isolation of cytoplasmic membranes of *B. subtilus* and procedures for measuring the intracellular accumulation of purines by the organism have been established. Using, 8-azaquanine resistance as a means of selection, several mutant stains have been isolated following ultraviolet light irradiation and have been found to be deficient in their ability to transport guanine but not adenine. It is hoped that direct comparisons of transport behavior and of enzymes derived from the cellular membrane fractions of the mutant and wild-type stains will provide significant data relevant to the mechanism of purine transport.

Hydrogen Activation

Previous studies in this laboratory have shown that cell-free extracts of *Clostridium kluyveri* catalyze the reduction of DPN by molecular hydrogen. Partial resolution of the hydrogenase system responsible for this reaction has led to the discovery that hydrogen-linked DPN reduction requires the presence of Fe^{++}, FAD, and an unidentified thermal stable cofactor which is present in boiled extracts of the organism. Research on this problem has now been resumed; the ultimate objective is to isolate and identify the thermal stable cofactor and explore its role in other biological transport systems.

Optimum conditions for the preparation of cell-free extracts of *C. kluyveri* have been determined and a new manometric assay of hydrogenase activity has been developed. The new method involves measurement of hydrogen uptake associated with the reduction of pyruvate to lactate when the hydrogenase-dependent reduction of DPN is coupled with pyruvate reduction in the presence of added pyruvate and lactic dehydrogenase. This method is superior to that formerly employed since the nonhydrogenase dependent blank reaction is negligible and it permits a continuous measurement of the time course of the reaction. The nonlinear time course of hydrogen uptake exhibited by most enzyme preparations has been shown to be due

to the presence of ferric iron and can be overcome by preincubation of the enzyme with glutathionine.

Ethylene Glycol Metabolism

Studies on the conversion of ethylene glycol to a equimolar mixture of acetate and ethanol have been continued. The organism responsible for this fermentation has been shown to be a new species of *Clostridium* and has been named *Clostridium glycolicum*. Acetaldehyde has been identified as an intermediate in glycol decomposition by cell-free extracts. Treatment of cell-free extracts with charcoal results in enzyme preparations that are markedly stimulated by additions of Fe^{++} pyruvate, α-ketoglutarate, serine, threonine, glutamate and boiled extracts of the organism. Lack of stimulation of dimethylbenzimidazole cobamide and the lack of inhibition by purified intrinsic factor appear to rule out the involvement of vitamin B_{12}-coenzyme derivatives. These properties indicate that the conversion of ethylene glycol to acetaldehyde in this organism involves a mechanism distinct from that discovered in *Aerobacter aerogenes* by Abeles.

Based on growth yield data it has been concluded that the dissimilation of ethylene glycol to ethanol and acetate is associated with the production of at least 0.85 moles of ATP per mole of ethylene glycol fermented. This is considerably in excess of that expected if the sole energy yielding reaction involves a dismutation of the acetaldehyde intermediate to ethanol and acetate. Attention is drawn to the possibility that the conversion of ethylene glycol to acetaldehyde may, in this organism, be an energy yielding process.

Section on Cellular Physiology

The program of the Cellular Physiology Section has continued in its fundamental objectives of investigating the structural basis of the biochemical activity of proteins and their functional relationships in the integrated activity of cellular structures.

The hypothesis that the three-dimensional structure of proteins is determined by their primary amino acid sequence has continued as a major stimulus of the research. This thesis, originally formulated as a consequence of observations on regeneration of native enzyme in the cyclic reduction and reoxidation of disulfide bridges in the enzyme ribonuclease, and subsequently fortified by similar reversible alterations in the three-dimensional structure of other enzymes, has been illuminated by the initiation of systematic studies on environmental factors and structural modifications influencing enzyme reactivation during the past year.

This hypothesis has also found support in investigations on the molecular structure of the fibrous proteins of the contractile mechanism of muscle, a program initiated some time ago and of growing emphasis in the research of the section.

In addition to studies focused on the structure of protein molecules, the program of the section, as reported below, includes investigations on more biological aspects of biochemistry such as the biosynthesis of proteins, the biochemistry and cytology of cell transport and the mechanism of fat transport.

Structure of Ribonuclease

Systematic study of the conditions for formation of native ribonuclease in the reoxidation of the reduced, inactive enzyme has demonstrated that the rate of reactivation is inversely proportional to the protein concentration, that the reactivation proceeds more rapidly at 24° C. than at 37° C. and is accelerated by increasing pH. Similar oxidation studies using reduced egg white lysozyme gave similar results for the influence of protein concentration on the rate of reactivation. However, in the case of lysozyme reactivation proceeded more rapidly at 37° C. than at 24° C.

While several differences were noted between the two proteins in the influence of environmental conditions on the rate of reactivation, the findings with both enzymes, coupled with observations on the reactivation of reduced polyalanyl trypsin, further substantiate the hypothesis that the tertiary structure of proteins is determined by their amino acid sequence and requires no additional genetic control. However, these kinetic experiments emphasize the fact that the formation of native enzyme from the reduced form during spontaneous reoxidation is not in keeping with the physiological requirements of protein synthesis.

If formation of disulfide bridges in these molecules is considered to be the last step in the biosynthesis of the enzymes, then this spontaneous oxidation process under optimal conditions is exceedingly slow in contrast to the apparent rate of complete synthesis of the enzyme in the pancreas—and the optimal conditions in themselves are not uniformly "physiological." As a consequence of these considerations, the possibility of enzymic reoxidation was investigated and it was discovered that a system could be prepared from rat liver consisting of a washed microsomalfraction and a heat stable, dialyzable component which together lead to extremely fast reactivation of ribonuclease under conditions which result in virtually no reactivation in the absence of this microsomal system—conditions more in keeping with physiological requirements. These results suggest that a system may exist *in vivo* for the catalysis of the conversion of newly synthesized polypeptide chains to the corresponding native proteins.

Investigation of the role of side chain interactions on the reoxidation process has demonstrated that 8 of the 11 amino groups in the ribonuclease molecule may be modified by the introduction of alkyl or polypeptide chains without altering the activity of the enzyme or its capacity to undergo reversible reduction-reoxidation. Three of the amino groups are resistant to polyalanylation and are presumed buried in the structure. These residues have now been identified in the amino acid sequence, and it has been observed that if the alanylation reaction is carried out in bicarbonate rather than phosphate buffer one of these three, identified as lysine #41 in the amino acid sequence, is also reactive. Since alanylation of the epsilon amino group of this lysine residue leads to a parallel loss of enzyme activity, it appears that lysine #41 is critically involved in the active center of ribonuclease.

It has long been believed that rupture of the disulfide bridges of ribonuclease leads to conversion of the native three-dimensional structure of the protein to a randomly coiled polypeptide chain. This conclusion was based primarily on optical rotatory changes. Observations during the past year on polarization of fluorescence measurements of ribonuclease indicate that the native molecule undergoes a subtle reversible structural transition above 36°C.—the temperature where it becomes susceptible to tryptic digestion and below the temperature at which optical rotatory and ultraviolet spectral changes are observed. Similar changes with reduced, carboxymethylated ribonuclease are of interest in that they detect structural changes that may have significance in the mechanism of regeneration of active enzyme in the reoxidation process — that reduced ribonuclease may not be a random coil, strictly speaking, but may have residual structural elements that considerably restrict the possibilities in reformation of the four disulfide bridges in the molecule. However, this does not influence the genetic control hypothesis mentioned earlier for it is known that reoxidation of reduced ribonuclease in strong urea solution, where all evidence indicates a random coil exists, leads to inactive enzyme which can, however, be transformed to active enzyme in normal aqueous solution by providing conditions for disulfide interchange. Under these conditions, all structural tendencies inherent in the amino acid sequence cooperate to produce the thermodynamically most stable form.

It has been known for some time that limited digestion of ribonuclease with the proteolytic enzyme subtilisin leads to no loss of enzyme activity. However, when precipitated in acid solution the enzyme separates into two parts RNase S protein and RNase S peptide, the latter representing 20 amino acids from the amino terminal end of the molecule. These fragments, inactive by themselves, can be recombined to give a fully active enzyme. Since they are more susceptible to selective degradation by exopeptidases and endopeptidases, they offer an opportunity to study the essentiality of some portions of the structure for enzyme activity. At 25° C. RNase S protein treated with carboxypeptidase lost valine and serine from the C terminal end. The resulting product was active when combined with RNase S peptide but physical studies and observations on enzyme activity with respect to temperature indicated a lack of stability. If RNase S protein was digested with carboxypeptidase at 37° C. more extensive digestion was observed and the product was inactive in the presence of RNase S peptide and unable to combine with the latter judging from spectral studies. On the other hand, RNase S peptide was extensively digested by carboxypeptidase converting it from a 20 to a 15 amino acid peptide without loss of ability to reactivate RNase S protein. Thus it appears that extensive

portions of the molecule, while essential for meeting the configurational requirements of the enzyme, are fundamentally without influence on its activity.

The ready formation of native ribonuclease from the reduced form has suggested the possibility of total synthesis with some chance of success. As an approach to this, experiments were designed for attacking the partial synthesis of the molecule. However, the chemistry of the process of recombination of fragments requires the blocking of all amino and carboxyl groups other than those involved in the desired reaction, and this blocking must be reversible. It has been found that the trifluoracetyl derivatives of amino groups and methyl esters of carboxyl groups can be readily formed and subsequently cleaved by treatment of the blocked enzyme with piperidine.

Structure of Lysozyme

Studies on the structure of egg white lysozyme have been completed. Techniques for peptide separation which have previously been perfected in this laboratory have been used to obtain a series of tryptic peptides from lysozyme. The sum of the amino acid compositions of these peptides is equal to the total composition of the enzyme, and thus the entire molecule can now be represented by this set of "subunits." Use of selective blocking for sites of trypsin cleavage and use of other cleavage agents has yielded additional information which allows a tentative assignment of each "subunit" to a specific region of the enzyme. The characterization of the various peptide fragments of lysozyme has now been completed, and the accumulated information makes possible the complete reconstruction of the amino acid sequence of this protein.

Following short incubations of oviduct with radioactive leucine, the newly synthesized lysozyme (containing the radioactive leucine) was isolated, and different leucine-containing peptides have been obtained by using techniques previously employed in the amino acid sequence determination. Variations in the specific activity of the leucine, when compared with the peptide alignment for lysozyme, are consistent with a model which depicts protein biosynthesis as a process of unidirectional growth (along a special template) of a polypeptide chain beginning at the amino and terminating at the carboxyl end.

Myosin Structure and Activity

Studies on the structure and function of the fibrous proteins involved in muscular contraction are continuing. As a first approach to this problem earlier reports presented successful efforts directed toward exact measurements of the molecular parameters of myosin. These efforts demonstrated that the long myosin molecule is composed of three polypeptide chains of identical structure wound together in the form of a three-stranded rope. This complex can be dissociated into single strands and reassociated by removal of the dissociating agent. Though enzyme activity has not been regenerated in these experiments, the results demonstrate the great tendency of this particular sequence of amino acids to form the fundamental α-helix structure in aqueous solution and to associate into a three-stranded structure. The failure to recover enzyme activity appears to be due to disulfide bridge formation; methods for preventing the latter have not yet been devised.

Other experiments demonstrated that a portion of the enzymically active center of myosin (ATPase) could be selectively blocked using radioactive sulfhydryl reagents. Enzymatic digestion followed by peptide separations have provided a means of isolating those portions of the molecule involved in the active center of myosin ATPase. During the past year methods have been developed for preparation of these fragments in pure form and in sufficient quantity for the determination of structure.

By very carefully controlled digestion of myosin by trypsin, it has been possible to isolate a portion of the myosin molecule, previously identified as H-meromyosin, which retains the ATPase and actin-binding sites of the original molecule. The new procedure developed provides material devoid of internal fragmentation by trypsin. An earlier proposal placed this portion as a thickening of one end of the myosin molecule. Electron microscope observations elsewhere have now verified the existence of these club shaped molecules. End-group analyses of our new H-mero-

myosin lead to the tentative conclusion that the "club" represents the amino terminal ends of the polypeptide chains.

Lipopeptides in Protein Synthesis

Studies on the involvement of lipid compounds of amino acids in protein synthesis have been pursued further during the past year. A more extensive purification of some of the lipid amino acid compounds has been achieved. Some of these materials appear to be lipopeptides with specific fatty acids attached to the amino group of the peptide. Further structure studies indicate that the peptides are also bound in ester linkage to a polyglycerol phosphate.

Studies initiated to examine more closely the involvement of the membrane structures of cells in protein biosynthesis have suggested on the one hand that a direct utilization of the energy available from the electron transport network may be possible without the mediation of ATP. On the other hand, studies on the relationship between the nucleoprotein granules, ribosomes (known to be active in protein biosynthesis), and the cell membranes suggest that the ribosomes attached to the membranes are more active than the free ribosomes.

Cell Transport of Lipids

Studies on the biochemistry and cytology of cell transport have proceeded along two main pathways. Investigations on the uptake of fatty acids and chylomicrons by lactating mammary gland have suggested an involvement of lipoprotein lipase with the uptake of fat by this organ and support the hypothesis of involvement of this enzyme in the transport of triglycerides. The enzyme appears in the mammary gland only hours before parturition and disappears abruptly on cessation of suckling. Further studies on the lipid metabolism of mammary gland have demonstrated that this is one of the few tissues possessing glycerol kinase and is thus able to synthesize triglycerides from glycerol.

In the course of studies designed to consider the function of cell membranes in cell transport using a mutant of the slime mold *Dictyostelium discoideum* it became apparent that lipids of this organism are quite unusual. These lipids contain about 80% unsaturated fatty acids of which the two major ones are a previously undescribed diunsaturated 16-carbon acid and a diunsaturated 18-carbon acid. Studies with radioactive precursors have demonstrated the pattern of fatty acid synthesis. Myristate and palmitate are elongated to C_{16} and C_{18} fatty acids. The organisms have enzymes that then introduce double bonds specifically and sequentially at positions 9–10 and 5–6.

LABORATORY OF CHEMICAL PHARMACOLOGY

A. Mobilization and Utilization of Metabolic Substrates

Function in the living organism is mediated through biochemical reactions; rapid adaptation to a changing environment is possible because certain enzymes are activated by the action of the nervous system. All kinds of behavior require an increased utilization and consumption of metabolic fuel-free fatty acids (FFA) from adipose tissue and glucose from glycogen. It seems logical that adjustment to these demands is mediated by the CNS since regulation of the supply of metabolic fuel is as an integral a part of behavior as regulation of breathing and cardiac output.

We have shown that adipose tissue contains much more norepinephrine (NE) than needed to control the circulation, suggesting that control of the output of FFA, as well as glucose, is a function of the sympathetic nervous system. Last year we presented some evidence of this when we showed that stimulation of sympathetic fibers innervating adipose tissue *in situ* can increase the FFA in effluent blood.

Evidence of an absolute requirement for the sympathetic system in mobilization of metabolic substrates was obtained by showing that exposure of rats to 4° C., body temperature is maintained by production of heat through mobilization and burning of additional FFA and glucose. However, if animals are pretreated with Ecolid (ganglionic blocking agent), they no longer can mobilize FFA or glucose, and they die in a few hours with a reduction of body temperature to 13° C.; however, if they are also given epinephrine in oil, the rats maintain temperature and their ability to mobilize metabolic substrates.

Evidence of the importance of the sympathetic system in mobilization of substrates was obtained using new technics of chemical sympathectomy:

rats are adrenal demedullated and then given either a nonsedative dose of reserpine or BW 392C60, a potent bretylium-like compound which prevents release of NE by nerve impulses. On exposure of these animals to cold, FFA and glucose are not mobilized; the body temperature drops and the animals die at 15° C. in about 3 hours. However, pretreatment of rats with epinephrine in oil sustains body temperature and life.

The sympathetic nervous system regulates output of FFA and glucose through catecholamine-induced activation of adipose tissue lipase (ATL) and liver phosphorylase respectively. Thus, cold-exposure or the administration of catecholamines rapidly activates lipase and phosphorylase and increases FFA and glucose output. After chemical sympathectomy, cold-exposure no longer activates lipase and phosphorylase, or increases output of FFA and glucose.

Although lipase is activated by epinephrine (E) as well as NE, studies with adrenal demedullated rats exposed to cold show that NE released at nerve endings is adequate to cause a maximal mobilization of FFA; but that E from adrenal glands is needed to mobilize adequate amounts of glucose.

Chemical sympathectomy also prevents the mobilization of FFA and glucose in other situations. For example, sympathectomized rats do not activate ATL or increase the output of FFA (or glucose) after heavy muscular exericse, or after administration of a ganglionic stimulant, large doses of alcohol, morphine or depot ACTH.

These studies have reoriented our thinking about the essential role of the sympathetic nervous system in energy-producing processes, for the chemically sympathectomized animals, exposed to cold, exercise and other stresses, exhibit the classical shock-like response shown by adrenalectomized animals.

In fact, adenalectomized and chemically sympathectomized rats show identical responses to stress such as cold-exposure; in both types of animals the ATL and liver phosphorylase are not activated; FFA and glucose are not mobilized; and the animals die in 3 to 4 hours at a body temperature of 15° C. Pretreatment of adrenalectomized rats with epinephrine in oil does not benefit these animals but if they are pretreated with corisone they can now activate lipase and mobilize metabolic substrates. If the adrenalectomized rats are pretreated with aldosterone they can also mobilize metabolic substrates. Apparently the effects of adrenalectomy on mobilization of substrates stem from failure of sympathetic receptors to react to catecholamines possibly because of an unfavorable electrolyte environment.

FFA Mobilization in Starved Rats

Plasma FFA levels and adipose tissue lipase activity are markedly elevated in starved rats. These changes are not prevented by chemical sympathectomy, indicating that, in this circumstance, lipase in adipose tissue is activated by an entirely different mechanism from that involved in ordinary stress.

B. Control of Basal Metabolism

Treatment of mice with triiodothyronine doubles the O_2 consumption. This effect is prevented by ganglionic blockade or by chemical sympathectomy, indicating the close interrelationship of the thyroid and the sympathetic system. Since the turnover of NE in peripheral tissues is unchanged by triiodothyronine it seems probable that one of the effects of the hormone is to sensitize the sympathetic receptors to catecholamines.

C. The Neurochemical Transducer

Adaptation, or response to environmental stimuli, requires no new function but alterations in intensity of existing function and is mediated by changes in the level of hormones at reactive sites. We have introduced the term "neurochemical transducer" to describe those units at nerve endings that synthesize and store a neurohormone, and release it to receptor sites. (Biologically these may be considered as the basic units of behavior.)

The following model now best represents the NE neurochemical transducer: NE is isolated from receptors and inactivating enzymes by a lipoid membrane and is present in at least two pools—a mobile, readily available pool, and a reserve pool, presumably complexed with ATP in granules. NE in the mobile pool is sequestered within the membrane by active transport (a "pump and leak" system). Monoamine oxidase (MAO) outside the membrane metabolizes NE which diffuses out and ensures that, despite the continuous synthesis of amine, the steady-state level is maintained below that which saturates the transport system. The nerve impulse counteracts

the pump in front of the receptor, thereby releasing some NE. Between each nerve impulse, the pump is restored and since the receptor is extremely close to the storage membrane, the NE released onto the receptor is pulled back into storage depot by the action of the pump.

A picture of the NE neurochemical transducer is built on the basis of the following data:

(1) Uptake of $C^{14}NE$ by tissue slices shows that the amine is taken up against a concentration gradient by a process blocked by reserpine and metabolic inhibitors.

(2) The scheme requires that NE be continuously formed irrespective of nervous activity. On injection of H^3NE (i.v.) the label is rapidly taken up by various tissues. A plot of radioactivity against time shows that the H^3NE in tissues first declines rapidly but within 12 hours declines exponentially. The exponential decline is the classical picture of a substrate store turning over at a constant rate. Complete blockade of sympathetic tone (ganglionic blockade) does not appreciably reduce the turnover rate. These results show that NE is formed in excess and is either lost by release onto receptors or by leakage onto MAO.

(3) The turnover of H^3NE in rat tissues is slow, with a half life of about 15 hours for heart and about 40 hours for skeletal muscle. The failure of nerve stimulation to deplete NE is explained by the economy of amine loss at nerve endings. Sympathetic stimuli are pulsatile so that most of the NE released to receptors is pulled back into storage between each nerve impulse. Thus, continuous synthesis together with economy of release explains why nerve endings are not depleted by nerve stimulation.

(4) Preliminary results show that NE storage sites have a high specificity for H^3NE and rapidly reject E.

By regarding the synthesis, storage, physiological release, metabolism and the receptor as all parts of a single control unit a broad picture of drug action is permitted. Thus, the NE transducer can be affected by drugs in 8 ways: (1) a drug (e.g., neosynephrine) may mimic NE, (2) indirectly mimic NE by releasing NE (amphetamine), (3) block the action of NE (dibenamine), (4) block metabolism of NE (MAO inhibitor), (5) block synthesis of NE (dopa decarboxylase and dopamine-β-oxidase inhibitors), (6) block storage (reserpine), (7) block release by nerve impulse (bretylium), (8) activate process by which nerve impulses release NE (guanethidine).

We have demonstrated drugs that act in each of these ways; furthermore the effect of drugs has supplied us with considerable additional information about the transducer.

(1–2) Drugs like amphetamine act peripherally by releasing NE onto receptors but centrally they stimulate receptors by a direct action. In small doses, amphetamine prevents depletion of NE by guanethidine. This finding fits the view that guanethidine is a specialized sympathomimetic agent acting "irreversibly" on the same process normally activated by amphetamine and by the nerve impulse.

(3) Sympathetic blocking agents can have a highly selective action. For example, isopropylmethoxamine (BW 61–43) is an agent which has no obvious effect in normal animals but prevents mobilization of FFA from adipose tissue and glucose from glycogen by catecholamines.

(4) MAO inhibitors lower blood pressure because they also act like bretylium—that is, they prevent the release of NE from nerve endings by nerve impulses.

(5) Compounds that block synthesis of NE *in vivo* (dopamine oxidase inhibitors) do not necessarily lower the levels in tissues. This is further evidence that NE released at nerve endings is used over and over.

(6) Reserpine depletes NE by blocking the NE pump. As a result the amine diffuses out onto MAO and thus leaves the tissues as the deaminated form.

(7) Bretylium and a number of synthetic phenylmethylguanidines (especially BW 392C60) prevent nerve impulses from releasing NE at nerve endings. These compounds also prevent the release of NE induced by guanethidine and block the action of amphetamine.

(8) Guanethidine and a number of synthetic phenylethylguanidines deplete NE by a mechanism which is blocked by bretylium and is different from that of reserpine. The decarboxylation products of α-methyl-DOPA and α-methyl-m-tyrosine may also act like guanethidine.

NE seems to be released by guanethidine from the mobile pool directly onto receptor sites rather than onto MAO and thus enters the blood stream largely in the undeaminated form. Further evidence that guanethidine is released from the mo-

bile pool is shown by the kinetics of release which are first order until about 40% is depleted and then zero order presumably as release from granules becomes the rate limiting step. Finally, administration of guanethidine to rat 20 hours after giving H^3NE decreases specific activity of NE in heart indicating that there is a pool of endogenous NE which has not mixed with the label. In contrast, tyramine given at this time also releases NE but increases the specific activity of H^3NE in heart. This seems plausible only if guanethidine releases NE from the "mobile pool" and tyramine releases NE directly from the granules containing NE complexed with ATP.

D. Drugs That Interfere With Biochemical Control Mechanisms

Pituitary-Adrenal System

A misconception in endocrinology is that tranquilizing agents like reserpine, chlorpromazine and morphine, protect animals from stress by blocking the output of ACTH. These compounds in sedative doses actually produce a persistent pituitary-adrenal response so that the animals can no longer respond further to a stressful stimulus. Moreover, in large repeated doses the drugs can reduce the content of ACTH in the pituitary to about 25% of normal.

Lipid Transport

Each day a large amount of FFA is mobilized from adipose tissue; part is burned in tissues but most is re-formed in liver to TGL which is then secreted as a lipid protein complex and carried back to be restored in adipose tissue. Despite the large turnover of lipid the normal TGL in rat liver is only about 5 mg./g. A fatty liver might be produced by drugs that increase mobilization of FFA, or increase formation and deposition of TGL, or block secretion of liver TGL. Thus, large doses of alcohol and morphine produce fatty liver by increasing FFA mobilization through increasing sympathetic tone. The glucocorticoids also secreted in response to these drugs enhance deposition of TGL in liver. Drugs like CCl_4 act mainly by blocking the secretion of TGL from liver.

Factors Which Affect Duration of Drug Action

Enzymatic Mechanisms of Drug Metabolism

(a) Overwhelming evidence is now accumulating that nitro reductase oxidizes TPNH in air and is therefore a component of TPNH oxidase

(1) T P N H - oxidase + TPNH → reduced TPNH oxidase + TPN

(2) reduced TPNH-oxidase + O_2 → active hydroxyl donor

(2B) anaerobically reduced TPNH oxidase and nitro compound → TPNH-oxidase + nitroso compound.

(b) Studies of the mechanism of drug oxidation in liver microsomes are hampered by difficulties of solubilization. The claim by other workers that aniline hydroxylase is solubilized by a snake venom or by a pancreatic lipase appears to be an artifact since after these procedures the hydroxylase activitiy in supernatant appears to be due to suspended particles.

Distribution of Drugs

Two new ways by which drugs are reversibly bound to tissues have been disclosed:

(a) Imipramine, and perhaps the phenothiazines, are localized in various body tissues by reversible attachment to phospholipids.

(b) Guanethidine and perhaps other strong bases are not bound to plasma proteins but are strongly bound to various tissues especially heart *in vivo*. The extent of binding to heart is drastically reduced on homogenization of the tissue. Preliminary results suggest that guanethidine is taken up in tissue slices by a process having certain characteristics of active transport.

Activators and Inhibitors of Drug Metabolism

(a) In rats, methyltesetosterone and other anabolic agents induce drug enzymes. Phenobarbital and a large number of drugs, not anabolic agents, also induce drug enzymes. The two mechanisms have been shown to be different.

(b) Liver microsomes are present in two forms: rough (containing the ribonucleic acid) and smooth-surfaced, and drug metabolizing enzymes are present mainly in latter. After induction of enzymes by phenobarbital or 3,4 benzpyrene the

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number of smooth microsomes is markedly increased.

(c) A new phenomenon opposite to that of drug enzyme stimulation has been discovered. Morphine given to male rats in single doses depresses the activity of the microsomal enzymes which dealkylate morphine, demethylate aminopyrine and hydoxylate aniline and hexobarbital.

(d) "Heat-labile" endogenous inhibitors of drug metabolism have been reported to be present in liver nuclei and microsomes. A major part of this activity is due to presence of pyrophosphatases which destroy TPNH and TPN.

Mechanism of Action of Desmethylimipramine, a New Antidepressant

Previous studies have shown that imipramine (Tofranil) exerts its anti-depressant action through the formation of a metabolite — desmethylimipramine (DMI) a nonsedative, non-MAO-blocking agent. The action of DMI in the model system (reversal of effects of reserpine) is associated with the release of NE, thus: (1) Rats given DMI+reserpine show no excitation if animals have been selectively depleted of brain NE by α-methyl-m-tyrosine. However, the combination of drugs again produces excitation when the NE level has started to rise. (2) DMI produces excitation in rates only if given before reserpine, not if given after. (3) Further evidence that the action of DMI is related to catecholamines is the striking potentiation of DMI on the awaking action of DOPA given to reserpinized rats.

Species Differences in DMI Metabolism

Desmethylimipramine (DMI) shows an antidepressant action in rats and man, preventing and reversing the action of reserpine, but has little activity in mice, rabbits and cats. In rats and man the drug has a long half life but in mice and rabbits its half life is extremely short. In cats the half life of DMI is extremely long, but in cat brain NE is liberated by reserpine over a period of four or more hours compared to one hour in other species. This explains the failure of DMI to act in cats since we have shown in other studies that DMI acts only if NE is rapidly released.

Passage of Substances Across Membranes

(1) Membranes Within the CNS

Studies of the distribution of drugs after intracisternal or intraventricular injection are of value in identifying CNS compartments and in interpreting drug effects after injection directly into the CNS. As further evidence that substances leave the CSF by a process of filtration, the rate of efflux of inulin was shown to be proportional to the pressure difference between CSF and dural sinus blood. However, a number of quaternary ammonium compounds including N^1-methylnicotinamide, hexamethonium and decamethonium leave by active transport.

Incubation of various quaternary compounds with choroid plexus *in vitro* shows that they are taken up against a concentration gradient and that the process is depressed under anerobic conditions and by 2,4-dinitrophenol.

The passage of lipid-insoluble drugs across the ependymal lining of the ventricles was found to be rapid. This means that in studying the effects of a lipid-insoluble drug in the CNS, the blood-brain barrier may be by-passed by injecting the drug directly into the brain ventricles.

(2) Biliary Excretion of Drugs

The mechanism by which the liver secretes quaternary ammonium compounds such as procaine amide ethobromide (PAEB) seems to be specific for strong ions since it does not act significantly on the tertiary amine derivatives. The process is saturable and is antagonized by other quaternary compounds provided they are also excreted in bile. The process is not depressed by organic acids such as bromsulphalein which are transported by another mechanism.

For studies of bile secretion, an ideal substance should not be metabolized nor bound to protein or tissues and should be easy to assay. Such a substance has been found in N-acetyl-PAH.

(3) Penetration of Drugs Into Cells

Certain quaternary ammonium compounds appear to enter liver slices against a concentration gradient by a saturable transport process that is

blocked by anerobic conditions and by dinitrophenol or iodoacetate. Similarly, the uptake of guanethidine by heart slices is blocked anerobically and by dinitrophenol.

(4) Effect of Drugs on Uptake of Norepinephrine and 5HT

A number of drugs interfere with uptake of NE and 5HT by various tissues. A number of these such as cocaine, chlorpromazine and imipramine are said to block the uptake of NE and 5HT but not to release them. Since desmethylimipramine (DMI) exerts no pharmacologic effects of its own, it provides a particularly useful tool for these studies. DMI does not change the rate of NE-C^{14} uptake into red cells but markedly depresses the active uptake of 5HT into platelets by competitive inhibition. However, after complete blockade of 5HT transport by N-ethylmaleimide, DMI does not block the passive uptake of 5HT.

Unlike reserpine which completely blocks 5HT uptake, DMI only slows the rate of uptake. This may explain the failure of DMI to deplete endogenous NE.

Other effects of DMI are: (1) it interferes with uptake of H^3NE *in vivo*; (2) blocks release of NE by guanethidine and tyramine but not by reserpine. These effects seem paradoxical and they do not explain the drug's antireserpine action nor its extraordinary potentiation of administered NE.

Development of New Drugs

(1) Antidepressants

Clinical trials show that DMI is active as an antidepressant in primary depression and acts more rapidly than the parent compound. In collaboration with Geigy, Switzerland, we are looking for the structural characteristics of active antidepressant DMI analogues. A number of analogues of DMI and of desmethylchlorpromazine have proved potent enough in preventing the action of reserpine to warrant clinical trial.

(2) Dopamine Hydroxylase Blockers

Collaborative studies with Smith and Nephew, England, have yielded structural characteristics for inhibitors of dopa decarboxylase and dopamine hydroxylase. Thus far we have found extremely active dopamine hydroxylase inhibitors but they have too short a half life *in vivo* to merit clinical trial.

(3) Bretylium-Like Compounds

In collaboration with Burroughs Wellcome we have shown that BW 392C60 (N-*o*-chlorbenzyl-N′,N″-dimethylguanidine) is the most potent bretylium-like compound yet found in antagonizing the guanethidine-induced release of NE. This drug is now in clinical trial as a hypotensive agent. The greatest value of the compound is as a tool to produce chemical sympathectomy. Use of this compound has shown that completely sympathectomized rats cannot respond to a stressful stimulus.

Development of New Methods of Analysis

(1) Methods for analysis of dopamine and NE have been simplified and their sensitivity increased. (2) A new method for assay of FFA involving formation of lipid-soluble Cu^{++} complex and assay of lipid-soluble copper has been applied to plasma; results to date indicate that the problems of present titration methods may be obviated. (3) The level of NE in tissues where the amounts are too low to be assayed by current methods can be assayed from the uptake of highly labeled H^3NE tracer. (4) A sensitive coupling method to determine dopamine oxidase inhibitors has been developed. (5) Methods for determining whether enzymes are functionally blocked *in vivo* now include those for MAO, dopamine oxidase, and dopa decarboxylase.

LABORATORY OF TECHNICAL DEVELOPMENT

Gas Chromatography

The major emphasis in the developmental part of this work continued to be on the development of methods of microanalysis using the techniques of gas-liquid and gas-solid chromatography. This included the development of methods of detection, methods for measurement of radioactivity in gas chromatographic effluents and procedures for applying these techniques toward the solution of a variety of biochemical and physiological research problems.

Five distinct but related methods for radioassay of labeled compounds analyzed by gas chromatography have been developed, each was explored and the usefulness and limitations of each defined.

Cumulative Collection on Anthracene

The previously developed method of accumulating the entire effluent in one detector filled with anthracene crystals while monitoring the radioactivity accumulated by scintillation counting was found to have the limitation that resolving power decreased during the course of the analysis.

Fraction Collection on Anthracene

The method of fractionation of the effluent into a number of separate anthracene filled vials for subsequent scintillation counting was developed and applied to a number of biochemical metabolic studies and evaluated. It became apparent that this technique offered the possibility of measuring very small quantities of radioactive tracers with a resolution limited only by the frequency with which the effluent was fractionated. This method has proved useful in many studies conducted at NIH and has been adopted elsewhere as well.

Ionization Chamber

The method of measurement of radioactivity using an ionization chamber, started last year, was completed, evaluated, and the results published. The vapors in the effluent were combusted to carbon dioxide and water, the water converted to hydrogen gas and the gases led to a room temperature ionization chamber for measurement of radioactivity.

Flow-through Scintillation

A flow-through method for scintillation counting of Carbon-14 and tritium has been developed. A cartridge filled with anthracene crystals is used as a "flow-through" radiation detector. Upon leaving the column, the effluent is passed over hot copper oxide, which converts the organic materials to carbon dioxide and water. If tritium is to be counted, the water is reacted with hot iron to release hydrogen and tritium gases. The effluent is then passed through the anthracene cartridge. The counting rate of the cartridge is monitored continuously during the analysis by a highly efficient, low-background, scintillation counter to yield a record of eluted radioactivity that resembles the conventional mass detector record.

The detector is simply constructed, easily maintained, insensitive to change in gas composition but highly sensitive to radiation. High efficiency (more than 70% for Carbon-14 and 20% for tritium) is coupled with low background counting rates (ca. 15 cpm at Carbon-14 settings, 50 cpm at tritium settings). The counting rate returns to this low background rate following the detection of a labeled compound.

This method has been published, was used extensively in studies of the specificity of the esterification mechanism involved in fatty acid absorption in our laboratory, formed part of the methodology used in the "derivative ratio analysis" method for measurement of steroids, and was made available to many investigators at NIH.

It possessed the advantage over the cumulative method of higher sensitivity without sacrifice of resolution, and the advantage over ionization chamber and geiger counters of insensitivity to change in gas composition.

Tritium Assay

Two methods of assaying tritium in compounds analyzed by gas chromatography were developed. One represented an improved method for converting the tritium in the samples to tritium gas for assay by scintillation counter or ionization chamber, and the second a modification of the method of fractionating the effluent for subsequent radioassay. When the samples contain sufficient tritium in each component for accurate assay in less than 10 seconds, the assay is performed during the analysis. The column effluent is passed through a combustion train consisting of: a heated tube containing copper oxide in which organic materials are converted to carbon dioxide and water; a second tube containing heated iron, maintained in the reduced state by a stream of hydrogen gas, in which the water reacts to liberate tritium labeled hydrogen gas; a magnesium perchlorate water trap; and either an ionization chamber or a transparent tube filled with anthracene crystals for scintillation counting. When the samples contain insufficient tritium for radioassay during the analysis, the effluent is fractionated by being passed through a succession of cartridges containing p-terphenyl crystals coated with silicone oil. High boiling materials in the effluent are trapped and retained in these cartridges. Each cartridge is then transferred in its entirety to a vial containing DPO-toluene for radioassay by liquid scintillation counting for whatever time is required for statistically accurate results.

Determination of Blood Gases

The micro-method for blood gas analysis was developed further with a new system for rapidly evolving the gases from the sample. The results agreed very well with Van Slyke determinations in the hands of the developer, but in the hands of a technician performing routine analysis the results are not yet satisfactory.

The D.C. Discharge Detector

This detector has been shown to have very high sensitivity without the need for radioactive ionizers, and is the basis for the micro-method for blood gases. Its high sensitivity and ability to measure atmospheric gases have led to its application in the gas chromatograph mounted in space and lunar exploration capsules.

Quantitative Microdetermination of Lipids

The effluent of a liquid-liquid or silicic acid chromatographic column may contain materials of interest dissolved in mixtures of solvents. Since the chemical nature of the solvents and solutes are often very similar, and since the composition of the solvents may be varied intentionally ("gradient elution") during the chromatography detection of the solute in solution may be difficult.

The method is based on the difference in volatility between the lipid and the solvent. Aliquots of the solution are injected into a miniature gas chromatographic column operated at room temperature but containing only uncoated, solid refractory material. Carrier gas is passed through this column to a hydrogen flame ionization detector. When the response of the detector indicates that the solvent has completely evaporated, the column is heated rapidly to 600° C., pyrolyzing the remaining material. The pyrolysis products are delivered to the flame ionization detector until the detector response again returns to baseline, at which time the heat to the column is turned off. The system is then ready for another injection.

The electric charge carried during the passage of the pyrolysis products through the detector has been found to be a function of the total lipid injected.

Application of Gas Chromatography Methods

A method has been developed for microassay of complex biological molecules, such as sterols having specific functional groups. An unknown is acetylated with C^{14} labeled acetic anhydride along with known material bearing the same functional group. When the two substances are separated by the gas chromatograph the ratio of the radioactivity is used to determine the ratio of standard to unknown. Sensitivity to nanograms of material is obtained.

Collaborative studies utilizing the methods developed were carried on in cooperation with other laboratories of NHI and other laboratories at NIH and elsewhere. Included in these are studies of the active agents in poison ivy for development of methods of standardizing the therapeutic extracts, studies of the fatty acid composition of chyle and serum in relation to ingested fats, and studies of the part played by enzymatic activity in determining the lipid composition of chyle after ingestion of various fats.

Ultra Microanalysis of Sodium and Potassium

A method for measuring the content of sodium and potassium in micropuncture samples obtained from renal tubules has been developed and applied. The method is based on the effectiveness of a helium plasma excited by a radiofrequency field to excite characteristic emission of Na and K. A sample of the order of several millimicroliters is suitably buffered with cesium and phosphate and pipetted onto a fine wire filament which is used to volatilize the sample into the glow. The characteristic emission is selected by an interference filter and measured with a photomultiplier photometer. A peak reading voltmeter registers the response of the photometer which is linear over a range of 10^{-11} to 10^{-12} moles of sodium or potassium per sample. In application a standard error of $\pm 2\%$ has been obtained on single determinations.

Ultra Microfreezing Point Depression Apparatus

An apparatus for observing the thawing point of samples of biological fluids of the order of a few millimicroliters has been constructed. A thermoelectric refrigeration system consisting of a two stage Peltier effect thermocouple is controlled by a feedback servo that maintains the temperature of the samples at whatever level is set on the control dial. The rapidity of action, which is made possible by the electronic control and low thermal mass of the cold stage, allows

one to control the rate of thawing so well that the point of disappearance of the last crystal can be approached rapidly—reversed and equilibrated under visual control. At present eight samples under oil in individual holes in a silver block are observed under a microscope. Samples and standards can be interspersed in the same block to calibrate or provide interpolation points.

Blood Flow Measurement

The ultrasonic system for measurement of flow described in the previous report has been improved and somewhat simplified in a second model which utilizes sum frequencies for the intermediate frequency instead of requiring the method of multiplication and conversion previously used. In addition, a method of establishing the zero flow point electrically has been developed. This obviates the necessity of occluding the flow to obtain the zero flow calibration point. The high frequency 20-mc sound carrier used in this system makes it possible to utilize transducers of the order of 0.050″ in diameter. A system for catheter tip flow measurement is currently being constructed. In performance, the system has demonstrated superior frequency response and ability to resolve lower flows than other systems. Some spurious temperature sensitivity is also being investigated.

Nuclear magnetic resonance techniques for flow measurements offer the unique advantage of being able to measure flow without mechanical or electrical contact with the blood vessel. It can, therefore, measure total flow in an appendage or total number of protons in the sensitive region and can measure the passage of other resonant nuclei such as that of the fluorine atom. The apparatus has been tested for measuring continuous, pulsating and complex flow patterns, such as two coaxial flow streams in opposite direction or two separate tubes in the same sensitive area. Sensitivity sufficient to determine flows to reasonable accuracy in small vessels has been obtained by highly stabilized electronic circuits and determination of optimum sensor coil geometry.

Fast Reaction Methods

Fast reaction studies have added greatly to our basic knowledge of chemical and biological systems. It was felt for some time, however, that in any reaction in which several steps were present, more than one detection method would add greatly to an understanding of these steps. The most obvious combinations are optical-thermal, optical-electron spin-resonance, plus adding electron conductivity to either of the two. Then thermodynamics and kinetics could be studied together. The final two combinations have been built.

The work of the past year has gone mainly into greatly improving the optical-thermal apparatus for use in the study of the mechanism of the hemolysis of red blood cells. It will be extended to the detailed study of the model reactions of catalase, peroxidase, and other heme or hemocyanin reactions. A special flow apparatus has been built which mixes the reactants in 0.1 millisecond even when viscosities are as great as 1000 centipoise, and stops the flow in less than 8 milliseconds. About 2 ml. of solution are needed for experiment. The optical system will respond to changes of 0.0001 optical density units even to 3.0 O.D. in less than 1 millisecond. The thermal system responds to 0.00002° C temperature changes in about 8 milliseconds. This is presently being reduced to less than one millisecond and a CoOp student from University of Cincinnati is improving the photomultiplier tube to see 0.0001 O.D. or better in 0.1 milliseconds, the latter improvements are necessary for the enzyme reactions. A 12-volt high-current tungsten light source stable to one part per million in intensity has been built as well as an all solid state temperature regulator which can detect less than 0.0001° C. temperature change.

The special mixers that have been developed are of particular interest due to their fast mixing time and ability to handle solution of grossly different visocosities, i.e., water plus packed red blood cells (25,000 g's).

Details of the mechanism of hemolysis have indicated that the cells may first go to hemolytic volume and then open allowing hemoglobin to diffuse out. Further work on this point is being done with the faster system. The earlier model also proved too slow to discriminate between the various steps in the carbonate reaction, and this is being repeated in the fast apparatus to explain the discrepancy between the optical and thermal data found in the literature.

Calorimetry of Intact Cell Metabolism

A differential calorimeter using 5 ml of solution and capable of measuring 0.00001° C. temperature changes has been built to follow the heat of reac-

tion of the sodium-potassium ATPase activity in human red blood cell membranes. A very high heat of absorption was observed, about 100–300 Kcal/mole of PO_4 released, when the ATPase is poisoned by a cardiac aglycone such as strophanthidin. Heat evolution appears to occur with the uninhibited system to the extent of 700–1000 Kcal/mole of PO_4 released. Further work on this system may lead to an understanding of the mechanism of this reaction.

Photochemistry of the ATPase System

The irradiation of the intact red blood cell ghosts with 366 mμ has shown that the free radical produced in riboflavin upon irradiation can react with the enzyme which hydrolyzes ATP when Mg is present and thus produce almost complete inhibition. When Mg is removed and the irradiation carried out, the riboflavin radical is even more efficient than Mg in acting as an activator for the hydrolysis. In fact, while it is known that Mg alone will hydrolyze ATP without the enzyme, the process is very slow and the riboflavin radical acting without enzyme produces a reaction almost as fast as the enzymatic one. Preliminary trials using the enzyme alkaline phosphatase yielded similar results. A very crude trial to observe the free radical was carried out at Varian Associates with good results indicating a radical associated with the riboflavin and one associated with the enzyme.

During the past few months a U.V. irradiator producing 10^{19} photons per cm^2 second has been constructed and tested, riboflavin purified, the electron spin resonance spectrometer operated and finally riboflavin irradiated in the cavity. Rather large drifts were produced in the spectrometer during irradiation and it is still unclear whether or not radicals were observed. Calibrating radicals indicated the machine was operating well, so that considerable work will need to be done to stabilize the system during irradiation.

Similar work using gamma rays is being done in cooperation with the Greek Atomic Commission.

Fluorescence and Phosphorescence

Excitation of fluorescence and phosphorescence by electrons is well known and is highly efficient. Electron excitation of materials of biological interest was investigated by exposing about 50 luminescent of potentially luminescent materials to a beam of low energy electrons accelerated through a thin aluminum window directly into the sample. The low energy electrons were obtained from a simple electron gun provided with a pulsed high voltage system. Luminescence was measured by means of a photomultiplier photometer. In general, minerals and crystalline scintillators and scintillators in toluene were effectively excited to luminesce but aqueous solutions and most fluorescent organic materials were not much greater than the blank. The blank was shown to be in part due to luminescence of the nitrogen in the air and presumably could be reduced. The luminescence although not much greater than the blank would be useful if the blank could be eliminated by optical means. The apparatus has been rearranged to analyze the emission spectra of these materials and compare them with the fluorescence spectra.

Measurement and Intracellular Localization of Tetracycline

An assay method for the tetracycline antibiotics has been developed, based on fluorimetric measurement of the substituted anhydrotetracyclines formed when these antibiotics are heated in strongly acid media. This method has greater sensitivity and freedom from interference than the available photometric methods of analysis and greater simplicity, rapidity, and reproducibility than the existing fluorimetric method. For oxytetracycline it is also more sensitive than that method.

Application of this method to the study of binding of tetracycline to several tissues has shown that the ratio of drug concentration in the tissue to that in the suspending medium is about 3 for Earle's strain L cells in tissue culture, of the order 10 for isolated mitochondria from rat brain and liver, and 150–350 for *E. coli* taken as a typical, tetracycline-sensitive, Gram-negative rod. When the bound material, which is mostly bound reversibly, is washed out it appears both chemically and biologically to consist of unchanged tetracycline even after several hours exposure to the binding tissue.

Experiments are partially completed to determine the effect of various media and drug concentrations on binding by bacteria and to explain the fluorescent microscopic observation that in living tissue culture cells intracellular bacteria are left unstained by the antibiotic at a concentration at

which extracellular bacteria and intracecellular mitochondria are strongly stained. The importance of the latter point to the question of carrier states following clinical use of tetracycline is apparent.

Theoretical Analysis of Biological Transport Problems

In the general analysis of input-output systems, it has been shown that the experimental assumptions that are customarily made in carrying out any tracer experiment can be directly interpreted in terms of the axioms which characterize a linear vector space and a linear operator or mapping from one linear space to another. Hence, tracer input and output can be represented as vectors in a normed linear space and the relation of output to input is a continuous linear mapping of input space into output space. An immediate consequence is that if input space and output space are the same (i.e. the set of all continuous function of time) the set of all continuous linear transformations is a normed operator algebra, when addition and multiplication are appropriately defined. Much of the analysis of particular problems can be carried out within this algebraic framework.

Particular problems studied have been the time dependent counterflow problem and diffusion in non-homogeneous systems. An interesting result obtained in the counterflow problem is that for high membrane permeability the equation for the system reduces to

$$\frac{V^2}{h}\frac{\delta^2 C}{\delta X^2} = 2\frac{\delta C}{\delta C}$$

where V is the velocity of flow and h the permeability. This is the diffusion equation. This equation as one very interesting immediate result, namely that the diffusion coefficient depends on v2. This demonstrates that in a region supplied with blood by a counterflow system, small changes in velocity can make very large changes in washout time.

LABORATORY OF CARDIOVASCULAR PHYSIOLOGY

Studies on the Atrium

The outcome of some of the studies done in this Laboratory in the past several years has been such as to indicate a substantial contribution of the atria to the regulation of the circulation. Two studies were completed this year which shed further light on this general subject.

Atrial Fibrillation

The question quite naturally arose as to what the effect of atrial fibrillation, per se, might be. A review of the literature did not uncover any satisfactory data. The lack of a satisfactory analysis of this question was attributable to the fact that when atrial fibrillation was induced ventricular irregularities also occurred and to the fact that reflex compensatory mechanisms, such as baroceptor activity, tended to obscure the results. Accordingly, experiments were designed to circumvent these difficulties. Autonomic activity was abolished by vagotomy and ganglionic blocking agents. The ventricular irregularities were obviated by preliminary section of the bundle of His and then sequentially pacing the atrium and ventricle so that the normal temporal relationship between these atrial and ventricular excitation was re-established. In such a preparation when the atria were fibrillated, the hemodynamic consequences of the fibrillation alone could be assessed since neither ventricular irregularities nor reflex buffering occurred. Fibrillation was electrically induced and verified with bipolar electrograms.

Several phenomena of interest were noted. When fibrillation was induced the atrial contribution to ventricular filling was absent, mean left atrial pressure rose, left ventricular end-diastolic pressure fell as did aortic pressure and cardiac output. Forward flow fell by between 10 and 20%. Analysis of pulse contours revealed the stigmata of mitral regurgitation to be present during atrial fibrillation, a finding not present in the control period. The presence of regurgitation during fibrillation was confirmed by the intraventricular injection of ascorbic acid with a recording platinum electrode in the atrium.

These experiments may help in the evaluation of clinical atrial fibrillation with special regard to the question of why certain people with this phenomenon do well and others are obviously hurt by it. It seems clear that in the absence of other disease, atrial fibrillation simply removes one of the contributory elements of a properly regulated circulation which is not, of itself, essential to an adequate (albeit a suboptimal) performance.

However, in the presence of other disease, atrial fibrillation can, by imposing an additional impediment, produce a substantial increase in the symptomatology of circulatory failure. This comes into clearer focus if one examines the data from two other points of view. First, mean atrial pressure always had to be appreciably higher to produce a given hemodynamic stimulus to the forward propulsion of blood, i.e., left ventricular end-diastolic pressure. Further, the larger the ventricle and the stroke volume the greater was this difference. This does not occur with a normal sinus rhythm. Secondly, the pulse contours indicate that the same generality holds with regard to the volume of mitral regurgitation during atrial fibrillation, i.e., the larger the ventricle and the stroke volume, the larger was the mitral regurgitant contour in the atrial pressure tracing.

The Timing of Atrial Systole

Further attempts were made to define the role of atrial function by use of the preparation described above. In this preparation the absence of atrial systole could be studied by turning off the atrial pace and observing the hemodynamic consequences of atrial asystole while pacing of the ventricle was continued at its previous rate. Further, the effect of changing the time of atrial systole could also be systematically studied. When atrial systole occurred in the range of 60 to 120 msec before ventricular systole (AS–VS interval of 60 to 120 msec), the atrial contribution to ventricular systole was effective and a mitral regurgitant wave was not seen in the atrium. When the AS–VS interval was longer than 120 msec, the mitral valve was apparently reopened by continued inflow and a regurgitant atrial pressure pulse contour was observed with the subsequent ventricular systole. When the AS–VS interval was shorter than 60 msec a prominent pressure wave was observed in the atrium with ventricular systole but it is not possible to be certain whether this is the effect of continuing atrial systole alone or this effect plus mitral regurgitation.

As with the studies on atrial fibrillation, the dispacement of atrial systole resulted in a higher mean left atrial pressure relative to any given left ventricular end-diastolic pressure. Both this gradient and the magnitude of the regurgitant pulse contour were greater when the heart and stroke volume were larger. With a properly placed atrial systole, ascorbic acid injected in the left ventricle did not appear in the left atrium. With an improperly placed atrial systole, the platinum electrode revealed clear evidence of mitral regurgitation.

Mechanism of Closure of the Mitral Valve

The two series of experiments described above strengthen the position arising out of previous work in this Laboratory indicating that the mitral valve can be preclosed (closed prior to the onset of ventricular systole) solely as a consequence of the activity of the atrium. This will certainly necessitate a reevaluation of certain phonocardiographic criteria which purport to use heart sounds as an indicator of the time of valve closure and also introduces the necessity for a reevaluation of the first heart sound.

As far as the significance of these findings is concerned, they help to explain certain clinical observations such as the appearance of an early systolic murmur with atrial fibrillation when this occurs. Further, it now appears that an augmentation of atrial activity, such as occurs with sympathetic stimulation or during exercise, provides a mechanism as a result of which the total left ventricular stroke volume is propelled in a forward direction rather than allowing some of it to be devoted to mitral valve closure at the onset of ventricular systole.

Studies on Homeometric Autoregulation

The experiments which have been conducted in the last year relating to the phenomenon of homeometric autoregulation will be considered under two headings. One group of experiments had as its objective a further dynamic characterization of the phenomenon. A second group of experiments was aimed at a further examination of the possibility that the compensatory response to an increase in activity is in some way related to the efflux of potassium that generally occurs when this phenomenon is observed.

Characterization of the Dynamics of Homeometric Autoregulation

The adaptive response of the heart to changes in rate was observed in experiments that were conducted in the areflexic dog, right heart bypass preparation. Heart rate was increased in a stepwise manner with mean aoritic pressure held con-

stant. At each step, cardiac input and thus output were increased so as to hold stroke volume and stroke work constant over the entire range of heart rates studied. Quite remarkably, it was observed that left ventricular end-diastolic pressure remained essentially unchanged, i.e., the left ventricle (as a result of increasing stroke power) performed the same stroke work from the same end-diastolic pressure regardless of the rate and of the fact that a substantially shorter period of time was available to accomplish that work. Taken in its broadest meaning these data appear to indicate that when heart rate is increased at each rate the timing of the various phases of systole, i.e., shortening of duration, are such as to compensate precisely for the altered time available for each phase and thus produce the same stroke work from a given end-diastolic pressure. The existence of this rate-induced homeometric autoregulation makes unnecessary an invasion of the heterometric (Starling) type of autoregulation and the latter is thereby preserved for increases of stroke volume which cannot be accomplished in any other way (without the operation of external influences such as sympathetic stimulation).

Similar experiments were conducted to determine the effect of increasing aortic pressure on the phases of ventricular systole. It was found that when aortic pressure was increased, as with an increasing heart rate, the heart adapted itself in such a manner that even though isovolumic systole was prolonged, the duration of ejection shortened by an equal amount so that total systole remained unchanged. As a result of this type of homeometric autoregulation the heart pumping against a higher resistance performed much more stroke work, again without a change in left ventricular end-diastolic pressure, and thus held in reserve the heterometric (Starling) type of autoregulation for increases in stroke volume.

It thus became possible to make two generalities about the performance characteristics of the heart. The first is that the heart is the kind of a pump which will, within limits, eject whatever is fed into it; it accomplishes this by some presently unknown physicochemical phenomenon associated with an increase in fiber length (heterometric autoregulation). The second is that, whatever stroke volume is being ejected by it, the heart can continue to eject this volume against a wide range of resistances and over a wide range of heart rates without invading more than briefly the fiber length mechanism (homeometric autoregulation). An attempt is being made, as described below, to characterize aspects of the chemical changes associated with the latter type of phenomenon.

Potassium Efflux During Homeometric Autoregulation in the Metabolically Supported Isolated Heart Preparation

Potassium values in coronary arterial and venous blood were determined during pressure induced homeometric autoregulation and in some instances a net loss of K^+ was observed which exceeded that observed after a large dose of acetyl strophanthidin. These K^+ losses were up to 2.5% of the total calculated intracellular K^+. The amount of the K^+ loss appeared to be related to the increase in O_2 consumption caused by the increased aortic pressure. Since left coronary blood flow was held relatively constant in such experiments, the A–V O_2 widened and coronary venous blood content was lower. We therefore examined the possibility that a lower myocardial pO_2 might be the stimulus which promulgated the net K^+ efflux. This does not prove to be the case since, when we restricted coronary blood flow to a point where the A–V O_2 was even wider and the coronary venous O_2 content lower than during pressure-induced homeometricity, a net efflux of K^+ was not observed. The possibility was also examined that a particular type of effort the heart makes might be the stimulus for the net K^+ efflux independently of changes in O_2 consumption. Accordingly, experiments were done in which, simultaneously, aortic pressure was lowered slightly and cardiac output increased greatly (work increasing four to five-fold) in such a way that O_2 consumption remained unchanged. A net loss of of K^+ was not observed. It appears from the data obtained thus far that while a lowering of myocardial pO_2 is not the immediate cause of the loss of K^+, something associated with the increase in the O_2 consumption when this occurs as the result of increased activity, does stimulate the loss of this electrolyte.

The hypothesis has been suggested that an increase in O_2 consumption results in an increase in CO_2 production and that the consequent increase in intracellular pCO_2 or hydrogen ion concentration in some way influenced the membrane of the cell so as to result in a K^+ loss.

Studies on Myocardial Metabolism

Three types of study were done in this general area. The metabolically supported isolated heart preparation was used in each. The first was an examination of the influence of coronary blood flow on myocardial O_2 consumption. The second was an examination of the effect of acetyl strophanthidin on myocardial O_2 consumption; observations were also made of the effect of this agent on the heart during myocardial hypoxia. The third not sufficiently advanced to merit more than mention here, was an examination of the influence of restricting coronary blood flow on lactate and pyruvate metabolism in the heart.

The Influence of Changing Coronary Blood Flow on Myocardial O_2 Consumption

Studies made elsewhere suggested the possibility that the amount of O_2 consumed by the heart is in some way influenced by the amount supplied to it. The studies alluded to were of a type that did not make it possible to ascertain whether the activity of the heart remained constant when the coronary flow was varied. Since we knew from previous experience that a variation in certain aspects of the heart's activity could, of itself, cause a change in O_2 consumption, it seemed to us worthwhile to examine this relationship while the heart's activity could be examined and held constant. Such an examination was possible in the isolated supported heart preparation.

It is a reasonable assumption that if coronary flow is stopped, O_2 consumption will cease soon thereafter. It is also reasonable to assume that above a certain level, even though one perfuses more blood through the myocardium, it will not use any more O_2. The matter of interest is, of course, what happens in the area in between. This has been determined. Varying coronary blood flow from about 100 to 600 ml per minute does not influence myocardial O_2 consumption in this preparation. In the range just below 100 ml per minute a slight decrease in O_2 consumption (about 15%) can occur but this is always accompanied by evidence of deterioration of the heart's performance, i.e., rising left-ventricular end-diastolic pressure while heart rate, aortic pressure and stroke volume are held constant. If one attempts to reduce coronary flow any further a runaway rise of end-diastolic pressure occurs, mitral regurgitation appears and ventricular fibrillation will occur unless coronary blood flow is promptly augmented.

In addition to the above, one interesting and unanticipated finding which appeared in each experiment was that, as coronary flow was gradually diminished, left ventricular end-diastolic pressure rose prior to any decrease in O_2 consumption. It, therefore, appears that the performance characteristics of the heart are influenced by the rate of supply (or washout) of some other substance before the influence of myocardial hypoxia is felt.

The Effect of Digitalis on Myocardial O_2 Consumption

Varying results have been reported concerning the influence of digitalis on myocardial O_2 consumption and efficiency. It has been reported that when digitalis is given to normal man, myocardial efficiency falls. When given to the patient in congestive failure, myocardial efficiency rises. Such data have been used to support the position that digitalis acts differently in the normal heart than in the failing heart. We believe this to be an incorrect assumption and that closer examination of the meaningful relationships would reveal that the observed difference of the effect on efficiency is not based on any basic difference of the mode of action of the drug under the two sets of circumstances. When digitalis was given to normal man, cardiac output fell, calculated work fell and efficiency therefore fell. In the patient with congestive failure, cardiac output rose, calculated work rose and efficiency therefore rose. What has been missing is an adequate appreciation of the absence of any meaningful relationship between work and O_2 consumption. The meaningful experiment is in our view, one in which the effect of the drug on O_2 consumption is examined under circumstances wherein the activity and the type of activity of the heart is held constant. This has been determined. With the activity held constant, doses of acetyl strophanthidin which produced a marked increase in contractility had no discernible effect on myocardial O_2 consumption.

Remarkably, no data seem to be available on the question of whether digitalis increases contractility in the severly hypoxic heart. Accordingly isolated supported hearts were rendered hypoxic by restrictions of coronary blood flow severe

enough to produce marked elevations of left ventricular end-diastolic pressure. The administration of acetyl strophanthidin under these circumstances was followed by marked improvement in contractility without any change in myocardial O_2 consumption.

Studies on the Synchronicity of Ventricular Contraction

Previous experiments from this Laboratory demonstrated that changes in the pathway of activation and the consequent change in the synchronicity of ventricular contraction could modify the external work produced from any given end-diastolic pressure. This was shown by contrasting the ventricular systole observed during atrial pacing with that obtained during ventricular pacing. There is also a profound augmentation of ventricular systole during cardiac sympathetic nerve stimulation. The juxtaposition of these two findings gave rise to a potentially important question, namely, does cardiac sympathetic nerve stimulation achieve a portion of the augmentation effect by altering the pattern of myocardial activation in addition to the effect of the norepinephrine which is known to be liberated under these circumstances?

Accordingly, studies were initiated to determine if, as a result of cardiac sympathetic nerve stimulation, the pathway or time required for ventricular activation is altered in such a way as to have meaningful mechanical sequellae. Experiments were designed to measure conduction time through the A–V node, conduction velocity in the right bundle branch, surface activation of the left ventricle and also to estimate total ventricular activation time. For these purposes bipolar electrodes were implanted on the atrium, bundle of His, right purkinje papillary muscle junction and on the surface of the ventricle at multiple points. Conduction time between atrium and His bundle (an index of A–V nodal delay) is shortened by 50–60% as a result of sympathetic nerve stimulation. Conduction time between His bundle and right purkinje spike is either unchanged or shortened by less than 3% as a result of sympathetic stimulation. The pattern of surface activation of the left ventricle is not modified by sympathetic stimulation. The interval between activation of the right anterior papillary muscle and the base of the interventricular septum (an estimate of total ventricular activation time) is reduced by 5–10% as a result of sympathetic stimulation. These data are taken to indicate that sympathetic nerve stimulation reduces the time for ventricular activation 5–10% by a mechanism apparently not related to its effects on the major distributions of the specialized conduction system and therefore by exclusion either in peripheral purkinje tissue, purkinje muscle junction or muscle.

Studies on Reflexes Arising From the Heart

In this general area attention was focused on the question of why, when a patient sustains a coronary infarction and hypotension results, there is not a greater and more consistent increase of peripheral vascular resistance. Experiments were conducted in which a hind limb was perfused through its isolated vessels at constant flow while changes in pressure were measured as an indicator of changes in resistance. With occlusion of the left anterior descending coronary artery or by interruption of blood flow to the left main coronary artery, neuronally mediated vasodilation was frequently seen even though carotid sinus pressure fell. This response was abolished by the interruption of impulses in the vagi (either section or cooling) but not by atropine, thus establishing that the vagi carry the afferent limb of this reflex. Confirmation of the fact that the efferent limb of the reflex consists of a dimunition of sympathetic activity was obtained by the recording of electroneurograms of the inferior cardiac nerve of the cat before and during occlusion of a coronary artery. The impulse traffic was observed to diminish in spite of a lower arterial (and baroceptor) pressure.

This work received recognition in the form of the Young Investigator's Award of the American College of Cardiology.

Studies on Renal Function

Extrinsic (Autonomic) Factors

As described in detail in earlier reports, the studies in this laboratory concerning various factors which modify renal function were initiated when it was observed that stimulation of the stellate ganglion of the dog produced a striking diuresis. Since the results of these studies indicated

that the diuresis was reflex in nature and possibly the result of stimulation of arterial baroreceptors by an increase in arterial pulse pressure, further studies were undertaken to determine the effects of changing arterial pulse pressure by carotid artery occlusion. In the early carotid occlusion studies renal blood flow (PAH), glomerular filtration rate (inulin), and electrolyte excretion did not show consistent changes during carotid occlusion. A perfused kidney preparation was therefore developed in which renal blood flow could be controlled and monitored.

The specific details of the stellate ganglion stimulation experiments have been discussed in earlier reports. It appears that infusion diuresis may be mediated at least in part by the same mechanism as stellate stimulation diuresis, i.e., by withdrawal of renal vasoconstrictor tone.

With the perfused kidney preparation it was possible to analyze those factors which contribute to the net response of the kidney to carotid occlusion. Using this preparation it has been established that carotid occlusion is always associated with an increase in renal vascular resistance, a change which can be substantially diminished by the intrarenal injection of an adrenergic blocking agent. This increase in resistance is observed whether or not renal blood flow is controlled by a pump. At the same time, it is well known that urine flow increases when renal arterial pressure is increased, independently of a change in renal blood flow or a change in renal vasoconstrictor nerve activity. Consequently, the rise in arterial pressure associated with carotid occlusion can contribute directly to the response of the kidney during occlusion. Therefore, the variability of the changes in water and electrolyte excretion by the kidney during carotid occlusion may represent a varying contribution of direct and reflex mechanisms to the total response. Thus, during carotid occlusion it is possible that total renal blood flow can decrease and urine flow and free water clearance increase with no change in the level of circulating antidiuretic hormone simply as a result of an increase in medullary blood flow subsequent to the increase in renal arterial perfusion pressure.

The results of the stellate stimulation and carotid occlusion experiments, when put in proper prospective indicate that reflectes emanating from arterial baroreceptors represent a potentially important mechanism whereby body salt and water homeostasis can be achieved.

Intrinsic Factors (Autoregulation)

In the course of these studies, the pressure-flow characteristics of the preparation could be ascertained. At the same time, it was of interest to determine those factors which may or may not contribute to the pressure flow relationships. As many have found previously, renal resistance was observed to increase as renal perfusion pressure was increased, i.e., the preparation autoregulates. Of interest, however, was the finding that little change in needle pressure (tissue pressure) is observed during autoregulation; sudden maintained increases (70–90 mmHg) in ureteral and needle pressure produced a transient decline in renal blood flow and then a subsequent rise to near the control level indicating that the renal circulation autoregulates in spite of, rather than because of, an increase in tissue pressure. In contrast to findings of previous workers it was found that the decapsulated kidney also *does* autoregulate. These experiments therefore show, we believe, that a change in tissue pressure or the presence of the renal capsule is not essential for the phenomenon of autoregulation.

Renal Blood Flow Distribution

It is generally accepted that renal medullary blood flow is only a few percent of total renal blood flow and that the medullary circulation does not autoregulate, the latter observation presumably accounting for pressure diuresis. At the same time it has been shown that the innervation of the renal medulla is scarce compared to the renal cortex. Experiments were initiated to determine if significant changes in renal blood flow distribution take place during such situations as changing renal nerve discharge, increasing renal perfusion pressure, etc. The transit time of cold saline is presently being used to estimate blood flow distribution changes. Although the experiments are in a very early stage, the results to date indicate that such transit times are much longer in the deep portions of the kidney than in the superficial portions. It is hoped that this approach combined with the perfused kidney preparation will provide a means whereby greater insight can be achieved with respect to those factors modifying both total renal blood flow and renal blood flow distribution.

Kallidin and Renal Function

Over the past several years, studies from this laboratory have contributed significantly to the purification and identification of the hypotensive proteinases, called Kallikrein and the polypeptides kallidin. Partially as a result of these studies synthetic polypeptides are now available. Current evidence suggests that these polypeptides may be concerned with local blood flow regulation and/or distribution. Since the kidney excretes large amounts of both the enzymes, the polypeptide studies have been undertaken to determine their effect upon renal hemodynamics as well as upon renal water and electrolyte excretion. When infused directly into the renal artery of the dog, kallidin produces an increase in renal blood flow and a striking diuresis and natriuresis, the latter characterized by both the increase in urine flow and an increase in urinary sodium concentration. Only a modest kaluresis occurs since urinary potassium concentration decreases. These experiments suggest that kallidin increases renal blood flow, that it may have a direct effect on electrolyte excretion and thus represent another endogenous mechanism which contributes to salt and water homeostasis.

Studies on Cathechol Amines

Norepinephrine Refractoriness

The role of catechol amines in circulatory regulation has been of interest to this laboratory for a period of years. The potent influences exerted on the myocardium have been discussed in detail in previous reports. Although there is little doubt that catechol amines exert a beneficial effect upon the myocardium it has also been established that when an animal is maintained on a constant infusion of norepinephrine, refractoriness develops, the mechanism of this is not yet known. It has been established that tissues can store large amounts of catechol amines and it has been suggested that the responsiveness to injected catechol amines may be diminished as a result of high tissue catechol amine content. Experiments were undertaken to determine whether agents which decrease tissue catechol amine concentration can reverse the refractoriness observed on constant norepinephrine infusions. The results to date indicate that ephedrine sulphate, a drug believed to release catechol amines from tissue can reverse norepinephrine refractoriness. This reversal can take place at a time when there is no significant change in arterial pH or arterial plasma catechol amine levels.

Mechanism of Action of Ephedrine Sulfate

During the above studies it was of interest that although ephedrine could increase arterial blood pressure substantially (100 mm Hg), there might be little or no change in arterial catechol amine levels. On the other hand, tyramine produces approximately the same increase in pressure but a significant increase in arterial catechol amine levels. More detailed studies were therefore undertaken to determine the mechanism of action of ephedrine. The results of this study indicate that the extent to which ephedrine releases catechol amines is small, even in circumstances in which the catechol amine storage has been increased by catechol amine infusions. Thus it appears that although part of the effect of ephedrine is to decrease tissue catechol amines, this effect alone can not explain the potent pressor responses observed.

LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM

The Laboratory of Kidney and Electrolyte Metabolism is pursuing a wide variety of activities. These may be subdivided into four major areas which will be discussed separately.

I. Renal Physiology

a. Micropuncture Studies in the Dog

In the last report, the development of a micropuncture technique for the study of renal tubular function in the intact dog was described. In the past year considerable progress has been made. It has now been established with certainty that fluid throughout the accessible portion of the proximal nephron (the first 60%) is isotonic to plasma. U/P osmolality ratios approximating 1 have uniformly been observed under circumstances in which the final urine is either hypotonic or hypertonic. These findings are consistent with results in other species and have been interpreted as indicating that the luminal membrane in the proximal nephron is freely permeable to water. Net reabsorption of fluid, as evidenced by a progressive in-

crease in the concentration of inulin in the luminal fluid, is considered to be effected by active sodium reabsorption and osmotic flow of water along the gradient established by the prior solute removal. It had been assumed that this process is uninfluenced by antidiuretic hormone and that approximately the same fraction of glomerular filtrate is reabsorbed proximally both in water diuresis and in antidiuresis. However, the results of the most recent studies in the dog are inconsistent with this thesis. The U/P inulin ratio has been noted to be greater in antidiuresis than in water diuresis, indicative of enhanced reabsorption of proximal fluid in antidiuresis when ADH is maximal. Since, as indicated above, the U/P osmolality is the same in both situations, it is probable that the increase in the U/P inulin in antidiuresis is provided by an acceleration in the transport of sodium and chloride out of the nephron into the surrounding cortical tissue. Vasopressin is known to stimulate sodium transport in other epithelial tissues (frog and toad skin and bladder). Consequently, it has been concluded tentatively that an analogous effect occurs in the proximal nephron and accounts for the increased reabsorption noted in antidiuresis in the dog. This is an important observation which requires further study.

In association with the above studies bicarbonate reabsorption has also been examined. In the dog the concentration of bicarbonate in the most distal portion of the accessible part of the proximal segment is slightly less than that of plasma. Apparently, reabsorption of bicarbonate exceeds water reabsorption slightly if at all. This contrasts with observations in the rat in which the concentration of bicarbonate falls progressively throughout the nephron. These studies are also being pursued as is an examination of potassium reabsorption under a variety of experimental conditions in the intact dog.

b. The Influence of Nonelectrolytes on Urine Concentration

Urea is known to play a unique role in the mechanism of urine concentration. Its administration results in the attainment of a higher urine osmolality than with other solutes. This enhancement of urinary concentrating ability is thought to depend upon the permeability of the luminal membrane of the collecting duct to urea and its accumulation in medullary tissue. In order to obtain further support for this thesis, the effect of other relatively diffusible nonelectrolytes on the concentrating mechanism was examined in the dog and correlated with the degree of accumulation of the nonelectrolyte in the medullary region of the kidney. Earlier work had established that certain urea analogues do, in fact, increase the ability of the rat to elaborate a hypertonic urine, though to a lesser degree than does urea. Similar studies in the dog have now been concluded, and it has been established that not only urea, but also methylurea, acetamide and 1,2-propanediol produce enhancement of concentrating ability in this species. As with urea, all of the latter compounds diffuse across the luminal epithelium and accumulate in highest concentration in the medullopapillary region of the kidney. Propanediol was the least effective compound studied with respect to enhancement, and in accordance with theory, accumulates in the medulla to the smallest extent. On the other hand, its clearance relative to inulin is the lowest of the compounds studied, indicating that it must be reabsorbed to a considerable degree high in the nephron, presumably in the proximal segment.

c. Measurement of Medullary Blood Flow

For some time efforts have been directed at developing a method for the measurement of medullary flow. The purpose of these studies was to examine the effect of changes in medullary blood flow on urine concentration in an effort to study the influence of the countercurrent system on the process. A number of methods have now been tested and discarded. Unfortunately, the characteristics of the countercurrent system of itself preclude use of the presently available methods, all of which are based on the Fick principle which requires constancy of arterial concentration of the test subject. However, in the course of these studies, a precise method for the measurement of regional tissue flow has been developed. The method utilizes the Fick principle and requires administration of hydrogen gas and estimation of the curve of desaturation of tissue hydrogen gas with time. From the slope of this curve blood flow may be calculated. Hydrogen gas is measured by use of a platinum electrode. Its concentration in tissue is equal to that in the vein, thereby eliminating the necessity of simultaneous venous determinations. Furthermore, the

arterial concentration approaches zero within seconds after cessation of either intra-aortic administration of hydrogen or administration by respiration. Consequently, simultaneous arterial concentrations are also unnecessary. The simplicity of the method due to elimination of the necessity of simultaneous arteriovenous differences is remarkable. Thus far, the method has been tested successfully in cardiac and skeletal muscle, brain, renal cortex, etc. The results to date are in excellent agreement with those obtained by other conventional methods.

II. Electrolyte and Water Transport Across Biological and Artificial Membranes

a. Electrolyte Fluxes in Separated Renal Tubules of the Rabbit

Fluxes of sodium and potassium have been estimated in the past in thin slices of renal cortical tissue. Data from such studies have been difficult to interpret, since the anatomic arrangement of the cells in tissue slices complicates precise analysis. The fluxes obtained in this manner are merely gross estimates of the exchange of isotope into and out of the whole slice and provide no direct information concerning the actual fluxes across the individual cell membranes. Although this difficulty has been recognized by most workers in the field, it had not been generally appreciated that the fluxes across whole slices may vary not only in a disproportionate manner from those across the cell membranes, but may also vary in direction during experimental manipulation. Last year a method was designed which eliminated some of these difficulties, and permitted relatively direct estimates of fluxes of electrolytes across individual cell membranes. The cortical tissue of rabbits was treated with collagenase, the resulting separated segments of proximal tubules were suspended in appropriate media, and flux measurements performed using sodium^{24} and potassium^{42}. Using this technique, a number of interesting and important observations have been made within the past year.

In the first place, the cell suspension as noted earlier, was shown to be viable as indicated by adequacy of respiration, maintenance of steady state concentrations of sodium, potassium, and tissue water, and the maintenance of the ability to transport and accumulate PAH actively. It has also been shown that potassium within the tubule cells may be divided kinetically into a minimum of two distinct compartments. Addition of the cardiotonic steriod strophanthidin, a known inhibitor of active transport in other tissues as well as in the kidney, reduces the potassium content of the cell suspension. These results are similar to those observed in slices. The reduction in potassium content has been shown to be due, in part at least, to a specific decrease in the influx of potassium into the cell. In contrast to the results of slice studies, in which the efflux rate constant was either unaffected or actually increased following addition of strophanthidin, it has been demonstrated unequivocally that the efflux rate constant actually falls following addition of the drug. This difference in the results of the studies using tissue slices and cell suspensions emphasizes both the difficulties involved in the interpretation of flux studies in whole slices and the unique potentiality of the tubule suspension system for the analysis of fluxes across individual cell membranes. Most recently, it has also been established that at 0° C., a temperature at which it is generally assumed active transport ceases, all of the tissue potassium within the cell suspension remains exchangable. This, too, is at variance with earlier studies in slices. Of greater importance and also in contrast to the results of slice studies, it has been possible to demonstrate the persistence of active transport at this temperature.

Studies Concerning the Exchange of Na^{24}. Earlier slice studies had not revealed the presence of a tissue sodium compartment responsive to known inhibitors of cation transport. This observation, together with other negative findings, had led to the conclusion that sodium and potassium transport may not be linked in renal tubular cells. Using the present technique, it has been possible to analyze the tissue sodium compartments and exchange rates with greater precision. At least two compartments are present in the tubule cells, one extremely rapid, equilibrium being virtually complete within 60 seconds, and the other a slow and more readily discernible compartment. The fast compartment, which exchanges at a rate of approximately five times per minute, is responsive to strophanthidin and other inhibitors such as anoxia. The former compound increases the sodium content of the tissue by reducing the rate constant

for sodium efflux. In view of this demonstration, it is probable that this rapid compartment, not clearly discernible in kinetic studies in slices, is of physiological significance. However, a 1:1 link between sodium and potassium transport, which had been assumed, was not demonstrable. Instead, sodium efflux appeared to be five to seven times greater than simultaneously estimated potassium influx. Despite this lack of stoichiometry, sodium efflux was shown to be dependent upon the presence of potassium in the bathing solution. Efflux was significantly reduced when the medium potassium was decreased from 5 to .3 mM/L.

Para-aminohippuric Acid Studies. PAH transport has also been examined in the tubule cell suspension system. These studies are incomplete but, as with the others reported above, the results differ significantly from those obtained in slices. Thus, for example, diodrast, a competitive inhibitor of PAH accumulation, had been assumed to lower the concentration of PAH in renal tissue by increasing the "run-off" or efflux of PAH from the cells. In view of the difficulties in interpretation of efflux measurements in slices with respect to K alluded to above, it was not surprising to note in the present studies that diodrast clearly reduces the active uptake of PAH and does not influence the efflux rate constant.

b. Studies in Red Cell Ghosts

The red cell ghost system has been described in detail in earlier reports. Its advantage as a model for the study of electrolyte transport has been discussed. The most recent experiments were designed to determine whether the red cell membrane, prepared by fragmenting hemolyzed red cells, selectively binds electrolytes and nonelectrolytes. The fragmented membrane was dialyzed against various solutions at 37°. Radioactive tracers of sodium, potassium, chloride or glucose were added to the outside bathing medium and binding estimated from the ratio of the counts within the dialysis bag to those outside. Electrolyte "binding" was studied in rat red cell membranes, glucose "binding" in human red cell membranes. Although in the last report it had been suggested that the rat red cell may selectively "bind" potassium on the outer surface, no selective binding was observed in the present studies. Instead a Donnan distribution of sodium and potassium was noted. Of interest, however, was the fact that the Donnan ratios were reduced by the addition of the divalent cations, magnesium or calcium. The magnesium and calcium effect is inhibited by the addition of EDTA, a potent chelating agent, to the medium, as well as by the addition of ATP. The effect of the ATP is presumably due to its ability to act as a chelating agent and is not specifically related to the provision of high energy phosphate, as has been suggested by others.

The experiments involving glucose binding in human red cells are in a preliminary form at the present time. Since the isotope method selected for the demonstration of binding assumes no metabolic transformation of glucose, final interpretation of the observations will require additional study. Recognizing this importnat qualification, a number of observations of interest have been made. Glucose (or at least label) is bound or accumulates within the dialysis bag in association with the fragmented membrane. The "binding" requires the presence of ATP and magnesium. Unlabeled glucose or sorbose, but not fructose, arabinose, 2-deoxyglucose or mannose prevent or decrease the apparent glucose "binding." It is of interest of that C^{14} sorbose also "binds" in the presence of ATP and magnesium. No interpretation of these studies is as yet available, since it depends, as indicated above, on the final determination of the metabolic state of the administered C^{14} glucose.

Measurement of Cytoplasmic and Nuclear Membrane Properties of Amphiuma Erythrocytes. The purpose of these studies was to measure the potential difference across both the cytoplasmic and nuclear membranes of Amphiuma red cells. Amphiuma red cells were selected because of their large size and consequent suitability for micropuncture analysis. The cells contain approximately 70% water, 11.0 mM of sodium, and 87 mM of potassium per liter. As a preliminary to the electrical measurements, fluxes of potassium and sodium have been estimated. Sodium efflux is inhibited by cardiac glycosides, by dinitrophenol and by removal of potassium from the medium. The inside-outside concentration ratio for chloride has been established, and it has been assumed that the potential across the cytoplasmic membrane would be a reflection of the chloride diffusion potential. However, to date it has not been possible to obtain data in support of this assumption. Whether the potentials measured are ac-

curate reflections of the true potential or represent methodological difficulties has not yet been established.

III. Mechanism of Action of Vasopressin and Other Hormones

a. Vasopressin

Antidiuretic hormone increases the permeability of a number of biological membranes to water. Its role in the elaboration of a hypertonic urine of low volume is well known, and depends upon hormone-induced enlargement of aqueous channels or pores in the tubular epithelium, which permits osmotic flow of water out of the distal nephron into the surrounding interstitial tissue. On the basis of studies presented last year, it has been proposed that the hormone exerts its effect in responsive tissues (toad blader, kidney) by stimulating the production and/or accumulation of cyclic AMP in the tissue. The nucleotide is thought to alter membrane permeability either directly, or more likely, by initiating a series of unknown reactions in the tissue. Much of the work in the past year has been directed at developing additional evidence in favor of the thesis.

In collaboration with a group at Western Reserve University, the activity of cyclic AMP in toad bladder tissue has been measured with and without hormone. Thus far it has been shown that the nucleotide is present in the epithelial tissue of the toad bladder. Although antiduretic hormone increased the concentration of cyclic AMP in some studies, it has not yet been possible to demonstrate a reproducible rise in the concentration of the nucleotide in the toad bladder tissue following incubation with hormone. On the other hand, theophylline, which prevents the degradation of cyclic AMP to its inactive form, 5′ AMP, uniformly increases the concentration of the cyclic form in the tissue. The methylxanthines had been shown to exert in toad bladder effects on water and sodium transport which are indistinguishable from those produced by antidiuretic hormone.

In association with the above studies the metabolic effects of vasopressin and of the proposed intermediate in its action, cyclic AMP, are being examined in both toad bladder and renal tissue. Others have shown that the hormone increases oxygen consumption and glycogenolysis in toad bladder when incubated in sodium-containing medium. These results have been confirmed. In addition, cyclic AMP increases oxygen consumption and glycogenolysis, whereas the degradation product, 5′AMP, has no effect or depresses glycogenolysis. Similar studies are now being carried out using dog renal cortical tissue.

Phosphofructokinase activity has also been assayed in the toad bladder. Cyclic AMP is known to stimulate the activity of this enzyme in tissues in which it is the rate-limiting enzyme in glycogenolysis. The enzyme apparently is not rate-limiting in the toad bladder, and although some stimulation is produced by both cyclic AMP and its degradation product, 5′AMP, vasopressin is without effect.

The most interesting evidence concerning the relationship between cyclic AMP and antidiuretic hormone concerns their respective effects on phosphorylase activity in toad bladder and renal tissue. In virtually all tissues examined, cyclic AMP increases the activity of glycogen phosphorylase. It has now been shown that a similar effect occurs in toad bladder, and of greater significance, it has been possible to demonstrate a uniform increase in glycogen phosphorylase activity in toad bladder following incubation with vasopressin and other neurohypophyseal hormones. Epinephrine and ACTH, which increase phosphorylase activity in muscle and adrenal respectively, are without effect in toad bladder. Further evidence concerning the relationship between the hormone and its supposed intermediate has been developed by showing that acidification of the bathing medium, a procedure which inhibits the permeability effect of ADH also prevents the hormone-induced increase in phosphorylase activity. Most recently it has been possible to show that phosphorylase activity in both rabbit renal medullary tissue and dog cortical and medullary tissue are significantly increased by ADH. This represents the first demonstration of a specific metabolic effect of the hormone in mammalian renal tissue. Although these results lend support to the view concerning the intermediacy of cyclic AMP in ADH action, they do not afford any insight into the precise mechanism of action of the hormone on membrane permeability.

Other investigators have suggested that the action of vasopressin is dependent upon prior linkage of the octapeptide via its disulfide bridge to sulfhydryl groups on the membrane. Support for

this hypothesis derives from studies in which certain reducing agents (cysteine and thioglycollate) have been shown to prevent the permeability effect of the hormone. These results are difficult to interpret, however, since the octapeptide remains in the reduced form only in the presence of the reducing agent. Consequently, it is not possible to distinguish between an effect of the reducing agent on the tissue or on the octapeptide or on both. The interpretation is further complicated by recent results obtained in this laboratory. Both cysteine and thioglycollate interfere with the permeability effect of vasopressin and theophylline, but do not alter the response to cyclic AMP. Clearly the effect of theophylline cannot involve the postulated disulfide bond, nor can significant alterations of the sulfhydryl groups on the membrane account for the persistence of the cyclic AMP effect. It has been suggested tentatively that these reducing agents may actually interfere with the production of cyclic AMP in the tissues, Both antidiuretic hormone, which is thought to stimulate its production, and theophylline, which prevents degradation of the intermediate, would be expected to be unaffected in the presence of an agent which limits the enzymatic conversion of ATP to cyclic AMP, whereas the effect of exogenous cyclic AMP should be unaltered by the reducing agent.

b. Aldosterone and the Renin-Angiotensin System

An examination of the factors involved in the control of aldosterone secretion by the adrenal continues. Last year it was unequivocally demonstrated that the kidney secretes a substance, now known to be renin, which stimulates aldosterone release from the adrenal. During the past year further support for this observation has been obtained. Angiotensin-like activity in lymph from normal dogs has been assayed and compared with similar analyses of lymph from dogs with experimental hyperaldosteronism due to caval constriction. Angiotensin-II activity, a reflection of the amount of renin released from the kidney, was considerably greater in lymph from caval animals than from normals. A double assay system was used in these studies, the first, the pressor effect of the extract in appropriately treated rats, and the second, the steroidogenic response of the isolated adrenal of hypophysectomized-nephrectomized dogs. The results were similar using both methods of assay, in that lymph of the caval animals increased blood pressure in rats and increased aldosterone secretion of the isolated adrenals to a greater extent than did lymph from normal animals.

In association with members of the Cancer Institute, it has been proven that Sprague-Dawley rats bearing Walker's carcinosarcoma–256 secrete aldosterone at an excessively high rate. These rats were noted in the past to retain sodium, leading to the suspicion, now confirmed, that aldosterone secretion is elevated. On the other hand, corticosterone secretion was unaltered in these animals.

Alterations in plasma sodium and potassium concentrations are also known to affect aldosterone secretion in a number of species. Observations similar to those noted in other laboratories have been extended in an effort to characterize the mechanism more clearly. Thus, it has been observed that the intravenous injection of potassium chloride or of K_2SO_4 into hypophysectomized dogs results in a striking increase in aldosterone secretion. Corticosterone secretion also rose in these studies. In three of six animals, despite nephrectomy, hypersecretion of aldosterone continued during maintenance of hyperkalemia, indicating that in these animals neither the renin-angiotensin system nor the presence of an intact anterior pituitary was essential for aldosterone release. Furthermore, in agreement with other studies, the direct injection of potassium chloride or potassium sulfate into the isolated adrenal also increased aldosterone release, supporting the view that potassium exerts a direct effect on steroidogenesis within the adrenals. Preliminary studies have been interpreted as indicating that a reduction in the plasma sodium concentration effected by dilution may also stimulate release of aldosterone from the adrenal.

The effect of prolonged and continuous intravenous infusion of angiotensin-II in unrestrained normal dogs has been studied. The most important observation thus far noted is that rates of angiotensin-II infusion, insufficient to elevate blood pressure in the unrestrained animal, result in enhanced urinary aldosterone excretion. However, angiotensin also produced transient renal hemodynamic effects which apparently exerted a direct influence on electrolyte excretion. These studies are being continued.

IV. Cardioglobulin

As discussed in detail in previous reports, plasma is known to contain a protein system composed of at least three globulin fractions, which exerts an inotropic effect on the isolated frog heart. This system is present in mammalian plasma and may play an important physiological role in the maintenance of normal cardiac contractility. In the past year the chemistry of this system has been intensively studied. It has been observed that one of the components of this system, cardioglobulin A, is rapidly inactivated by a variety of tissue homogenates as well as by plasma. The inactivation has the characteristics of an enzymatic process, and has been shown to involve the release of inorganic phosphate. Inactivation is prevented by the addition of certain high energy phosphate compounds, including ATP, ADP and creatinine phosphate. On the basis of these observations it has been tentatively concluded that cardioglobulin A contains a high energy phosphate which is removed by a plasma and/or a tissue enzyme, thereby accounting for its inactivation. Furthermore, on the basis of other information, it has been suggested that the high energy phosphate in cardioglobulin A may provide energy, not for the contractility effect directly, but for the transport of a cardioglobulin C calcium complex into heart muscle. Further support for the view that a high energy phosphate is associated with cardioglobulin A was the observation that the gradual and progressive decline of activity of the entire cardioglobulin system in the assay system in frog heart is associated with release of inorganic phosphate into the medium and a disappearance of cardioglobulin A activity.

In association with the above studies, an attempt at obtaining stable fractions of the three cardioglobulin components is under way. It has been observed that a cardioglobulin inhibitor is elaborated in plasma during the usual preparative separation of the three compounds. The formation of the inhibitor has unfortunately further complicated the fractionation system, and studies at the present time are directed at eliminating this particular problem. It is of interest that the formation of the so-called inhibitor in rat plasma is prevented by the addition of sodium ascorbate, but not by the addition of other reducing agents. Vitamin C, on the other hand, does not prevent the development of the so-called inhibitor during the fractionation process itself.

LABORATORY OF METABOLISM

Before presenting details it may be useful to call attention to some selected areas in which particularly significant progress has been made during the past year.

An important advance has been the definitive demonstration of a hormone-stimulated lipase in adipose tissue. The magnitude of the effects obtained adequately accounts for the observed increases in rate of release of fatty acids during hormonal stimulation. At the same time, using a newly developed balance method, it has been shown that fatty acid esterification is actually increased during hormonal stimulation of fatty acid release. Consequently, it can now be concluded that the primary mechanism for increasing rates of fat mobilization lies in a system capable of rapidly activating one of the lipases of adipose tissue (sec. 2).

On the basis of previous studies we have suggested that basal metabolic rate might be significantly influenced by rates of free fatty acid (FFA) mobilization and we put forth the tentative hypothesis that the hypermetabolism of the hyperthyroid state might be secondary to an abnormally high rate of fatty acid mobilization. Important new evidence has now been obtained from clinical studies that supports this hypothesis. It has been shown that the administration of hexamethonium, which inhibits FFA release from adipose tissue, has a small but definite depressing effect on the metabolic rate of normal subjects that have been made hyperthyroid by treatment with triiodothyronine. It has also been shown that intravenously administered norepinephrine raises the metabolic rate of normal subjects while simultaneously increasing the turnover of serum FFA. Pretreatment with an adrenergic blocking agent abolished both the stimulation of FFA turnover and the hypermetabolic effect of the norepinephrine (Section #4).

Further studies on cholesterol biosynthesis have shown that reduction of the side-chain double bond probably occurs at several points. Dihydrolanosterol and desmosterol appear to be relegated to relatively minor roles in the normal pathway. From studies with triparanol and other similar inhibitors and from studies of the proper-

ties of "desmosterol reductase" it has been concluded that a single enzyme in liver particles is actually responsible for reduction of the side-chain independent of the structure of the sterol nucleus. In other words, we now believe that liver contains a "side-chain reductase" with only limited specificity. Triparanol inhibits this reductase and thus blocks reduction of the side-chain at all stages, leading to the previously reported accumulation of desmosterol (sec. 1–B).

1. Pathway of Cholesterol Biosynthesis and Inhibitors of Cholesterol Biosynthesis

(*A*) *Normal Pathway of Cholesterol Biosynthesis*

Development of a powerful technique for fractionation of sterols by thin layer chromatography has made possible a series of studies of sterol metabolism in the liver and in the skin. This thin layer technique permits rapid and quantitative resolution of a number of sterol intermediates that previously could be isolated only by laborious and time-consuming column techniques or by gas-liquid chromatographic analysis of only very small samples.

Studies of the time course of incorporation of radioactive mevalonic acid into the liver sterols of intact rats have yielded valuable evidence regarding the normal pathway of cholesterol metabolism. One school of investigators has maintained that the side-chain double bond of lanosterol is retained until the very last step in cholesterol biosynthesis. Others, on the basis of isotopic data, have shown that sterols with saturated side-chains can be converted to cholesterol and suggested that reduction of the side-chain of lanosterol might occur as the very first step after cyclization of squalene. The present studies show that very little radioactivity is observed in dihydrolanosterol and likewise very little is observed in desmosterol during the first 30 minutes after injection of labeled mevalonate. Since major amounts of radioactivity were found in Δ^7 $(+\Delta^8)$-cholestenol, it is concluded that side-chain reduction occurs prior to the shift of the nuclear double bond to the Δ^5 position. A fraction of C_{28} sterols was isolated and found by chromatographic analysis to contain a mixture of molecules with saturated and unsaturated side-chains. Since this fraction contained considerable radioactivity, it is probable that side-chain reduction occurs to some extent at the C_{28} stage. In summary, the evidence obtained supports the suggestion previously made that there is not a single unique pathway of cholesterol biosynthesis in normal rat liver. Instead it appears that reduction of the side-chain double bond can and does occur at several different stages during modification of the sterol nucleus. The present findings indicate that reduction at the very first step (lanosterol to dihydrolanosterol) and at the very last step (desmosterol to cholesterol) are relatively minor pathways.

(*B*) *Desmosterol Reductase*

Studies completed this year support the conclusion, made previously, that the enzyme responsible for reduction of the side-chain double bond in desmosterol also catalyzes the reduction of the side-chain double bond in lanosterol, $\Delta^{7,24}$-cholestadienol and probably in other intermediates. In other words, it appears that the enzyme, while apparently rather specific for the sterol side-chain is relatively nonspecific as regards the nuclear configuration of the sterol.

The evidence leading to this conclusion is briefly as follows: reduction of lanosterol to dihydrolanosterol in a cell free system was demonstrated for the first time. It was shown that "lanosterol reductase" activity was located in the particulate fraction of liver, both in microsomes and in mitochondria. The enzyme is TPNH specific and it is inhibited by reagents that interact with sulfhydryl groups. In all of these respects "lanosterol reductase" activity is essentially identical to "desmosterol reductase" activity. Furthermore, triparanol and two other inhibitors of desmosterol reduction were shown to inhibit lanosterol reduction when added to homogenates.

Evidence was obtained from *in vivo* studies that triparanol inhibits reduction of $\Delta^{7,24}$-cholestadienol and zymosterol. When labeled mevalonate was administered to triparanol-treated rats significant amounts of radioactivity were found in these sterols with unsaturated side-chains but in normal rats the radioactivity was mostly associated with the corresponding saturated side-chain sterols.

We are then led to conclude that what we have previously called "desmosterol reductase" should now be regarded as a generalized "side-chain reductase." Triparanol and several other inhibitors block the activity of this reductase and lead to the accumulation of desmosterol. As was pointed out

in earlier publications, the accumulation of desmosterol under these circumstances does not prove that it is necessarily a major intermediate in the untreated animal. The work described above does in fact appear to relegate desmosterol to a minor role while at the same time confirming that it is *one* of the precursors of cholesterol.

(C) Desmosterol as a Precursor of Adrenal Steroids and Bile Acids

As reported last year, clinical studies provided evidence that desmosterol could be directly converted by man both to bile acids and to adrenal steroids. Further evidence for this direct conversion has now been obtained in mice and rats by *in vitro* techniques. It has been shown that homogenates of adrenal gland can convert desmosterol to corticosterone (compound B) and that this conversion does not go through cholesterol. Using liver homogenates it has been shown that the side-chain of desmosterol can be readily oxidized. The rates of metabolism of cholesterol and of desmosterol were compared in both the adrenal homogenates and in preparations of liver mitochondria. The two sterols were metabolized at comparable rates. Whether or not desmosterol is an intermediate in the conversion of cholesterol to steroids and bile acids is not known but these findings are compatible with such a possibility.

(D) Sterol Metabolism in Skin and in the Optic Lens

A comprehensive survey of skin sterols in normal animals and in triparanol treated animals was carried out. It appears that the mechanism of action of triparanol in the skin is similar to that in the liver, that is, it exerts a general inhibition of side-chain reduction. Simultaneous studies of pathological changes in the skin were carried out in collaboration with Dr. Wertlake in the Pathological Anatomy Department. Whether the degeneration and atrophy observed is attributable to the desmosterol accumulation demonstrated cannot be stated but further studies along these lines may be fruitful.

It was shown that desmosterol accumulates in the optic lens of triparanol-treated rats. The structural changes in the lens have been studied by optical and electron microscopy by Dr. von Sallmann in the Ophthalmology Branch of NINDB.

(E) Atherogenicity of Desmosterol

It was shown for the first time that desmosterol is deposited in the atherosclerotic lesions and deposited to about the same extent as cholesterol. Because of the limited availability of desmosterol only one experiment was possible but the results of this critical study were clear cut. A rabbit was put on anatherogenic diet containing cholesterol and also desmosterol. After about four months on the diet the animal was found to have gross atheroscelrotic lesions. At the time of sacrifice desmosterol accounted for about one-third of the circulating sterols. Again, one-third of the sterols in the atherosclerotic lesions was found to be desmosterol and two-thirds cholesterol. Thus it has been shown that if desmosterol is present during the genesis of an atherosclerotic lesion it will be deposited at no greater and no less a rate than cholesterol. Postmortem studies reported from other laboratories have been interpreted to show that desmosterol might be less atherogenic than cholesterol because only a small amount of desmosterol was found in lesions of patients that had been treated with triparanol for a few months before death. Because the lesions in these cases were undoubtedly well advanced at the time triparanol treatment was instituted it would be anticipated that desmosterol would constitute only a small fraction of the total sterol in the lesions, inasmuch as the increment in sterols laid down during the relatively short period of triparanol treatment must have been quite small. The present animal study must give a more relevant result since desmosterol was present in the serum throughout the period during which the lesions developed.

2. The Metabolism of Adipose Tissue as Influenced by Hormones

The mobilization of fatty acids from adipose tissue is a key process in the control of energy metabolism in the fasting state and in response to exercise. An understanding of the hormonal factors controlling this process will undoubtedly be as important as the understanding of factors controlling glucose mobilization and utilization.

An important advance has been made in this area during the past year. It has been shown that under the proper conditions a lipase system in adipose tissue can be dramatically activated when

the tissue is exposed even for only a few minutes to epinephrine, norepinephrine, glucagon or ACTH. Previous reports have appeared from two laboratories showing that 25 to 50% increases in lipase activity could be effected with these hormones but the rate of fat breakdown in the intact tissue increases by several fold. The quantitative disparity left doubt as to the significance of this hormone-stimulated lipase activation. We have now been able to show increases in lipase activity of a magnitude adequate to explain the increased rates of fat mobilization.

A finding of great value in the further development of this problem is that the major fraction of lipase activity in adipose tissue homogenates is intimately associated with fat and can be separated from the bulk of the cell material by floating it to the top of a centrifuge tube. This should make the further purification and characterization of the enzyme a much easier problem.

A method has been developed for simultaneously determining rates of lipolysis and rates of esterification of fatty acids in intact adipose tissue. This method appears to be free of the ambiguities associated with isotopic techniques for measurement of the rate of these processes. By applying this method to tissue stimulated by the lipolytic hormones it has been shown that parallel with the increase in lipolytic activity there is a marked increase also in the rate of fatty acid esterification. The latter may be an indirect effect secondary to the increase in intracellular fatty acid concentration but this is not yet firmly established.

It has been demonstrated for the first time that adipose tissue contains, in addition to the hormone-sensitive lipase discussed above, lipase with very high activity in the splitting of monogycerides. The latter enzyme is *not* affected by the hormones that increase fat release from adipose tissue.

Prostaglandin is an acidic lipid first identified in extracts of seminal fluid and prostate gland which has potent vasodepressor activity. Last year it was obtained in crystalline form and its structure determined by Dr. Sune Bergstrom and coworkers at the Karolinska Institute in Stockholm (2-(6-carboxyhexyl)-3- (3-hydroxyocten-1-yl)-4-hydroxycyclopentanone). Several prostaglandin derivatives were obtained from Dr. Bergstrom and examined for possible effects on adipose tissue metabolism. It was shown that prostaglandin E has a slight inhibitory effect on lipolysis in adipose tissue. More striking was the ability of prostaglandin to counteract the stimulatory effect of epinephrine and other lipolytic hormones on fat breakdown in the epidymal fat pad. It was further shown that in intact dogs prostaglandin counteracts the vasopressor effects of epinephrine and norepinephrine. These findings suggest the possibility that the biochemical basis for the vasodepressor effects of prostaglandin and its ability to partially block the action of lipolytic hormones may be related. Further studies are in progress along these lines. The physiological role of prostaglandin remains to be established but the fact that it has now been shown to occur in other tissues as well as in the accessory sexual glands makes it possible to visualize a hormonal function for this material.

3. Studies on the Factors Controlling Mobilization and Utilization of Free Fatty Acids (FFA) *in Vitro*

Previous work from this laboratory showed that the rate of utilization of labeled fatty acids by muscle and by liver *in vitro* increases with the concentration of fatty acids in the medium. These studies have now been extended by using a perfused liver system. It was shown that the uptake of FFA was greatly increased when the concentration of FFA in the perfusing fluid was high. An important additional observation was that the oxygen consumption by the perfused liver increased when it was perfused with high concentrations of FFA. All of these studies suggest that utilization of this substrate is a function of the concentration at which it is offered to the peripheral tissues. On the other hand, the *in vitro* studies, while they show an increased utilization of labeled fatty acids, fail to show any effect of FFA concentration of Q_{o2}. Attempts are being made to determine whether serum factors or hormonal factors are necessary for optimal FFA utilization.

4. Clinical Studies of the Relation Between FFA Utilization and Metabolic Rate

A series of clinical studies has now been completed with respect to the importance of FFA concentration as a factor determining basal metabolic

rate. These studies were done in collaboration with Dr. Elsworth Buskirk and Dr. Ronald Thompson, making use of the metabolic chamber. It has been shown that intravenously administered norepinephrine consistently produces an increase in oxygen consumption—10 to 20% above control values. This increase in oxygen consumption is associated with the expected rise in serum FFA concentrations and an increase in serum FFA turnover, as determined from measurements of palmitate-1-C^{14} turnover. When an adrenergic blocking agent (pronethalol) was given just prior to the intravenous administration of norepinephrine, the rise in serum FFA levels was prevented and there was no increase in oxygen consumption. In fact, under the influence of the blocker, norepinephrine actually caused a slight but definite *decrease* in oxygen consumption.

We have previously postulated that the hypermetabolism seen in hyperthyroid patients might be secondary to a high rate of FFA mobilization. It has been previously shown that the adipose tissue of hyperthyroid animals releases fatty acids at a higher than normal rate and that adipose tissue from hyperthyroid animals shows a much greater response to lipolytic hormones. The question posed was whether interference with FFA mobilization in the hyperthyroid patient would influence his metabolic rate. Normal controls were made hyperthyroid by treatment with triiodothyronine (T_3). In the metabolic chamber the oxygen consumption was measured before and during the intravenous administration of hexamethonium. Although the effects of hexamethonium were small, they were remarkably consistent, causing in every case a decrease in oxygen consumption of approximately 10%.

The results of these clinical studies coupled with the results of *in vitro* studies of FFA utilization are compatible with the hypothesis that mobilization of FFA may be one of the factors in determing basal metabolic rate. The general problem of how thyroid hormone increases body metabolism is now being reevaluated by this laboratory in the light of these findings.

5. Differences in the Metabolic Fate of Fatty Acids of Different Structure

It is now well established that dietary fatty acids of different structure influence serum cholesterol very differently. The biochemical basis for these effects of dietary fat has not yet been elucidated. Differences in the uptake of different labeled fatty acids are being explored.

(A) Chylomicrons containing a predominantly saturated pattern of triglycerides are removed from the circulation more rapidly than chylomicrons containing predominantly unsaturated triglycerides. This differential uptake was demonstrated in perfused livers as well as in the intact animal. On the other hand, there was no such differential uptake in perfused adipose tissue.

Livers were perfused with high concentrations of linoleate or palmitate or both. These perfusions caused a net increase in the triglyceride and phospholipid content of the liver. The net changes were independent of which fatty acid was used but the distribution of fatty acids in the liver lipids depended upon the nature of the fatty acid used. Thus, the triglyceride fraction in the liver showed a doubling of the percentage content of palmitate when palmitate alone was perfused through the liver; when linoleate was used there was some increase in the linoleate content of the triglyceride fraction but to a smaller extent. Evidently the liver is able to accommodate increases in the amount of palmitate delivered to it more readily. This may be related to the specificity of the enzymes involved in triglyceride biosynthesis. In most animal species both the α and the α' positions tend to contain predominantly unsaturated fatty acids.

C^{14}-labeled fatty acids were used to compare the fates of linoleate and palmitate. Linoleate was preferentially incorporated into the phospholipid fraction, while palmitic acid was preferentially incorporated into triglycerides and cholesterol esters.

(B) The unsaturated fatty acids synthesized by normal animals are of the *cis* and *trans* isomers of oleic acid and of linoleic acid. It was found that absorption from the intestinal tract in the form of chylomicron triglycerides occurred at the same rate for each member of the isomeric pairs. Similarly, the rate of disappearance of the isomers from the circulation after injection of the free fatty acid occurred at essentially identical rates. Liver lipids were analyzed and again no difference was found for the rates of incorporation into cholesterol esters, triglycerides, diglycerides, monoglycerides or phospholipids. In summary, then, oleic acid and its *trans* isomer (elaidic acid) and

linoleic acid and its *trans*, *trans* isomer appear to be indistinguishable, at least in terms of the rates at which they are metabolized *in vivo*.

6. Clinical Studies of Serum Lipoproteins and Their Metabolism

(A) The discovery of a syndrome characterized by almost complete absence of high density lipoprotein (HDL) from the serum affords an opportunity to explore the function of this lipoprotein. The first patients were found living in an inbred population on the island of Tangier and the syndrome has been named Tangier disease. Epidemiologic studies suggest that a single pair of allelic genes may exert major control over plasma HDL concentration. Patients with the rare homozygous defect have little or no plasma HDL, low serum cholesterol levels and infiltration of the RE system with cholesterol esters. The question of whether HDL plays a role in hypertriglyceridemia is being explored.

(B) A standard procedure has been developed for determination of lipoprotein lipase levels in human serum. This procedure gives reproducible results and eliminates errors due to effects of endogenous substrate and nonlinearity that have been encountered with other methods. Applying this procedure to heparin-stimulated lipoprotein lipase release, it has been shown that most hyperlipemic subjects have a normal response. The 10% with abnormal responses were patients having the "fat-induced" form of hyperlipemia.

Serum glyceride levels determined by a direct method have been analyzed in over 300 fasting subjects to obtain satisfactory reference levels. The upper level of normal is established at 180 mg/100 ml.

A pedigree has been identified with hyperglyceridemia that falls neither in the fat-induced nor carbohydrate-induced category. The patients have normal cholesterol levels and normal lipoprotein lipase activity.

(C) There is good evidence that the triglycerides of lipoproteins or chylomicrons taken up by the liver are hydrolyzed and the free fatty acids subsequently incorporated into liver esters. Studies of lipase activity in the liver have been initiated. It has been shown that at least two different lipases are present. The lipases split triglyceride emulsions in the absence of any serum activator. Partial purification of a lipase from the soluble fraction has been accomplished.

7. The Metabolism of Cholesterol Esters

Studies done in collaboration with Dr. Arthur Karmen of the Laboratory of Technical Development were undertaken to establish whether or not the formation of cholesterol esters during absorption from the intestine showed selectivity. Mixtures of labeled fatty acids were fed and the lipids recovered from chyle were extensively fractionated and studied. It was shown that the different fatty acids were incorporated in a nonselective way into triglycerides. On the other hand, oleic acid was used to a greater extent in the formation of cholesterol esters than was palmitic acid, linoleic acid or stearic acid. Lecithin synthesized during absorption preferentially incorporated stearic acid, whereas oleic acid and palmitic acid were incorporated to a much smaller extent.

The cholesterol esterase activity of rat liver has been studied with regard to cellular distribution and fatty acid specificity. Most of the enzyme was found in the soluble fraction with 11 to 30% in the microsomes. The hydrolysis of various cholesterol esters was studied and it was found that cholesteryl oleate and cholesteryl linoleate were hydrolyzed most rapidly, cholesteryl acetate occupied an intermediate position, while cholesteryl palmitate and cholesteryl stearate were least well hydrolyzed. The enzyme in the microsomal fraction appears to be different from that in the soluble fraction in certain properties, although the specificity of the two seems to be similar.

The cholesterol ester synthesizing system of liver was studied. It was shown that both ATP and Coenzyme A are essential and that the synthesizing system is entirely particulate, being found both in the microsomal and mitochondrial fractions of liver.

8. Studies on Experimental Nephrosis

It is well known that the injection of anti-kidney serum into experimental animals can produce a syndrome similar to clinical nephrosis. The nature of this process has been studied using antibody proteins that have been degraded by proteolytic enzymes by the methods described by Porter. The fragments obtained retain their ability to combine with kidney antigens but they do not in-

duce proteinuria. It has been reported that the combination of different univalent fragments with antigen does not result in fixation of complement. The findings suggest that complement fixation is somehow essential for the processes that lead to damage of the glomerulus and to the full blown nephrotic syndrome. If this explanation is correct, it should be possible to protect against kidney damage due to anti-kidney serum by lowering the levels of complement in the recipient animal. This possibility is being further explored. These studies are being carried out in collaboration with Dr. Parker A. Small of NIMH.

9. Studies of Protein Structure

Work on the structure of fibrinogen and of actomyosin has continued. Insight into the structure of fibrinogen has been obtained by coupling the molecule with a fluorescent dye: 1-dimethyl amino naphthalene-5-sulfonyl chloride. Studies of the degree of polarization of the fluorescent light from the complex in media of different viscosities leads to the conclusion that fibrinogen must contain subunits even smaller than those liberated by trypsin digestion. Essentially what is found is that there must be a great deal of flexibility in the molecule, more than would be expected on the basis of previously proposed subunit structures. The variation in fluorescent intensity with pH suggests tyrosine residues in the protein are involved in the complex formation.

A new form of actomyosin has been isolated from rabbit muscle. Hitherto actomyosin has been formed only with the filamentous (F-form) of actin and this preparation is a very highly viscous one. The new form of actomyosin now isolated appears to contain the globular or monomeric form of actin (G-form). Preliminary studies suggest that in the intact muscle it may be the G-form of actomyosin that is normally present.

10. Phospholipid Biosynthesis in Red Blood Cells

Red blood cell membrances (ghosts) have been shown to incorporate labeled fatty acids into phospholipids. Most of the incorporation occurs in lecithin and essentially all of that in the β position of the molecule. Evidently there is little or no *de novo* synthesis of lecithin but rather the labeled fatty acid is activated to the Co A derivative and incorporated into lysolecithin. The source of the acceptor lysolecithin is not clear since phospholipase activity has not yet been demonstrated in the ghosts. Similar results were obtained using intact red blood cells.

The above studies were carried out with red cell membrances obtained from two kinds of sheep. One variety has red cells characterized by a very high intracellular concentration of potassium while the other has red cells with a low intracellular concentration of potassium. Since the incorporation of labeled fatty acid into lecithin was comparable in the two kinds of red cells, it appears that the phospholipid turnover studied is not intimately related to the mechanisms of potassium transport in these cells.

11. Formation of Chylomicrons From Endogenous Sources

Dietary fat is absorbed primarily in the form of chylomicrons delivered to the blood stream by way of the thoracic duct. However, it is known that very low density lipoproteins can appear in the circulation during fasting. Studies were undertaken to characterize the lipoproteins of the chyle in animals on fat free diets.

Rats on a fat free diet continued to produce chylomicron-like lipoproteins demonstrate in thoracic duct chyle. The lipid in the chyle is predominantly triglyceride and most of it is associated with particles of density less than 1.006. The chyle obtained directly from intestinal lymphatics was similar to that obtained from the thoracic duct. It thus appears that the lipoproteins arise in the intestine. This lipoprotein continues to be produced even when only saline is given. It is suggested that endogenous substrates are utilized by the intestinal mucosa in order to form these "fasting" chylomicrons.

12. Studies on Structure and Mechanism of Action of Parathyroid Hormone

Dr. Gerald Auerbach of NIAMD has isolated and partially purified parathyroid hormone from phenol extracts of bovine parathyroid glands. Further purification has been achieved using Sephadex columns. Physical studies and end-group analysis show that the material, with a

potency of 3,000 U.S.P. Units per mg, is now pure. This material is being used for studies of the mechanism of action of the hormone.

Section on Chemistry

The work of the past year may be summarized in four areas. These are (1) the development of gas phase chromatographic techniques for the qualitative and quantitative analysis of biologically important substances, (2) investigation of the isolation, structure, properties and biogenesis of plant alkaloids, (3) studies of the components of the kallikrein-kallidinogen-kallidin system, and of the chemistry of human polypeptide vasodilators, and (4) consultative and informal collaboration with various intramural research groups of the National Institutes of Health seeking the specific knowledge and equipment of the Laboratory for application to their particular problems.

(1) Gas Phase Chromatographic Methodology

A method has been developed for the analysis of 17-hydroxycorticoids in biological fluids. A system has also been developed for the analysis of nucleosides as their acetates. Hydrogen flame detectors have been evaluated for high molecular weight substances and found to be useful. The deleterious effect of copper and metallic columns and fittings has been confirmed. The preparative technique previously outlined has proved to be of great utility in several problems. A column packing has been found (neopentyl glycol succinate) which is highly efficient for the separation of cholesterol and desmosterol.

(2) Alkaloid Work

Work has continued on minor alkaloids of *Ormosia panamensis* using gas chromatographic techniques. Three new bases have been isolated, one being identical with a synthetic transformation product of another. Several degradations were carried out on an alkaloid from *Astrocasia phyllantoides* but its structure is still unknown. Structural investigations on the three *major* alkaloids from the *Ormosia* species have been intensified. The problem has been found to be peculiarly refractory. The alkaloids resist nearly all classical degradations and it is felt that a new chemical linkage must be involved.

The structures of Amaryllidaceae alkaloids, ambelline and amaryllisine have been elucidated, employing modern spectral methods. Biogenetic experiments on the Amaryllidaceae alkaloids have uncovered a branching point in the biosynthesis between phenylalanine and tyrosine. A cell-free enzyme system, capable of methylating norbelladine to a known biogenetic intermediate has been discovered and partially purified. The cofactor is S-adenosylmethionine.

Spectral studies have been completed on hydrogen bonding phenomena in several types of compounds yielding detailed knowledge of the rotational configuration of hydroxyl groups in these natural materials.

The structure of the alkaloid, tecomanine, has been elucidated. It is the third known example of a monoterpenoid cyclopentanoid alkaloid and is chemically related to the ant oil, iridiomyrmecin.

(3) The Kallikrein-Kallidinogen-Kallidin System

The human vasodilating substance kallikrein owes its action to the formation of the physiologically active polypeptide kallidin. The latter compound is formed when kallikrein acts on kallidinogen, a component of human plasma. Work directed to the isolation of kallikrein, kallidin and kallidinogen has been carried on as a joint study with the Laboratory of Cardiovascular Physiology.

Samples of human urinary, pancreatic and hog pancreatic kallikreins described in the last report have been purified (~90%) and characterized.

Human plasma kallidinogen has been isolated in 28% yield and a 34-fold purification achieved.

A new technique for the preparation and activation of hydroxylapatite has been developed. With this adsorbent, kallidinogen has been separated into six components; the biological activity coinciding with one of them.

(4) Miscellaneous Informal Collaborative Research

In addition to conducting the research cited above, a significant amount of time and effort has been spent by several members of the laboratory to help other scientists of the National Institutes of Health with specific problems. Our help has been sought primarily in three areas: (1) Large scale processing of microorganisms, plant materials, biological fluids, glands, and culture media. There has been an increased utilization of the 100-

gallon fermenter for the growing of nonpathogenic microorganisms. As a result of the reorganization of the various laboratories, operation of the large scale laboratory as a joint NHI–NIAMD project has been discontinued although most of the facilities will still be available to the NHI investigators.

LABORATORY OF CLINICAL BIOCHEMISTRY

Amine Biogenesis and Metabolism

Work in this area has continued along several lines. Inhibitors of the enzyme, dopamine β-oxidase, have been investigated *in vitro* and *in vivo*. All benzyloxyamines tested produced inhibition by a mechanism which indicates competition with substrate. However, when inhibitor is preincubated with enzyme before adding substrate, the inhibition becomes noncompetitive. Three procedures for demonstrating the *in vivo* effects of these inhibitors have been utilized: (a) ability to block the conversion of dopamine to norepinephrine in the guinea pig heart depleted of norepinephrine with aramine; (b) ability to block restoration of norepinephrine levels in guinea pig heart following administration of a short-acting depleting agent; (c) ability to inhibit the guinea pig adrenal enzyme. Benzyloxyamine has been shown by all three criteria to be active *in vivo*. However, the *in vivo* inhibition with single doses as large as 200 mg/Kg is no more than 80% and does not last for more than a few hours. The *in vivo* effects of other inhibitors are more variable. All compounds of this class produce hemolysis, methemoglobin, enlarged spleen, and convulsions. More compounds are being investigated and further attempts are being made to evaluate *in vivo* effects.

Certain findings suggest the existence of self-regulatory mechanisms for norepinephrine synthesis. For example, conversion of administered dopamine to noradrenaline is negligible in normal animal tissues *in vivo*. Following depletion of norepinephrine stores with aramine or α-metatyrosine conversion is rapid and extensive. Isotopic experiments have been used to corroborate this. Even without isotopes rapid repletion of depleted stores can be observed readily. The possibility that norepinephrine biosynthesis may be regulated by norepinephrine itself (as a feedback mechanism) or by the action of some other humoral agent released by the norepinephrine is being investigated.

It has been shown that labeled tyramine and norsynephrine (octopamine) can be converted to urinary epinephrine and normethanephrine. The significance of this alternative route remains to be determined. However, some of the pharmacological properties of tyramine (at high doses) may be due to conversion to norepinephrine.

It has been possible to label the norepinephrine in heart, spleen, brain, as well as adrenal gland, by administering 100 microcuries of tyrosine-U–C^{14} to individual guinea pigs. This has made it possible to evaluate the turnover of the hormone in the peripheral tissues. Initial studies have proven beyond question that the labeled material in the tissues is indeed norepinephrine. Preliminary findings corroborate a half-life of several days for adrenal norepinephrine. By contrast, half-lives in other tissues are of the order of several hours. Additional animals are being carried through to obtain more precise data. The isolated perfused heart has been found to contain all the catalysts necessary to form noradrenaline from the dietary amino acid tyrosine. The radioactive hormone and its precursor, dopamine, have been identified by many procedures. Under the conditions employed as much as 0.05 μg of noradrenaline was formed per gram of heart per hour. The perfused heart will be useful in future studies of catecholamine biochemistry.

α-Methylamino acids, such as α-methyl-dopa and α-methyl 5-hydroxytryptophan, are excellent inhibitors of the decarboxylation of the naturally occurring aromatic amino acids. Some controversy has existed concerning the mechanism of this inhibition. It is now apparent that the kinetics of this inhibition vary with the experimental conditions employed. If the enzyme is preincubated with inhibitor before the addition of substrate the characteristics of the inhibition are those of a noncompetitive inhibitor, while if no preincubation is employed competitive inhibition kinetics are obtained. Pyridoxal phosphate, the coenzyme for this reaction, also plays an important role. It protects the enzyme against the inactivation which occurs during preincubation, as well as against the effect of the α-methylamino acids.

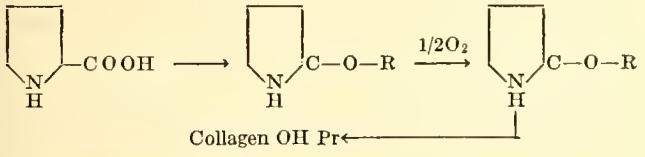
Once the enzyme has been inhibited, however, it has not been possible to reverse this inhibition by addition of coenzyme.

Studies with rat liver phenylalanine hydroxylase have shown that this enzyme can also convert tryptophan to 5-hydroxytriptophan (5HTP). The physiological significance of this reaction in the formation of serotonin has been under investigation. Inhibition of the enzyme *in vivo* causes little if any effect on serotonin levels in the tissues. This finding coupled with the distribution of the enzyme (found only in liver) makes it apparent that phenylalanine hydroxylase is not responsible for serotonin formation *in vivo*. Attempts to show the enzymatic conversion of tryptophan to 5HTP in tissues other than liver have not been successful.

The inhibition of monoamine oxidase (MAO) by iproniazid, labeled in the isopropyl moiety, has been investigated. It appears that isopropyl hydrazine is formed *nonenzymatically* by an oxidative cleavage of the iproniazid molecule. This volatile hydrazine appears to be an intermediate in the formation of the true inhibitor from iproniazid. Studies of the binding of inhibitor to enzyme have been performed, with emphases on the kinetics and type of inhibition. Attempts to solubilize or show a lipid requirement for monoamine oxidase have not succeeded.

Collagen and Hydroxyproline

Studies on the formation of collagen and hydroxyproline in cell-free systems have been continued. It has been possible to determine some requirements of the chick embryo ribosomal system and to show an RNA involvement. Attempts are now being made to determine the exact stage at which hydroxylation of proline occurs: whether it is at the stage of prolyladenylate, S–RNA proline, or a later intermediate. It has been found that the oxygen involved in the conversion of proline to hydroxyproline is derived from O_2^{18} and not H_2O^{18}. Thus the enzyme must be an oxygenase and the reaction for hydroxylation of proline would be written as:



R=adenylate, S—RNA, or other intermediate activated form

Proteins and Peptides

Studies have continued on the biosynthesis of the chromopeptide antibiotic actinomycin. Radioisotope experiments on the precursors of the actinomycin molecule have revealed that L-valine but not D-valine is used for synthesis of the D-valine in actinomycin. Evidence obtained with L-methionine-$C^{14}H_3$ and L-valine-1–C^{14} and glycine-1- and -2-C^{14} have indicated that *in vivo*, a direct methylation of L-valine and glycine is responsible for the synthesis of N-methyl-L-valine and sarcosine, respectively. A rapid method of assay for actinomycin, based on the selective solubility of the antibiotic in organic solvents has been developed. It was possible to demonstrate with the use of the assay procedure and radioisotopes that the incorporation of a precursor into actinomycin is generally a rapid, efficient and linear process. With inhibitors of protein synthesis such as chloramphenicol, puromycin or ethionine it was possible to show an inhibition of protein synthesis by *Streptomyces antibioticus* (as measured by incorporation of a C^{14} labeled amino acid) with a simultaneous stimulation (2- to 3-fold) of actinomycin synthesis. These results indicate that the mechanism of synthesis of the peptide antibiotic, actinomycin, differs from that postulated for protein synthesis.

Brain can actively synthesize γ-glutamylhistidine from histidine and a γ-glutamyl donor such as glutathione. The enzyme which catalyzes this reaction is γ-glutamyltranspeptidase. Up to this time the presence of this enzyme in brain and the ability of histidine to act as an acceptor of the γ-glutamyl group have not been ascertained. Unequivocal demonstration of the enzyme in brain in the present study was possible because of the development of a highly sensitive and specific fluorometric method of assay. That γ-glutamylhistidine is formed *in vivo* is suggested by its presence in human urine. It has not previously been demonstrated in animal tissues. Numerous γ-glutamyl derivatives have been isolated from plant tissues and certain microorganisms. Recently, additional γ-glutamyl and β-aspartyl peptides have been identified in human urine by another laboratory. Since these peptides were also found in the urine of fasting individuals their endogenous origin is strongly implicated but their immediate source is

still unknown. A possible source of γ-glutamyl and β-aspartyl peptides in urine is collagen. In keeping with this hypothesis was the finding, in humans, of a significant increase (at least 5-fold) in the urinary excretion of β-aspartyl histidine following an increase in dietary collagen and gelatin. This finding is of great interest and the presence in collagen of other β-aspartyl peptides such as β-aspartyl glycine and serine are currently under investigation. In line with this work, *in vitro* digestion of collagen with proteolytic enzymes is also under study. If such digests contain β-aspartyl and γ-glutamyl peptides (as others have suggested) then this will be strong evidence for the occurrence of these unusual linkages in this protein.

Studies have been carried out on the distribution of the dipeptides carnosine and homocarnosine. Only the brain was found to contain them, the white matter of human brain having from 2 to 4 times as much dipeptide as gray matter. This is just the reverse of the distribution of the precursor GABA, which is concentrated in the gray matter. Urine was found to contain homocarnosine with a daily excretion ranging from 2.0 to 9.8 μmoles per day. If it can be shown that the homocarnosine in urine does indeed come from the central nervous system and not from the diet, the level of this dipeptide in urine may serve as a useful index of brain metabolism. Concomitant with this work a systematic study was also made of the uptake of histidine, carnosine and homocarnosine by rat brain slices. Histidine and carnosine were accumulated by slices against a concentration gradient but homocarnosine was not. An examination of the slices after incubation revealed little or no metabolism of histidine or homocarnosine even after 3 hours of incubation. Carnosine, one the other hand, was hydrolyzed to histidine and β-alanine. The disappearance of carnosine could be accounted for entirely from the amount of β-alanine formed. It is interesting that sarcoma S–37 ascites cells could not take up carnosine or homocarnosine even though they showed a marked activity with respect to histidine uptake. The uptake of carnosine by brain slices was studied further with regard to energy requirement, efflux, and inhibition. The latter study revealed that the best inhibitors were other peptides, especially β-alanyl dipeptides. However, histidine and especially β-alanine were almost as effective inhibitors as were peptides.

The conversion of phenylalanine to tyrosine is being used in an attempt to gain information regarding the turnover of proteins in mammals and in bacteria. It was observed some time ago that an anomalous situation arose when one measured the specific activity of protein tyrosine after administering radioactive phenylalanine to a dog. The time course of decay of the tyrosine in plasma protein was different from that observed if tyrosine itself were administered. The origin of this effect is under study using the purified blood proteins of dogs and various combinations of tritium- and carbon-labeled phenylalanine and tyrosine. A similar study is being attempted in bacteria using a strain of Pseudomonas in which hydroxylation of phenylalanine to tyrosine is an inducible enzyme.

As part of a study on the over-all metabolism of proteins in the brain, a Ca^{++}-stimulated, soluble neutral protease has been extensively purified. A stable, active enzyme has been prepared using $(NH_4)_2SO_4$ fractionation, isoelectric precipitation, and DEAE-cellulose chromatography. Using casein as a substrate the characteristics of the enzyme have been determined. For maximal activity Ca^{++}, EDTA, sulfhydryl, and buffer, pH 7.4 are necessary in appropriate amounts. In search of the mechanism of action of the enzyme a series of model peptides was investigated with sensitive spectrophotometric methods. No action could be observed, indicating tentatively that the enzyme is unlike trypsin, chymotrypsin, carboxypeptidase A or B, papain or known cathepsins in its mode of action. Since the enzyme acts on oxidized ribonuclease, this well-characterized protein is being used in a study of the mode of action of the protease. End group analysis and finger printing techniques are being adapted for this purpose.

Amino Acid Uptake by Animal Tissues

A continuing study of the significance of aromatic amino acid uptake by brain has been pursued. Recently, earlier findings regarding the uptake of tyrosine by rat brain *in vivo* have been extended to phenylalanine and tryptophan. A comparison between brain and other organs has served to emphasize that brain is unique in this

regard. Competitive relationships previously demonstrated have been used to develop an hypothesis concerning the possible mechanism of mental retardation in conditions involving high blood levels of amino acids such as in phenylketonuria. Certain dfferences between amino acid uptake *in vivo* and *in vitro* led to suggestions about the anatomical location of portions of the uptake mechanism. Specifically, the marked stereospecificity found *in vivo* but not *in vitro* is considered to be a function of the "blood-brain barrier" or a related mechanism. To obtain verification of this hypothesis and to gain other information a study is in progress concerning the penetration of amino acids into the brain of newborn rats. This study is based on suggestions from other laboratories that newborn animals do not exhibit a barrier mechanism.

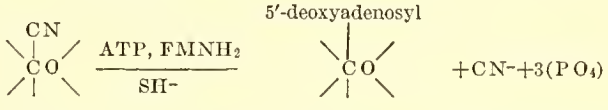
A study of the amino acid uptake of the Sarcoma 37 ascites cell has also been undertaken with the aim of developing a model system for the study of the effects of certain antitumor agents. In the initial phases of this program the transport of amino acids *in vitro* was investigated. With certain minor differences the transport of amino acids resembled the well-described case of the Ehrlich ascites cell. In addition, *in vitro* levels of alkylating agents which prevented the growth of these cells when the cells were reinoculated into mice had no effect on the transport system. An unusual effect of the antimetabolite p-fluorophenylalanine was noted in these studies and is being pursued at present. Simultaneous exposure of the cells to tyrosine and o-fluorophenylalanine led to significant, reproducible increases in tyrosine uptake of the order of 25 to 35%. Further, a much greater effect on the uptake of tryptophan, of the order of 200 to 300%, was noted. It has seemed appropriate to investigate this effect and to determine, if possible, its origin. Accordingly, labeled p-fluorophenylalanine is being employed to study the effect of amino acids on this compound and the conditions of the experiments are being altered to determine if this effect can be explained on the basis of counter flow or if some other explanation must be sought.

Biosynthesis of Phospholipids and Other Nitrogen Containing Lipids

Toward the end of 1961 studies on the biogenesis of phospholipids, which had been temporarily discontinued, were started again. During investigations on the incorporation of ethanolamine–C^{14} into phospholipid it was found that activity was greatest in microsomes fortified with fatty acids. However, the product formed on addition of palmitate and ethanolamine was found to be the corresponding amide, palmitoyl-ethanolamide and not cephalin. In fact, all the ethanolamine was incorporated into this amide. The reaction has been shown to be a net synthesis rather than some backward reaction of a hydrolytic mechanism. The fatty acid amides of ethanolamine have been reported to have anti-inflammatory and antiserotonin activities. The significance of these compounds and of the enzymes involved in their formation and metabolism are currently being investigated.

Studies Relating to Vitamin B_{12}

The conversion of vitamin B_{12} to B_{12} coenzyme is depicted in the following way:



In an attempt fully to understand this unique reaction, the enzyme was purified from extracts of *Clostridium tetanomorphum*. The reaction has been studied in a variety of ways, including (a) incorporation of the adenosyl moiety of ATP into coenzyme, (b) release of cyanide, (c) spectral changes, (d) PO_4 release from ATP, and (e) formation of enzymatically active coenzyme. It has not been possible to demonstrate a partial reaction, and the available data favor a concerted reaction, as presented in the above equation. The form in which the phosphates from ATP are released during coenzyme synthesis could not be studied until the high ATPase activity in the extracts was reduced. Once achieved it was possible to show that inorganic tripolyphosphate is released from ATP during the enzymatic reaction:

Adenine-ribose-P-P-P→5′deoxyadenosyl moiety
+tripolyphosphate

The role of vitamin B_{12} in one carbon transfer is also being investigated. The final reaction in methionine biosynthesis involves the transfer of the methyl group from CH_3 tetrahydrofolic acid (CH_3THF) to homocysteine. Adenosylmethio-

nine (AMe) and a B_{12} coenzyme analogue (CH_3B_{12}) have been implicated in this reaction. This conversion is being studied in both an animal system (pig liver) and in extracts of *E. coli*, using $C^{14}H_3THF$, methyl H_3-Ame, and $C^{14}H_3B^{12}$. The latter compound has been chemically synthesized. It is extremely light sensitive, similar to the B_{12} coenzyme, and during light inactivation the carbon to cobalt bond is broken via homolytic cleavage; CH_3O (35%) and CH_3OH (percentage not yet determined) being formed. The development of simple assays has facilitated studies on methionine biosynthesis. It has now been demonstrated that AMe is necessary for the reaction from CH_3THF in both systems studied, although the methyl group of AMe is not transferred to homocysteine. CH_3B_{12} is an absolute requirement in the bacterial system, not being replaced by vitamin B_{12} or B_{12} coenzyme. The CH_3 group of CH_3B_{12} is transferred during the course of the reaction, but AMe is not needed in this reaction. ATP and TPNH, which are needed along with AMe for the reaction from CH_3THF, are not necessary for the reaction from CH_3B_{12}. Although CH_3B_{12} does transfer its CH_3 group, the evidence indicates that free CH_3B_{12} is not an intermediate in the over-all reaction.

Development of Analytical Procedures

A procedure has been developed for the assay of N-dimethylaminoguanine in the presence of guanine. The former is a trace component in S-RNA. The method should make possible the evaluation of its significance.

A procedure for determination of phenethylamine in the urine of phenylketonurics has been developed.

Since the last report, attention has been given to those compounds which have been difficult to study by gas chromatography. Two approaches have been taken. The first has been a search for a suitable polar liquid phase and the second, the preparation of derivatives likely to chromatograph well. Efforts have been directed primarily to the 2,4-dinitrophenyl and phenylthiohydantoin derivatives of the hydroxy and basic amino acids. This has met with limited success but the work is continuing.

Section on Biochemical Genetics

RNA and the Genetic Code

This research encompasses the following aspects: (1) development of a cell-free assay for messenger RNA, (2) synthesis of a natural protein directed by purified preparations of messenger RNA, (3) investigating the characteristics of messenger RNA, and (4) the characteristics of the genetic code.

Cell-Free Assay for Messenger RNA. Protein synthesis was studied by following C^{14}-amino acid incorporation in cell-free *E. coli* extracts. Techniques were developed which stabilized such extracts so that they could be stored frozen for long periods of time without undue loss of activity. Small amounts of DNAase added to reaction mixtures were found to inhibit amino acid incorporation. Thus, the genetic material appeared to be directing protein synthesis in this completely cell-free system. Further experiments suggested that protein synthesis ceased because endogenous messenger RNA had been depleted. Purified preparations of messenger RNA then were added to reaction mixtures and were found to direct amino acid incorporation into protein with high efficiency. Such RNA fractions proved to be a new requirement for cell-free protein synthesis. This technique afforded a highly sensitive, cell-free assay for messenger RNA and provided the rationale for all subsequent work.

The Messenger Role of Viral RNA. A naturally occurring template RNA, tobacco mosaic virus RNA, was used to direct protein synthesis in this system. The protein synthesized under the direction of TMV–RNA was shown to have many characteristics of TMV-protein. Part of the protein formed could be purified with authentic TMV-protein by repeated isoelectric precipitations and DEAE column chromatography. TMV-protein is an excellent one to characterize for its entire amino acid sequence is known. Digestion with trypsin converts it into 12 peptides of established amino acid composition and chromatographic properties. Seventeen peptides were isolated after digesting with trypsin and each peptide was subjected to amino acid analysis and its radioactivity was counted. TMV–RNA was found to

direct C^{14}-amino acids into expected peptides. Thus, the cell-free synthesis of this protein and the messenger role of viral RNA was demonstrated.

Fate of Messenger RNA. Synthetic polynucleotides of known chemical constitution were assayed to see if they could direct the synthesis of simple proteins. Polyuridylic acid was found to stimulate greatly the incorporation of phenylalanine into protein. The product of the reaction was identified as polyphenylalanine and sRNA was shown to be an intermediate in its synthesis.

The effect of secondary structure upon the template activity of poly U was also investigated. Poly U in solution occurs as randomly coiled, single-strands. In the presence of polyadenylic acid, double- and triple-stranded polynucleotides are formed. Such double- and triple-stranded polynucleotides were unable to direct the synthesis of polyphenylalanine. In addition, different preparations of randomly-ordered polynucleotides with varying degrees of secondary structure were assayed for template activity. Preparations with considerable secondary structure had little template activity. These results strongly suggest that single-strandedness is requisite for messenger RNA activity. Another factor was found which influences the messenger activity of RNA; that is, its molecular weight. Poly U containing 300 or more uridylic acid residues had markedly higher template activity than shorter chains.

Radioactive poly U was synthesized and the fate of messenger RNA in this system was followed. Addition of poly U resulted in a specific aggregation of ribosomes which sedimented at 100–130 S. Protein synthesis occurred initially only upon heavy ribosomes. Thus, these ribosomal aggregates were shown to be the site of protein synthesis.

Evidence was obtained which strongly suggested that poly U acted catalytically in this system; that is, one molecule of poly U directed the synthesis of more than one molecule of polyphenylalanine.

Characteristics of the Genetic Code. Since the sequence of uridylic acid residues in poly U was the RNA codeword corresponding to phenylalanine, the use of synthetic polynucleotides afforded a relatively simple experimental means of determining the nucleotide compositions and characteristics of genetic coding units. Randomly-mixed polynucleotides of known base composition were synthesized and found to direct many other amino acids into protein in a highly specific fashion. The nucleotide composition of coding units corresponding to almost all amino acids was determined in this manner. Various characteristics of the code such as the coding ratio, degeneracy, specificity, efficiency, universality of the code, etc., were investigated. Evidence was obtained suggesting that the coding ratio (the number of nucleotides per codeword) is three. A degenerate code is one in which two or more coding units code for one amino acid. The code was shown to be highly degenerate. However, code words were found to display striking specificity for their respective amino acids. Comparison of the nucleotide composition of the coding units with amino acid replacements occurring in mutant proteins suggested that a large portion of the code was universal; that is, phylogenetically diverse species have similar genetic codes. Such experiments permit a detailed description of the nature of the code.

CLINICAL ENDOCRINOLOGY BRANCH

The work of the Clinical Endocrinology Branch has included chemical, physiologic and clinical studies related to the physiology of the adrenal cortex, physiologic and clinical studies related to phosphorus and calcium metabolism and to the function of the parathyroid glands, studies of renal mechanisms for excretion of salt and water, and studies not closely related to these.

Studies of adrenal steroid metabolism have included the development of a method for determining plasma androsterone and etiocholanolone, studies in the biogenesis of adrenal steroids *in vitro* by slices of rat and beef adrenals, studies on the role of the pituitary and of the renin-angiotensin system in control of adrenal steroidogenesis in the dog, in man and in the rat, studies on the effect of acute expansion of plasma volume on aldosterone secretion and excretion in subjects with primary and secondary aldosteronism and hypertension, and studies of the pathologic physiology of the kidneys and adrenal cortex in a new syndrome thus far observed in three patients.

Adrenal Function

In the course of studies in this Branch on adrenal physiology in general and on periodicity in adrenal function in particular, it was necessary to develop methods for measurement of 19-carbon

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adrenal steroids. A knowledge of behavior of such steroids is required to define the pattern of adrenal responsiveness to a series of agents known to influence steroidogenesis. It has been reported, furthermore, that periodic disease may be related to periodic increases in the secretion or decreases in the destruction of 19-carbon steroids. Samples of androsterone and etiocholanolone with high specific activity were obtained by the Wilzbach method. The steroids were separated and purified by a series of chromatographic steps of which a passage on thin layer proved essential to eliminate the "blank" susceptible to acetylation. Unknown steroids were extracted from blood and labeled, together with H_3 labeled tracer, with Carbon 14. With subsequent chromatography, methods were developed to measure C 19 steroids in blood. In current studies, normal ranges and circadian fluctuations are being determined. Methods for steroid analysis involving gas chromatography were further developed and patterns for several products of intermediary metabolism of steroids were determined. In cooperation with the Technical Development Branch, the gas chromatographic method was extended to include double labeling with collection of effluent material after the writing of the gas chromatogram and subsequent analysis of steroids by the double isotope derivative method.

Studies in the biogenesis of adrenal steroids by slices and sections of glands *in vitro* were continued. The ascorbic acid depletion test of Sayers and Sayers was used to distinguish ACTH from renin, in view of the known presence of both substances in, and their relative ease of extraction from renal tissue, and in view of the property of both to stimulate secretion of aldosterone, corticosterone and cortisol. It was found that whereas ACTH in single or sustained doses readily depletes ascorbic acid from the adrenal cortex of the hypophysectomized rat, angiotensin has no such property when given in either way.

Sections of beef adrenal cortex were shown to secrete aldosterone, corticosterone and cortisol *in vitro*. Secretion was gently stimulated by the addition to the medium of steroid precursors which appear beyond cholesterol in biosynthetic pathways. ACTH and angiotensin stimulate steroidogenesis from precursors preceding and including cholesterol. The results thus strongly suggest that ACTH and angiotensin act at or near a biosynthetic step involving cholesterol and that they act in a similar fashion. These studies have been extended to indicate stimulation of steroidogenesis by 3′ 5′ cyclic adenosine monophosphate. Current studies are designed to determine the similarity and differences between the stimuli provided by cyclic AMP and angiotensin or ACTH. Attempts to obtain similar results with rat adrenal sections continued to give equivocal results. Rat renin appeared to be entirely ineffective in stimulating steroidogenesis by rat adrenal cortex even in the presence of rat serum, presumably containing renin substrate. Factors influencing steroidogenesis by the adrenal cortex were studied extensively in the dog. The role of acute surgery was re-investigated by comparing studies done one or two hours after surgery with similar studies performed at least 18 hours following surgical intervention. In this manner it was shown that neither hypophysectomy nor nephrectomy lowered aldosterone secretion when studied in the resting conscious animal at a suitable interval after operation. The effects of synthetic ACTH were studied in hypophysectomized, nephrectomized dogs. Doses of synthetic ACTH with very marked effect on secretion of corticosterone and of cortisol had equivocal effects on the secretion of aldosterone. The effect of the renin-angiotensin system was further studied and dose response curves were prepared. At all doses at which angiotensin stimulated secretion of aldosterone, it could be shown also to stimulate secretion of corticosterone and of cortisol. The effect of constriction of the renal artery, presumably a stimulus to endogenous renin secretion, was studied by measurements of steroid secretion and of blood and lymph renin and angiotensin. Constriction of the renal artery regularly induced hypertension and increased secretion of aldosterone, hydrocortisone and of corticosterone. Whereas in some animals it produced transient increase in the secretion of renin, the latter response was not consistently present. The effect of low salt diet in stimulating steroidogenesis was studied further. Extracts of kidneys from dogs deprived of sodium regularly induced hypertension and stimulated steroidogenesis (aldosterone, corticosterone, cortisol) in hypophysectomized, nephrectomized recipients, whereas extracts from salt loaded dogs had no effect. The effect of constriction of the inferior vena cava on steroido-

genesis and on production of renin was examined. Secretion of renin into thoracic duct lymph could be regularly stimulated by caval constriction in salt depleted animals and this response was eliminated by nephrectomy. It does appear that secretion of renin is one means by which caval constriction induces increased steroid secretion. In these studies caval constriction had little influence on the secretion of corticosterone or cortisol, but critical evidence on this point could be derived only in the absence of the pituitary. The effect of carboline derivatives reported to stimulate secretion of aldosterone was studied in hypophysectomized, nephrectomized and in hypophysectomized, nephrectomized decerebrate dogs with negative results. The results of physiologic studies in the dog indicate that the renin-angiotensin system can stimulate steroidogenesis by the adrenal cortex. Furthermore, the stimulus of hemorrhage requires the presence of the kidneys for maximum efficacy. It is possible that the kidneys participate in the stimulus by releasing renin but this is not clearly established and other mechanisms are not excluded.

Clinical studies on the control of aldosterone secretion in primary and secondary aldosteronism and on the role of the adrenal cortex in hypertension were continued. The results of acute expansion of intravascular volume, of sodium deprivation and of pharmacologic agents which block the action of aldosterone were observed in a number of additional subjects and compared to those in patients with essential or malignant hypertension. The patients with hypertension were also evaluated by determination of renin in the renal vein by catheterization and by the radioactive renogram. Expansion of plasma volume with salt-poor human serum albumin continued to prove effective in distinguishing the patient with secondary aldosteronism (e.g., in cirrhosis), in whom aldosterone secretion and excretion are markedly diminished by the procedure, from the patient with primary aldosteronism (e.g., aldosteroma), in whom no change of aldosterone secretion or excretion is seen. In current studies the effect of this procedure on patients with unilateral renal disease, hypersecretion of renin, hypertension and aldosteronism is being studied. Observations include measurements of circadian fluctuation in aldosterone excretion and an attempt to develop a method for measurement of circadian changes in aldosterone secretion. It has thus been shown that, contrary to published reports, the magnitude of circadian fluctuations in aldosterone excretion is not diminished by keeping the patients in bed, although the timing of the peaks and troughs may be changed. Patients with primary aldosteronism from adrenal tumor continued to show marked circadian fluctuations in aldosterone excretion, albeit with a time pattern different from that of normal subjects. In a series of normal subjects, "maps" of circadian fluctuation of aldosterone excretion were greatly extended. As the pattern proved highly consistent from subject to subject (with high values in the morning and low values in the evening), addition of cases greatly increased the reliability of the estimate.

The relationship of aldosterone secretion to the juxtaglomerular apparatus and thus to renin and angiotensin secretion was further studied in three patients with a new syndrome. This syndrome was characterized by normal blood pressure, marked hypertrophy and hyperplasia of the juxtaglomerular apparatus, increased quantities of circulating angiotensin and marked increases in aldosterone secretion. The normal blood pressure and the hypersecretion of aldosterone persist despite expansion of intravascular volume with albumin. The hypokalemic alkalosis characteristic of the syndrome was readily reversible with aldosterone antagonists, thus indicating an aldosterone-dependent renal origin for this manifestation of the syndrome. The blood pressure response to infusion of synthetic angiotensin showed marked resistance to the pressor activity of angiotensin in all subjects. Two of the patients received human renin and its effects were compared with those in a normal subject. Both showed not only a marked resistance to the pressor effects of human renin but also a failure of aldosterone secretion to increase with renin. In a normal subject, in contrast, aldosterone secretion showed a graded increase with increasing doses of human renin.

Calcium and Phosphorus Metabolism

Studies of phosphorus and calcium metabolism and on the function of parathyroid glands included measurements of the effect of phosphate loading and of parathyroidectomy on renal tubular transport of phosphate, studies of the effect of parathyroid extract on bone *in vitro*, on the rachitic animal *in vivo*, and on electrolyte and hydroxy-

proline excretion in man, studies of tests designed to detect hyperparathyroidism and to correlate clinical, chemical and histological findings in this condition, and studies on the metabolic bone diseases of osteoporosis, sarcoidosis and renal failure.

Phosphate loading over prolonged periods of time was found to produce decreases in tubular reabsorption of phosphate, albeit never to a figure which would indicate phosphate secretion. In all studies there was a fall of filtration rate and the role of changes in filtration rate was independently studied. By direct comparison of maximal phosphate transport determined by increasing the serum phosphorus with that determined by increasing the filtration rate it was shown that filtration rate was slightly but significantly correlated with maximal phosphate transport. The decrease in transport observed with phosphate loading was significantly greater than that expected from the fall of filtration rate alone. The effect of parathyroid extract on renal electrolyte excretion was studied in parathyroidectomized dogs. The increase of phosphate excretion by decrease of reabsorption was regularly associated with a rise of urinary pH.

In vitro parathyroid extract was shown to increase the calcium content of the media surrounding slices of weanling rat calvarium and studies were extended in an attempt to establish a concentration-response relationship for this effect. Treatment of rats with vitamin D induced a similar increase in calcium in the medium when the calvaria were incubated *in vitro*. These studies were compared with similar ones in vitamin D deficiency where the opposite occurred; the studies are being extended to allow better evaluation of the role of parathyroid hormone and vitamin D in bone metabolism. Studies were carried out in puppies with rickets to elucidate the mechanism responsible for the hypophosphatemia characteristic of that condition. Preliminary results suggest that parathyroidectomy leads to rapid increase in serum phosphorus and in tubular reabsorption of phosphorus, results which suggest that secondary over-activity of the parathyroid and not a defect of the renal tubule is responsible for the hypophosphatemia of rickets. The effect of parathyroid extract was measured in normal human subjects and in human patients with hypoparathyroidism and special attention given to the balances of calcium and magnesium and to the urinary excretion of hydroxyproline. Parathyroid extract regularly increases urinary hydroxyproline and this was taken as an index of destruction of bone matrix as an early effect of parathyroid hormone. Urinary calcium and magnesium decreased initially and later increased with the extract. The findings suggest an early direct tubular effect of extract on calcium and magnesium with later hypercalciuria and hypermagnesuria resulting from elevated serum levels.

Studies designed to clarify the diagnosis of hyperparathyroidism were extended. The effect of a standard infusion of calcium on 24-hour phosphorus clearance was examined in a number of patients. The phosphorus clearance under basal conditions was compared with that found the following morning after a calcium infusion given by night. Both the newer and the older type of test gave essentially the same information; on the basis of positive results in these tests together with persistent hypercalciuria not responding to carbohydrate-active steroids, a number of subjects with minimal criteria for hyperparathyroidism were surgically explored. Histologic lesions of the parathyroids were found.

The present studies also include measurements of circadian fluctuations of urinary phosphate excretion over a thirty-hour period in normal men and women and in patients suspected of having diseases of the parathyroid. The normals show a highly repreducible pattern of phosphate excretion with relative retention during daylight hours and two peaks of relative phosphate loss during the night. Patients with hyper- and hypoparathyroidism are being studied in a similar fashion and their curves compared with those of the normal subjects. All the clinical, biochemical and histologic findings in patients explored for hyperparathyroidism have been reviewed and compared. A disorder, not previously defined, involving abnormalities of calcium and of phosphorus excretion and histological hyperplasia of the parathyroids without gross enlargement, was encountered in a number of patients. In addition, a patient with hyperparathyroidism resulting from lipomyoadenoma of the parathyroid glands was studied before and after operation. This lesion has not heretofore been reported. In several patients surgical intervention with partial removal of hyperplastic gland produced striking decrease in the rate of renal stone formation. Studies in

the metabolic bone diseases of idiopathic osteoporosis, sarcoidosis and glomerular insufficiency included measurements of calcium absorption with standard oral doses of calcium[47] measurements of bone formation with standard intravenous doses of calcium[47] and metabolic studies in which the effect of various agents were tested. The patients with idiopathic osteoporosis were studied for a reported effect of 6α fluorotriamcinolone in favoring calcium retention. Whereas the agent did not produce calcium retention it did indeed appear to produce less loss of calcium than that produced by a comparable dose of other carbohydrate-active steroids with different chemical configuration. Patients with sarcoidosis were shown by isotope studies to have marked increase in calcium absorption as originally detected by balance studies. It was found also that at the time they are suffering from hypercalcemia they show marked increases in the rate of bone formation. The increased absorption of calcium could be decreased with carbohydrate-active steroids such as prednisone and there is a suggestion from early results that the increased formation rate may likewise respond to the steroids. The disorder of calcium metabolism with glomerular insufficiency was further studied. Marked diminution in calcium absorption followed the onset of acute nephritis and was regularly found with chronic glomerular insufficiency. With the chronic disease, renal osteitis fibrosa was demonstrated byX-ray, by biochemical changes and by biopsy before treatment with vitamin D. With vitamin D the defect in calcium absorption was markedly improved or abolished and concomitantly the bone disease could be healed. Further studies are in progress to define the nature of the defect in calcium absorption with renal failure and to determine whether this bears any relationship to the remarkably high product of calcium by phosphate ions sustained in the serum of such patients.

Studies on renal mechanisms for excretion of salt and water have included measurements of the role of adrenal steroids on urinary concentration and dilution, studies on the role of the adrenergic nervous system on sodium and water metabolism. studies of circadian fluctuation in salt and water excretion in normal and in abnormal subjects with special reference to the effect of posture, and studies on the sodium metabolism of the fasting state. The role of adrenal steroids in promoting dilution and concentration of the urine was studied in patients with Addison's disease from whom treatment was temporarily withdrawn and in normal subjects receiving steroid therapy. It was found that the antidiuresis of untreated Addison's disease could be largely prevented by expansion of extracellular fluid volume without addition of steroid therapy. The expansion did not require the sodium ion, as expansion with albumin alone produced the same restoration of relatively normal diluting power as that induced by saline. Patients with Addison's disease were shown to have limited ability to produce concentrated urine and limited ability to reabsorb water under maximum antidiuretic hormone activity. The ability to concentrate and to reabsorb water against a concentration gradient could be restored with desoxycorticosterone in small doses or by simple dehydration, which presumably increased medullary solute concentration. Whereas maximal concentration of the urine could be shown with small doses of desoxycorticosterone, large doses did not restore concentrating power, a finding suggesting a role of desoxycorticosterone in promoting proximal tubular sodium reabsorption. The role of the adrenergic nervous system in retention or excretion of sodium and water was studied in view of indirect evidence that the sodium retention for cardiac failure may depend in part upon excess of catecholamines. In long term studies, the effect of standard doses of desoxycorticosterone on sodium balance were compared in the same patient on the same regimen with and without treatment with guanethidine at dosages which produced hypotension and thus presumably peripheral catecholamine depletion. The effects of small doses of norepinephrine were measured directly in normal subjects and compared with the effects of slightly larger doses. Results suggest that guanethidine diminishes the sodium retention resulting from a given dose of desoxycorticosterone and thus suggest that catecholamines are indeed involved in the pathological sodium retention of some patients with edema. Small doses of norepinephrine induced retention of sodium and water in normal subjects, a finding which supports the above conclusions. In large doses, norepinephrine induced excretion of sodium and water. Studies are in progress to define better the possible role of catecholamines in the states characterized by

sodium retention. Studies on circadian rhythms in secretion of salt and water included extensive transverse mapping of urinary sodium, potassium, phosphate, hydrogen ion (titratable acidity and ammonium), aldosterone, 17 ketosteroids and Porter-Silber steroids over 30-hour periods during which collections were made at 3-hour intervals in groups of normal subjects large enough to establish statistical significance for the fluctuations observed. Results of these and of longitudinal studies of body temperature over protracted periods were subjects to thermal variance spectrum analysis to evaluate the extent of fluctuations with circadian periods and that with other periodicities. Studies have been begun in which similar results are obtained from patients with disease. If the disease involves desynchronization of one body function from another, or if it involves a change of external timing of adrenal periodicity, it should be apparent from results of such analysis. In a patient with cancer and marked edema, for example, circadian periodicity of body temperature was completely lost despite absence of fever at any time.

Studies on the abnormal sodium metabolism of the fasting state were extended. The observation that the fasting subject may lose much more sodium that he loses when deprived of dietary sodium has been amply confirmed. It was shown that the extent of sodium loss appeared to be related to the degree of acidosis, and that a single patient might show wide differences in the extent of change of both these variables during different fasting periods. It was also shown that the degree of sodium loss was roughly correlated with the extent of increase of urinary ketone bodies and that it could be prevented by feeding of carbohydrate alone. Further studies defining the role of ketoacidosis in this abnormal sodium loss of the fasting state are in progress.

Studies not included in the above categories include investigations into the mechanism of the development of atherosclerosis by a study of the rate of passage of large molecules across the aortic endothelium. It was shown that labelled lipoproteins enter the arterial wall and that there are similarities in the dynamics of their passage into the wall and that of albumin and cholesterol shown for previous studies. It was clear that blood pressure was a prominent factor in determining the rate of transport and that stretching of the wall was essential for this effect. Current studies with lipoprotein include an attempt to avoid loss of label from a given molecule. It is anticipated that the role of hormones or of hormone deficiencies in promoting atherosclerosis can be further defined with this technique.

EXPERIMENTAL THERAPEUTICS BRANCH

Biochemistry of Aromatic Amines

Tyramine, a normal constituent of human urine and a vasoactive compound whose pressor effects are mediated by norepinephrine release, has been isolated and identified in mammalian tissues. With some exceptions, its occurrence in various species is confined to the central nervous system, the highest levels being in spinal cord (up to 5 μg/gm), brain stem and cerebellum. Preliminary experiments suggest roles for the amine in modulating synaptic reflexes in the spinal cord and involvement in central responses to certain drugs. Also, the possibility that it may serve as a natural precursor of norepinephrine is under study.

Sympathetic nerve and adrenal medullary tissues are known to be capable of synthesizing norepinephrine from its dietary precursor, tyrosine. This has not been shown previously for heart; indeed, there is a body of opinion that cardiac norepinephrine may be extracted from the circulating blood. Studies using isolated guinea pig hearts (Langendorff procedures) have shown synthesis of C^{14}-norepinephrine from C^{14}-tyrosine in the perfusing fluid. This finding indicates that extensive evaluation of factors affecting synthesis and metabolism of the amine in heart will now be feasible.

The final step in the synthesis of norepinephrine, the β-hydroxylation of dopamine, is under investigation in intact man. The impetus for development of sensitive techniques for quantifying dopamine-β-oxidase activity in man is the prospect that inhibitors of this enzyme will soon become available for clinical studies. The urinary excretion of norepinephrine and its metabolites may afford a useful, albeit rather insensitive, index. The basis of a more sensitive test has been developed with the finding that 4 to 9 percent of an oral dose of an artificial substrate of the enzyme, p-hydroxy-amphetamine (Paredrine), is excreted in the urine as its β-hydroxylated metabolite,

p-hydroxynorephedrine. It is of interest that an adrenalectomized patient showed one of the highest β-hydroxylating activities.

A veritable spectrum of catecholamine biochemistry was revealed by studies on the urine of 21 children with malignant tumors of the sympathetic nervous system. Elevated amounts of the following compounds were found: dopa, dopamine, norepinephrine, normetanephrine, and vanilmandelic acid. Tyramine levels were not increased. A hitherto overlooked aspect of norepinephrine metabolism is suggested by the finding that, while 95 percent of an intravenous dose of H^3-norepinephrine is excreted within 3 days as others have reported, the remaining radioactivity is excreted more slowly with a half time of about 8 days.

The carcinoid syndrome, with its metabolic and pathologic implications, is still receiving major attention. It has been shown that many sympathomimetic amines induce typical flushing in carcinoid patients, the most potent agents being epinephrine and isoproterenol. Flush induction with minute doses of these compounds given intravenously affords a useful diagnostic test at the bedside. Variants of the syndrome studied this year include: (1) a patient with metastases from a bronchial primary and with severe flushes, slightly elevated 5HIAA and normal blood serotonin. The flushes were controlled with prednisone. A kinin-forming system has been demonstrated in the plasma during flushes. (2) Another patient wtih metastatic gastric carcinoid with excretion of 5-hydroxytryptophan and large amounts of histamine in the urine.

The exact pathogenesis of carcinoid endocardial fibrosis remains an enigma though a relationship to excess serotonin appears likely. A somewhat similar pathologic situation is the so-called endomyocardial fibrosis (EMF) of Uganda. While the distribution of lesions differs from that in carcinoid, in both cases they are composed predominantly of fibrous, rather than elastic tissue. Pertinent in this connection may be the fact that bananas, a fruit which we have shown contains large amounts of serotonin, are a dietary staple of the African in Uganda. The epidemiologic correlation between EMF and banana intake was confirmed during a visit to Africa where discussions were held with personnel at the Makerere Medical College in Kampala with a view toward collaborative studies. We have shown in normal volunteers that the fate of serotonin taken orally is the same, whether contained in bananas or capsules, with 95 percent appearing in the urine as 5-hydroxyindoleacetic acid. A 4–6-fold increase in platelet serotonin levels was observed after 2 weeks' treatment with oral serotonin, 160 mg/day. On stopping treatment, platelet serotonin returned toward normal logarithmically, with a half life of about 3 days. This rather simple method of labelling platelets and measuring their turnover rate is being applied to study of platelet disorders.

The cause of elevated plasma tyrosine levels in thyrotoxicosis appears to be complex. The effect of thyroid hormone on tyrosine-α-ketoglutarate transaminase activity in rat tissues is to increase levels of the enzyme which would tend to decrease rather than increase tyrosine levels.

Methods for measuring urinary phenethylamine have been improved and observation of increased levels in phenylketonurics confirmed and extended. Since this metabolite might be a more sensitive index of impaired hydroxylation of phenylalanine than blood levels of the amino acid, it is planned to study formation of the amine in heterozygotes during phenylalanine loading.

Chemical Pharmacology and Therapeutics

An interaction between indoles and catechol compounds is suggested by such phenomena as flush induction with catecholamines in carcinoid patients and coexistence of serotonin and norepinephrine in certain areas of the brain. Because of this, naturally-occurring and recently synthesized indole compounds were studied for effects on tissue stores of norepinephrine in laboratory animals. Serotonin, α-methyl-serotonin, α-methyl-tryptamine and α-methyl-5-hydroxytryptophan were found to reduce the levels of norepinephrine in rat heart or brain, or both. In addition, a series of indole-phenylpiperazines were found to have similar activity. One of these, oxypertine, was studied in detail. It was found to decrease norepinephrine in rat heart and brain without affecting brain serotonin. Catecholamine levels returned to normal within 8–12 hours after injecting the drug suggesting a rapid turnover of the amine. The compound also has sedative properties and is an effective blocker of α and β adrenergic receptors, as defined by Allquist. In preliminary studies, the compound has been shown to be highly

effective in abolishing cardiac arrhythmias produced by chloroform-epinephrine as well as by coronary occlusion.

We are currently engaged in a long term, controlled study of the effectiveness of α-methyl-dopa (Aldomet) in 53 patients with severe hypertension. The drug is also being used frequently in hospitalized patients and has greatly simplified the management of many cases of malignant hypertension. In most patients (two-thirds), the drug is more effective than other potent antihypertensive agents though optimal effect usually requires concomitant use of a thiazide diuretic. Smoothness of effect, relative freedom from side effects, tranquilizing properties and frequent control of blood pressure in recumbent as well as standing positions make Aldomet the current drug of choice for many hypertensive patients.

Studies on the metabolism and mechanism of action of Aldomet have continued and permit the following conclusions: (1) The hypotensive effect is unrelated to the decarboxylase-inhibiting properties of the compound. (2) The drug exerts its action by being decarboxylated to α-methyl-dopamine which may in turn be β-hydroxylated to α-methyl-norepinephrine; the amine metabolites deplete tissue stores of norepinephrine and/or compete with norepinephrine at receptor sites, thereby lowering blood pressure. Alpha-methyl-m-tyrosine, a compound with similar biochemical properties to Aldomet but which like Aldomet does not lower blood pressure in animals, has been found to have weak hypotensive properties in human hypertensives.

We have continued to use monoamine oxidase inhibiting drugs, chiefly pargyline, in the management of hypertension in selected patients, either because of resistance to other drugs or need for antidepressive medication. Beneficial effects in patients with angina pectoris were previously ascribed to reduction of levels of blood pressure and pulse rate during exercise. It has now been shown in 7 patients that, as was presumed, the rise of cardiac output in response to standard exercise is reduced during pargyline therapy.

An interesting area of study on vascular responsiveness has appeared in studies of the pressor responses to tyramine in patients with pheochromocytoma, and hypertensive subjects receiving Aldomet or reserpine. A hypersensitivity to tyramine was found in three patients with pheochromocytoma. This may be of sufficient magnitude to warrant consideration of a tyramine-provocative test, analogous to the histamine test, but whose performance is not associated with any morbidity. Whereas, patients receiving reserpine exhibit diminished tyramine sensitivity secondary to presumed catecholamine depletion, several patients receiving Aldomet showed enhanced sensitivity. Our working hypothesis is that in patients with pheochromocytoma there may be increased levels of catecholamines, which are subject to tyramine release and an increased response. Similarly, α-methyl-amines, formed from Aldomet in tissues, could conceivably mediate enhanced tyramine pressor responses.

Metabolism of Hydroxyproline and Collagen

The urinary excretion of hydroxyproline (HOPr) peptides has been found to be a suitable index of the metabolism of soluble collagen in man, providing the subject ingests a gelatin-free diet. With dietary restriction, values in the range of 14–36 mg HOPr/day have been found in normal adults. During the period of rapid growth (adolescence), values may be as high as 260 mg/day. After growth ceases, there appears to be little change with increasing age; this is contrary to our previous impressions from studies in which diet was not rigidly controlled.

In addition to growth hormone, both thyroid hormone and parathyroid hormone have been found to increase HOPr excretion. This effect has been produced by administering the hormones to normal volunteer subjects and is also apparent in patients with thyrotoxicosis and hyperparathyroidism. No other abnormalities have been encountered in patients thus far; HOPr excretion is normal in the so-called "collagen diseases". Possibly, abnormalities will be detected in the latter states when the several HOPr peptides are isolated and quantified individually.

Studies in growing mice have shown that lathyritic factor does not alter synthesis of total collagen but does produce an abnormally large pool of soluble collagen. Several anti-inflammatory drugs have shown no preferential effect on collagen versus total protein metabolism. Studies in several species have shown that arteries contain large amounts of collagen. This protein constitutes about 30 per cent of the total protein content of aorta and up to 60 per cent of the protein

in muscular arteries. No change in ratio is seen during normal aging.

Miscellaneous

1. Studies are in progress to isolate and characterize the so-called angiotensinase(s) of blood. Angiotensin levels and disappearance rates are being determined by a complex method which terminates in measurement of pressor activity in the rat.

2. Gas-liquid phase chromatography has been used to detect minute quantities of the aglycones of digitoxin and digoxin. This, coupled with suitable hydrolysis of the glycosides, may provide a means of accurate chemical assay of cardiac glycosides in biologic media.

3. A large kindred has been studied in which primary pulmonary artery hypertension appears to be transmitted as a Mendelian dominant.

4. Although the pressor response to norepinephrine is increased in patients with hypertension, changes in plasma levels of free fatty acids following injection of the amine are of the same magnitude as that in normal subjects.

CARDIOLOGY BRANCH

An important objective of this Branch is to identify the various mechanisms which regulate the heart's activity, and to assess their relative importance. In previous years the greatest emphasis was placed on defining the role of the ventricular end-diastolic pressure and volume in the determination of the force of the heart's contraction. During 1962 greater attention was focused on the role of neurohumoral mechanisms in cardiovascular regulation. It is now clearly recognized that the adrenergic nervous system is capable of profoundly altering the mechanical activity of the myocardium, and for this reason, considerable effort has been directed toward defining the operation of this system.

I. Studies on the Adrenergic Nervous System

A. Biosynthesis and Metabolism of Norepinephrine by the Heart

Although the biosynthesis of norepinephrine has been demonstrated in vitro in tissue isolated from the adrenal medulla and from sympathetic nerve endings, it has recently been questioned whether the norepinephrine present in the heart is synthesized there or whether it is extracted from the blood by the sympathetic nerve endings in the heart. This problem was studied by perfusing an isolated dog heart with radioactive dopamine. Two to 12% of the added precursor was converted to norepinephrine in one hour. Thus, these studies show that the isolated heart is capable of synthesizing norepinephrine from dopamine.

Although two enzymes (catechol-o-methyl transferase and monoamine oxidase) have been shown to be capable of degrading norepinephrine, no evidence has been available regarding the relative importance of these enzymes within the heart. This problem was investigated using an isolated dog heart in which the norepinephrine was labeled by the administration of tritiated norepinephrine. The o-methylate metabolite, normetanephrine, was demonstrated to be a major metabolic product in the heart; this finding emphasized the importance of o-methylation in the metabolism of norepinephrine in the heart.

B. Definition of the Subdivisions of ther Norepinephrine Pool

Studies have also been continued to determine if the neurotransmitter is distributed homogeneously or whether it is distributed into multiple functional compartments. Infusions of tyramine were given until tachyphylaxis to further administration of the drug was obtained. Cardioaccelerator nerve stimulation was carried out before and after the tyramine infusion and no significant reduction in the inotropic or chronotropic responses to stimulation occurred. Since nerve stimulation was evidently capable of releasing norepinephrine at a time when the store of norepinephrine available to release by tyramine was depleted, it is clear that some of the "readily available norepinephrine" can be released by nerve stimulation but not by tyramine. Thus, it is concluded that the "readily available norepinephrine" stores may be further subdivided functionally.

Although radioactive norepinephrine can be extracted and stored at the sympathetic nerve ending, little information is available regarding its distribution within the neurotransmitter store. To investigate this problem radioactive norepinephrine was administered to dogs and the specific activity of norepinephrine was determined in coronary sinus blood before and during release of norepinephrine augmented by tyramine or car-

dioaccelerator nerve stimulation, and these values were compared with the values in myocardial tissue. It was found that upon augmented release of norepinephrine the specific activity of norepinephrine in blood fell. However, in all experiments the specific activity of norepinephrine in the blood released from the heart exceeded the specific activity in the cardiac tissue. These observations indicate that the radioactive norepinephrine is distributed unevenly within the endogenous store, initially mixing principally with the more exchangable norepinephrine.

C. Pharmacology of Adrenergic Blocking Agents

The function of the autonomic nervous system has also been studied with the aid of two anti-adrenergic drugs, reserpine and guanethidine. These agents are of interest since they are extensively utilized in clinical practice, yet their precise mechanisms of action have not been fully elucidated. It was concluded that the mechanisms of action of guanethidine and reserpine differ considerably. When guanethidine is administered the initial release of norepinephrine from the heart produces an adrenergic response, but this is associated with only minimal tissue depletion of norepinephrine. In contrast, resperpine produces more rapid depletion of myocardial norepinephrine stores than does guanethidine, but it does not result in the release of norepinephrine from the heart into the coronary sinus blood.

In other experiments the heart rate response to cardioaccelerator nerve stimulation and the corresponding levels of myocardial norepinephrine content were determined and correlated at various time intervals following the intravenous injection of reserpine and of guanethidine. Guanethidine produced complete blockade of the cardiac accelerator response before producing measurable depletion of myocardial norepinephrine content. In contrast, reserpine reduced the positive chronotropic response to cardio-accelerator nerve stimulation only after myocardial norepinephrine levels had been reduced to approximate 0.3 μg./gm. These observations suggest that the interference with adrenergic transmission produced by guanethidine is independent of changes in the level of stored adrenergic transmitter, but that almost complete depletion of stored adrenergic transmitter must occur before reserpine-induced adrenergic blockade develops.

Although the cardiovascular response to those vasoactive amines which act by releasing endogenous norepinephrine is profoundly reduced by the chronic administration of reserpine, the response of these amines immediately after the administration of reserpine has been poorly defined in the past. The cardiovascular responses to tyramine, tryptamine, metaraminol and amphetamine were found to be greatly potentiated for several hours after the intravenous administration of reserpine, while the responses to norepinephrine were essentially unchanged. The administration of reserpine increased the quantity of norepinephrine released from the heart by tyramine. It was concluded that the potentiated norepinephrine release was not due to an increase in the size of the "tyramine releasable" compartment of norepinephrine, but that the reserpine facilitated the release of norepinephrine by tyramine by preventing the reentry and/or binding of the norepinephrine.

Although it is well recognized that reserpine can produce total depletion of norepinephrine in the cardiovascular system in the experimental animal and its anti-adrenergic action has been attributed to this effect, no information has been available regarding the effect of reserpine administered clinically in man. In order to study this problem, biopsies of atrial appendages were obtained at the time of cardiac operation in 22 patients who were in normal sinus rhythm and who had never been in congestive heart failure. The average norepinephrine concentration in these tissues was 1.87μg./gm. In three patients who received 0.25–0.5 mg. reserpine orally for 30 days, the atrial norepinephrine concentrations were 0.36, 0.10, and 0.04 μg./gm. These results indicate that reserpine given orally to patients in the ordinary clinical doses significantly reduces the myocardial norepinephrine concentration. However, supramaximal electrical stimulation at the cardiac sympathetic nerves, at the time of operation, was still found to produce an adrenergic response.

Previous studies showing in anesthetized open-chest dogs that guanethidine and reserpine block reflex venoconstriction were extended to man. The effects of three commonly employed antihypertensive agents, reserpine, guanethidine and alpha methyl DOPA, on reflex venoconstriction were studied. In all cases reflex venoconstriction was abolished after chronic administration of

these drugs in therapeutic doses. These effects which result in a decrease in venous return to the heart, appear to be important in the production of orthostatic hypotension in patients undergoing therapy with these drugs.

D. The Autonomic Nervous System in Heart Failure, Shock and Anemia

It has been shown previously that pharmacologic blockade of the adrenergic nervous system in normal human subjects interfered with the circulatory response to muscular exercise. These studies have now been extended; an attempt was made to evaluate the contributions of adrenergic reflexes and of myocardial catecholamine stores to cardiac homeostasis in man by determining the effects of guanethidine in patients with borderline congestive heart failure. Guanethidine clearly increased the signs and symptoms of heart failure in five of 10 patients, suggesting that the adrenergic nervous system plays an important compensatory role in the circulatory adjustments of patients to congestive heart failure. These studies also emphasize the need for caution in the use of highly effective antiadrenergic drugs in the treatment of patients with limited cardiac reserve.

Although the sympathetic nervous system has been implicated as a major determinant of cardiovascular function during muscular exercise in normal man, its role in patients with congestive heart failure has not been defined. In an effort to asssay the activity of the sympathetic nervous system, arterial norepinephrine was measured in normal subjects and in cardiac patients both with and without congestive heart failure at rest and during exercise. These patients with congestive heart failure showed an augmentation of arterial norepinephrine which exceeded that of the normals in every instance. These results indicate that there may be an increased activity of the sympathetic nervous system during exercise in congestive heart failure and it was suggested that this activity may play an important supportive role to the circulation.

An investigation was carried out in order to determine whether hemorrhagic shock, which is known to produce an increased activity of the sympathetic nervous system, alters the release of norepinephrine from the "readily available" norepinephrine store. The cardiovascular responses to norepinephrine infusion and to infusion of tyramine, which acts by releasing endogenous norepinephrine, were compared before hemorrhage and at various time intervals after hypotension. The pressor effects of tyramine were found to be markedly reduced after several hours of hypotension. The responses to infusions of norepinephrine and angiotensin were only minimally attenuated during similar periods of hypotension. It therefore appears that prolonged activation of the sympathetic nervous system by hypotension alters the release of norepinephrine from the "readily available" store.

In order to determine whether the autonomic nervous system is important in mediating the circulatory response to acutely induced severe anemia, the effect of this stimulus was compared in normal unanesthetized dogs, and in unanesthetized animals with denervated hearts. These studies indicate that the unanesthetized dog, when subjected to acute anemia, can increase its cardiac output despite the absence of a nerve supply to the heart. This elevation in output was achieved in the denervated animals primarily by an increased stroke volume, whereas in control dogs it was attributable primarily to increased heart rate.

E. The Mechanism of Action of Sympathomimetic Drugs

A detailed investigation on the mechanism of action of metaraminol, a widely used vasopressor agent in the clinical management of hypotension, was carried out in experimental animals and it was concluded that the positive inotropic and pressor responses to metaraminol are due primarily to the release of norepinephrine at adrenergic nerve endings.

II. Studies on the Dynamics of Ventricular Contraction

A. Cineradiographic Measurements of Ventricular Dimensions

In order to analyze the mechanical activity of the left ventricle in man, a method for the precise, continuous measurement of ventricular dimensions in closed-chest subjects has long been sought. Silver-tantalum clips were sutured to the surface of cardiac chambers in patients undergoing cardiac surgery. After recovery cineradiograms were obtained and the distances between clips were measured on each individual frame of the film.

There was an increase in right ventricular size with inspiration and a decrease with expiration. Changes in left ventricular dimensions were less marked and lagged 3–4 cardiac cycles behind the changes in the right ventricle. The administration of isoproterenol and norepinephrine resulted in a decrease in the ventricular end-diastolic dimensions while methoxamine produced an increase in end-diastolic dimensions. Exercise also produced a consistent decrease in right and left ventricular dimensions, at a time when the rate of pressure development in the right ventricle was increased. This cineradiographic technique permits, for the first time, accurate and reproducible measurements of ventricular dimensions in man throughout the cardiac cycle and over the course of a number of cardiac cycles.

B. Left Ventricular Function

Although the response of the right heart to exercise has been well documented, the study of left heart dynamics during exercise has not been possible, until recently. The development of the transseptal method of left heart catheterization has permitted the introduction of flexible catheters into the left heart by the venous route, and using this technique a study of the left heart responses to standard exercise on a bicycle ergometer has been initiated. It appears that in patients with aortic stenosis a moderate increase in cardiac output is accompanied by little if any change in the pressure gradient across the aortic valve, by an elevation of left ventricular end-diastolic pressure, and by only a slight increase in left ventricular systolic pressure, suggesting that in such patients the left ventricle has little "functional reserve."

Ventricular function was also studied in patients by infusion of graded doses of angiotensin. If a response which is typical of the normal left ventricle could be established, the method could provide a readily controlled stress mechanism for the evaluation of patients with compromised ventricular function. In the patients studied so far, angiotensin infusion has resulted in minimal changes in cardiac output, but sizeable increases in left ventricular stroke work and left ventricular end-diastolic pressure. It is anticipated that this method will have clinical value in the assessment of left ventricular function.

III. Studies on Digitalis

The mechanism of action of digitalis has been of considerable interest to investigators in this laboratory for several years. These studies were continued in two general directions:

A. Effects of Digitalis on the Nonfailing Human Heart

Patients with acquired valvular heart disease and cardiac enlargement who were able to perform normal everyday activity without difficulty in the absence of digitalis therapy were exercised while receiving a placebo and again while receiving digoxin. Varying degrees of exercise were performed on a treadmill in a metabolic chamber and oxygen consumption was measured continuously. In all patients the oxygen debt was smaller during the period of digoxin administration although the external work performed was identical, indicating that the functional status of their circulatory system was improved by the drug. On the basis of this study it appears that digitalis administration is beneficial to at least some patients who have cardiac disease and enlarged hearts and some decrease in cardiac reserve without signs or symptoms of heart failure.

Since digitalis glycosides fail to elevate the cardiac output in patients who are not in heart failure, it has been suggested that these drugs do not stimulate myocardial contractility in nonfailing hearts. Accordingly, an investigation was carried out in order to determine whether ouabain, administered intravenously, modifies the force of contraction of normal, or near normal, nonfailing hearts. Changes in myocardial contractility were assessed by continuously recording the rate of change of intraventricular pressure (dp/dt). In 10 patients without heart disease peak dp/dt increased by 10 to 49 percent of control values. In the absence of changes in intraventricular systolic and diastolic pressures and in heart rate, the observed increases in dp/dt may be assumed to reflect increases in myocardial contractility. These observations in intact, unanesthetized subjects therefore indicate that ouabain is capable of stimulating the contractility of the nonfailing and the normal human heart.

The effects of digitalis on the peripheral vascular system of man were investigated. In normal

subjects ouabain resulted in an increase in both arterial and venous tone, while in patients with overt cardiac failure there was a decrease in both arterial and venous tone. These observations help to explain a number of the actions of digitalis which have puzzled physicians for many years.

B. Determinants of the Effectiveness of Cardiac Glycosides

Others have suggested that the positive inotropic effect of ouabain is dependent upon an intact myocardial catecholamine store and that the amount of ouabain required to produce fatal arrythmias is influenced by myocardial norepinephrine content; the exact mechanism by which glycosides increase the refractory period of the atrioventricular conduction system has been unknown. We have shown that intravenous ouabain infusion produced comparable increments in myocardial contractile force in control dogs, in dogs with chronic cardiac denervation, in dogs with myocardial catecholamine depletion, in vagotomized, reserpinized dogs, and in dogs with acute section of preganglionic sympathetic pathways. It was also determined that there were no significant differences in the toxic doses of ouabain in each of these four groups of animals. In addition, it has been shown that ouabain infusions produce the greatest prolongation of the A–V functional refractory period in control dogs, and that this effect was reduced in vagotomized dogs and reduced further in the dogs with chronic cardiac denervation. These observations indicate that the positive inotropic and arrhythmic doses of ouabain are independent of myocardial catechloamine stores, but that a large portion of the prolongation of the A–V functional refractory period produced by ouabain is dependent upon intact cardiac adrenergic innervation.

The difficulties encountered in the management of thyrotoxic heart disease with digitalis have suggested that changes in metabolic activity induced by the thyroid state may condition the myocardial response to digitalis. It was observed that the increase in ventricular contractile force produced by ouabain was not significantly different in euthyroid and hyperthyroid animals. However, ouabain produced a significantly greater increment in the ventricular contractile force of hypothyroid animals. The toxic doses of ouabain were identical in euthyroid and hypothyroid dogs, but were substantially greater in hyperthyroid dogs. In addition, hyperthyroid dogs were more resistant than euthyroid or hypothyroid dogs to the prolongation of the refractory period of the A–V node. These observations indicate that the three myocardial effects of ouabain studied were not altered in a uniform manner by changes in the thyroid state. In addition they help to explain some of the problems encountered by the physician in the management of the thyrocardiac patient.

IV. Cardiovascular Physiologic Studies

As before, the cardiovascular physiologic investigations have centered on the coronary circulation. The effect of varying coronary blood flow on myocardial oxygen consumption was studied in 23 experiments. The ventricles were kept empty, developed no pressure and performed to external work, while their temperature was held constant. A comparison of myocardial oxygen consumption at two levels of coronary blood flow (and oxygen delivery) was made in 42 instances, and in 32 of them myocardial oxygen consumption increased substantially as coronary blood flow was elevated. The ten exceptions all occurred when oxygen delivery greatly exceeded myocardial oxygen consumption, with oxygen extraction ratio below 35%. The energy derived from anaerobic metabolism at low levels of coronary blood flow could not account for the lower values of myocardial oxygen consumption which occurred under these circumstances. These observations show that in a nonworking heart, when myocardial oxygen extraction is in a physiologic range, myocardial oxygen consumption is intimately dependent on coronary blood flow and suggest that this dependence should be considered in the interpretation of experiments on the determinants of myocardial oxygen consumption. In seven experiments performed on hearts which developed pressure during systole, myocardial oxygen consumption remained constant in spite of large changes in coronary blood flow. Further experiments are planned to define the mechanisms responsible for the differences observed in the empty heart and the heart which is forced to develop a systolic pressure.

Efforts have also been directed toward the development of methods for the continuous measurement of local myocardial blood flow. Progress has been made in the construction of a heated thermistor probe, with which changes in

perfusion modify the rate of heat transfer from the probe. A series of experiments have been initiated together with investigators in the Laboratory of Kidney and Electrolyte Metabolism, NHI. Gaseous hydrogen, dissolved in saline, is injected into the coronary circulation, and the rate at which the hydrogen is removed from the heart determined by means of platinum electrodes introduced into the myocardium as well as into the coronary venous blood. Preliminary results indicate that both the thermistor and hydrogen techniques show considerable promise, and it is hoped to perfect these methods so that they can be employed for the measurement of myocardial blood flow in man.

Nonartifactual pressure tracings were obtained at catheterization in intact, unanesthetized human subjects. These tracings were analyzed for their first 50 harmonic components by the Computation and Data Processing Branch. It appears that the major harmonics of such tracings are within the first five to six calculated harmonics, and these harmonics are included within the frequency response (8 cycles/sec.) of standard catheter-external manometer systems.

V. Studies in Clinical Cardiology

A number of clinical investigations on patients with congenital and rheumatic heart disease were completed in 1962. As before, many of these studies were carried out in conjunction with the Clinic of Surgery, NHI. During the past year, attention was focused on patients with left ventricular hypertrophy, with and without obstruction to left ventricular outflow. The clinical features, phonocardiographic, roentgenologic and electrocardiographic findings in 100 patients with congenital aortic stenosis were correlated with the severity of obstruction as determined by left heart catheterization. In addition to defining the specific value of each clinical finding in the assessment of the severity of obstruction, indications for surgical correction of this lesion were established and the clinical and hemodynamic effects of operation were analyzed.

Although the definitive determination of the site and severity of obstruction to left ventricular outflow must be left to catheterization techniques, a simple and innocuous test would be of considerable value in the evaluation of such patients. The first derivative of the pressure pulse was continuously calculated by an electronic differentiating analogue computer. Patients with hypertrophic subaortic stenosis were found to have a brachial artery peak dp/dt greater than that found in normal objects. This appears to be due to the fact that little or no obstruction exists in early systole. In contrast, patients with fixed obstruction to left ventricular outflow (valvular and discrete membranous subvalvular stenosis) exhibited obstruction to left ventricular outflow throughout all of ventricular systole and the brachial artery peak dp/dt proved to be lower than normal. It is anticipated that the technique described should prove to be a simple and practical one in the recognition and differentiation of the various forms of obstruction to ventricular outflow.

It has recently been found that a unique feature of the disease in patients with idiopathic hypertrophic subaortic stenosis is the inconstant nature of the obstruction. Prominent among the determinants of the severity of obstruction is the contractile state of the myocardium. It therefore seemed likely that the administration of sympathomimetic drugs could exert profound effects on the circulatory dynamics of patients with hypertrophic subaortic stenosis. The intravenous administration of isoproterenol generally lowered the cardiac output and the systemic arterial pressure, while elevating the left ventricular systolic and end-diastolic pressures. Thus, this drug consistently decreased the effective orifice within the left ventricular outflow tract. In contrast, methoxamine abolished the left ventriculo-arterial pressure gradient in four patients.

The clinical, hemodynamic, and angiocardiographic findings in a group of 11 patients with idiopathic myocardial hypertrophy were described. These patients were either asymptomatic or their symptoms showed little if any progression over many years. The clinical findings and evidence of left ventricular hypertrophy on electrocardiographic, roentgenologic and angiocardiographic examinations were similar to the findings in patients with idiopathic hypertrophic subaortic stenosis. In contrast, none of these patients exhibited any hemodynamic evidence of obstruction to blood flow during the control state. The close relationship between this clinical syndrome and hypertrophic subaortic stenosis was shown by the finding that a gradient between the left ventricle and a systemic artery could be provoked by the administration of isoproterenol in these patients.

It has been generally thought that significant elevations of the left atrial and plumonary vascular pressures occur in patients with mitral regurgitation of sufficient severity to produce serious disability and gross enlargement of the left atrium. A group of patients with severe mitral regurgitation in whom gross left atrial enlargement was accompanied by normal left atrial and pulmonary artery pressures was described. The observed discrepancy between left atrial size and pressure must reflect a disturbance in the compliance of the left atrial wall. It was suggested that long-standing mitral regurgitation modifies the mechanical characteristics of the atrial wall and that the presence of a normal left atrial pressure must not be assumed to exclude the presence of severe mitral regurgitation.

Other Remarks

A substantial fraction (approximately 25%) of the professional and technical efforts of the Cardiology Branch are devoted to clinical activities which are not directly related to the direct aims of its research program. These nonresearch activities include: (1) Recording, mounting and interpretation of all of the electrocardiograms for the Clinical Center. Approximately 6,000 tracings were handled in 1962. In addition, a course in electrocardiographic interpretation was given and personal instruction in ECG interpretation was provided to Clinical Associates from other laboratories of NHI and from other institutes. (2) Clinical cardiology consultations for the Clinical Center. (3) Cardiology consultations to the Clinic of Surgery, NHI. (4) An average of 3 post-operative cardiac catheterizations weekly, carried out for the Clinic of Surgery. (5) Consultations in pulmonary physiology, and performance of pulmonary function tests for the Clinical Center.

Section on Clinical Biophysics

A. Cardiovascular Activities

The immediate objectives of the Section on Clinical Biophysics have been to learn as much as possible about the variables controlling the mechanical behavior of the various components of the cardiovascular system. The ultimate objective is synthesis of this information into an integrated picture of overall cardiovascular system behavior with emphasis on the interaction of its components. This implies the necessity of clearly identifying unique sets of controlling variables experimentally so that appropriate systems of mathematical equations may be developed.

The experimental studies of these systems of variables may be categorized in the following 5 groups: (1) Heart, (2) Great Vessels, (3) Small Vessels, (4) Veins, and (5) Control Systems. Although certain exploratory studies under (4) and (5) have been carried out, the major activities over the past year have been concentrated in the first 3 categories:

(1) Heart: Studies designed for the preliminary identification of variables that uniquely describe myocardial mechanical behavior have been interesting and rewarding. The general approach to this problem has been to hold all parameters (e.g., temperature, metabolic milieu, etc.) constant, measure all variables (e.g., ventricular pressure, ejection rate, etc.), and then analyze the relationship between any two variables for various selected constant values of the remaining variables. If unique relationships emerge between variables taken in this way, (two at a time) one has then identified primary variables describing the system. Specifically, the following studies have been done.

An animal preparation was devised in which it was possible to study the myocardial mechanical behavior in an intact, metabolically controlled, normally beating heart by the use of a heart-lung machine. Instrumentation was developed which made possible the computation of instantaneous tension and shortening velocity or rate of strain of the muscle elements contained in a selected circumference of the myocardium at different instantaneous heart volumes or fiber lengths.

Using this methodolgy, the tension-velocity data obtained were analyzed by computing regression equations relating wall tension to rate of strain at selected constant instantaneous heart volumes. A reciprocal relationship between the wall tension and rate of shortening of the wall emerged from this analysis. It therefore appears that 3 primary variables describing myocardial mechanical behavior are (1) instantaneous fiber length, (2) instantaneous rate of shortening and (3) instantaneous wall tension. It is interesting to note that during these studies many different stroke works would occur from identical initial fiber lengths. These findings are not consistent with the concept

that Starling's law uniquely describes myocardial behavior. These findings are consistent with the concept that myocardial function is determined by intrinsic muscle laws perhaps similar to those described by A. V. Hill for skeletal muscle.

There was sufficient variance in the foregoing data to make premature any statement that all of the primary variables had been identified. In view of the indirect approach that was necessary to measure these variables, it was highly desirable to confirm the above findings by direct measurement of wall tension, shortening velocity and fiber length in the normal beating heart. Transducers for this did not exist and therefore a program of instrument development was instituted. Transducers have just been developed in this section which can measure elongating strains on the surface of the heart as well as elongating strains through the heart wall (that is the increase in wall thickness as a function of time). Transducers were also developed to measure shearing strains in the wall, that is, the angular displacement of one muscle layer with respect to the other. Finally, a force or tension measuring transducer was devised. Although physiological studies using these transducers are just now getting under way, preliminary results indicate that the heart muscle undergoes relatively small shearing strains during the ejection phase of systole. The degree of shear is somewhat greater in the axis from apex to base than circumferentially about the lesser radius. The usual magnitude is of the order of 1 degree maximum shear over the ejection period. This is considered small. The change in wall thickness during ejection phase is of the order of 5%. Circumferential and longitudinal elongating strains are of the same order of magnitude. These studies should culminate in a body of knowledge which will allow precise statements regarding the relationships among the primary variables controlling the mechanical behavior of the myocardium. Such statements open the way to mathematical generalizations regarding myocardial function.

(2) Great Vessels: From a purely physiological point of view the information obtained in the foregoing studies indicate that the response of the heart from a given end-diastolic fiber length depends on the time course of the load presented to the ventricles. This raises the question as to the nature of the load presented to the myocardium by the great vessels. Since the vascular system contains inertial, viscous, and elastic components the load presented by the great vessels to the heart will contain components of force related to these properties. Loads of this nature cannot be defined by the simple concept of peripheral vascular resistance, but must be defined with the use of hydraulic impedance functions. Hydraulic impedance functions relate the instantaneous value of pressure to flow. This is done by resolving the respective pressure and flow curve into its Fourier series and then calculating the complex ratio between each pressure harmonic and its corresponding flow harmonic. The impedance patterns in the pulmonary artery and the aorta appear qualitatively similar. The impedance magnitudes initially diminished rapidly with frequency to wax and wane in magnitude thereafter. Such patterns are consistent with the view that both systems are distributed wave transmitting systems. The waxing and waning of amplitudes is consistent with significant reflections of energy from the periphery and other vessel junctions.

Carrying these studies one step further, the instantaneous relationship between the flow and the pressure-gradient was determined. In this case the calculation of the impedance series relating the pressure gradient to flow yields the "distributed longitudinal impedance" of the system, a property independent of a wave reflection and depending only on the local properties of the blood and vessel. It follows that knowledge of these relationships should permit calculation of instataneous blood flow from more simply obtained pressure-gradient information which would be of obvious value in clinical cardiovascular dynamic studies. The distributed impedance of the major vessels was analyzed not only with respect to the behavior of the impedance magnitudes with frequency but also with respect to the behavior of the real and imaginary parts of each term. A detailed analysis of these data indicate a striking agreement with previous theoretical considerations, a finding representing a major advance in the field of circulatory dynamics.

To supplement the foregoing studies detailed measurements of the instantaneous relationship between blood vessel diameter and instantaneous lateral pressure have been carried out again using the mathematical techniques outlined above. The impedance series so calculated will represent the

"distributed shunt impedance" of an energy transmitting system. The frequency spectrum of these impedance functions indicates that the blood vessel wall acts as a viscoelastic body containing inertial components also. Similar patterns have been found from the ascending aorta down to the lower abdominal aorta. Certain mathematical relationships exist between this "shunt impedance" and the "longitudinal impedance" of a blood vessel (discussed above) which permit one to relate precisely the pressure gradient, the flow, the pressure, and the pulse wave velocity to the dimensions and physical properties of the vascular bed. Studies to permit us to take this final step in establishing the mechanical behavior of the great vessels are under way, however, without results as yet.

(3) Small Vessels: Study of the fluid dynamics of the small systemic blood vessels has been only exploratory in nature and has been confined primarily to the "waterfall effect" in the lung. The "waterfall" effect in the lung refers to the finding that blood flow through the lung for a given pulmonary artery pressure will be independent of the left auricular pressure until the left auricular pressure equals the airway pressure. A mathematical theory based on certain assumptions has been evolved to explain this phenomenon. Studies are at present being done in a physical model to establish the validity of the theory in the model and to define critical parameters that will be necessary to be measured in the living pulmonary circulatory system. The results to date are in the process of analysis; however, qualitatively it has been demonstrated that the pressure-flow relationship in the model behaves like that in the pulmonary circulatory system.

B. Studies on Pulmonary Mechanics

It has been shown previously from this section that a functional relationship exists between the transpulmonary pressure, respiratory gas flow, and the degree of lung inflation. The relationship between the maximum expiratory flow and the degree of lung inflation (or volume) is of special interest and has been termed the maximum expiratory flow volume curve (F–V curve). Over the upper part of the vital capacity the relationship between the maximum expiratory flow and the degree of inflation is effort dependent and influenced primarily by the resistance of the upper airways. Over the lower segment of the curve, which has been termed the a F–V curve, the flow is not dependent on either maximum expiratory effort or on upper airway resistance. Theoretically this part of the curve depends upon the resting dimensions and physical properties of the lung and intrathoracic airways as well as the physical properties of the alveolar gas. Any acceptable mathematical model of the lung must be able to predict the changes in the a F–V curve that would be brought about by specified changes in the physical properties of the alveolar gas. Therefore, studies were designed in which the physical properties of the alveolar expired gas could be measured. Various different mixtures of gas were given so that wide extremes of both viscosity and density were achieved. It was found that the maximum flow that can be achieved over the lower part of the vital capacity is significantly more dependent on gas viscosity than on density. The converse was true for the peak expiratory flow which can be achieved over the upper part of the vital capacity. Thus, peak flow is relatively independent of viscosity but depends heavily on density. Certain problems related to computer technology have arisen that have prevented further progress on the detailed "fitting" of the a F–V curve to various mathematical models. Nevertheless, it has been possible to study certain limiting conditions predicted by some of these mathematical models. One of the models predicts that the maximum slope of the a F–V curve will vary inversely with the cube root of the density of the gas breathed and be relatively independent of the gas viscosity. "Blind" studies were done in which various observers were requested to measure the maximum slope on the a F–V curves. The measurements from each curve were then averaged. The average slope measurement from each of these curves was found to vary closely with the reciprocal of the cube root of the density. If a valid mathematical model can be developed, the way is open for applying computer analysis to mass surveys using these simply obtained a F–V curves. The potential importance of this to public health and air pollution matters is obvious.

CLINIC OF SURGERY

The investigative projects of the Surgery Branch have, as in past years, centered largely around development of new or improved methods for the surgical treatment of patients with congenital or acquired heart disease. In general, these projects which have been carried out at both

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the experimental and clinical levels, have been designed to elucidate the physiologic changes which occur as the result of various malformations and the alterations which accompany complete (or incomplete) surgical correction. The results of appropriate work in the experimental laboratory are applied in the clinical program where opportunity is taken to make physiologic observations before, in the course of, and following cardiac operations.

Within the past year, an increasingly large proportion of patients operated upon have been those with acquired rather than congenital heart disease. This difference in patient material probably reflects the development of satisfactory operations for the correction of acquired stenotic and regurgitant lesions of the mitral and aortic valves. A recent analysis was made of the operative results in 50 patients with acquired calcific aortic stenosis who were subjected to detailed hemodynamic studies both before and after operation. The results of the study indicated that debridement of the calcific valve followed by commissurotomy is the surgical procedure of choice in those patients in whom it is applicable. On the other hand, partial or total replacement of the aortic valve was found to be necessary in a significant proportion of all patients with this disease.

Total prosthetic replacement of the aortic valve has been carried out in nearly 50 patients with congenital or acquired valve disease. A tricuspid valve made of Teflon has been most often employed and during short periods of followup has been shown to afford complete relief of outflow obstruction and to be entirely competent. At present valves constructed of plain Teflon fabric and fabric which has been coated with a Teflon dispersion are being used in patients to determine if coating will delay or prevent stiffening of the leaflets.

In patients with predominant aortic stenosis before operation we have observed large pressure gradients between the left ventricle and aorta in the early postoperative period. It is unlikely that stiffening of the prosthetic valve can occur within a few weeks and it seems likely that patients demonstrating this phenomenon may have hypertrophic subaortic stenosis secondary to massive hypertrophy of the left ventricle caused by the valvular lesion. A physiologic assessment of this lesion may provide us with further information about the hypertrophic form of subaortic stenosis which is, of course, also seen as an isolated lesion. The operative treatment of the primary form of hypertrophic stenosis has been improved within the last year and in the last three patients operated upon large masses of the enlarged muscle have been removed by means of a new and specially designed instrument and immediate correction of the hemodynamic abnormality has been achieved. This is in contrast to the earlier patients in whom a less radical procedure was carried out and hemodynamic improvement appeared to occur gradually.

In virtually all patients with calcific aortic stenosis the valve is immobile and the fixed orifice of the valve, in addition to being stenotic, permits a greater or lesser degree of aortic regurgitation. This clinical observation gave rise to speculation concerning the magnitude of regurgitation through various fixed orifices and suggested an experimental study in which regurgitant flow through the aortic valve was directly measured. Regurgitation was acutely induced in dogs by means of special cannulae which permitted a fixed regurgitant orifice to be created and closed at will. Retrograde flow, as well as the various components of forward flow, were measured with an electromagnetic flowmeter in the aorta and femoral artery. It was found that the area of the regurgitant orifice bore an almost linear relationship to the volume of regurgitant flow and that the changes in the pattern of flow in the aorta were accurately reflected in the flow pattern recorded in the femoral artery. The observations have been extended in 25 patients in whom the femoral flow pattern was determined directly at the time of operation. In patients without aortic valve disease there was no retrograde flow in the femoral artery at any time. In all patients with clinical evidence of aortic regurgitation, however, retrograde flow could be measured during diastole and its magnitude correlated well with the area of the orifice through the incompetent valve which was measured at subsequent operation.

A clinical and hemodynamic analysis was made of the results of closed mitral commissurotomy in an unselected group of 35 patients subjected to operation. The status of the valve was assessed by left heart catheterization before and one year after operation. The results of the study indicated that the principal factor which determined

the extent to which closed mitral commissurotomy restored normal hemodynamics was the anatomic status of the valve encountered at operation. When a flexible, mobile and noncalcified valve was present, virtually all patients received dramatic hemodynamic benefit and quite often entirely normal valve function could be restored. When, however, immobility of the posterior leaflet or both leaflets was present closed operations afforded little or no benefit in the majority of the patients. The various clinical factors indicating preoperatively that such an unfavorable valve might be present were also determined and the principal one was radiographic evidence of calcification of the valve. These findings have strengthened our impression that all predominantly regurgitant lesions of the mitral valve must be treated by valve replacement and, at this time, it is also felt that the calcified stenotic valve can be corrected only by this means. Within the past year 15 patients with regurgitant or stenotic lesions of the mitral valve have been operated upon and the mitral valve replaced with the ball prosthesis devised by Starr. Thirteen of the fifteen patients have survived operation and all have shown striking clinical benefit. A number of them have also been subjected to detailed postoperative hemodynamic assessment and, although they are found to have normal left atrial pressure at rest, the prosthetic valve which has been employed has been shown to be stenotic. The small gradient evident across the prosthetic Starr valve may be responsible for the occurrence of embolization in patients who are not given anticoagulants and the hemodynamic data will give impetus to a modification of the design of the valve which will permit it to have a larger effective orifice.

A significant proportion of the work in the experimental laboratory has centered around the problems of materials for prosthetic heart valves. Evidence has been collected, from the use of prosthetic materials in vascular grafts, that the size of the pores in the prosthetic material may determine the degree of fibrous tissue ingrowth and consequently the stiffening of such materials. Since stiffening must be avoided in prosthetic valves, the relationship of pore size to tissue ingrowth is under investigation in dogs. A new fabric composed of alternating fibers of collagen and dacron is used to replace part of the heart wall or a portion of a mitral or tricuspid valve leaflet. This fabric, when implanted, has a small pore size but as the collagen is absorbed a large pore size results. Applications of such combined natural and prosthetic materials for the use in heart valves will undoubtedly be forthcoming.

Any material used for a prosthetic valve is subjected to stress, and fracture of leaflets due to flexing is a problem which has been encountered clinically. A plastic known as polypropylene is known to have an extremely long flex life but little information has been available concerning its biologic characteristics or its suitability for the use in heart valves. This material is being woven into suitable fabrics which are being tested as to their promotion of blood clotting, foreign body reaction, and possible toxic manifestations. The surface of a prosthetic material used for heart valves is also of importance and this is being investigated by the implantation, in animals, of dacron sheets which have been coated with other polymers so that some of them have slick (closed cell) surfaces and others porous (open cell) surfaces. The materials are being tested by implanting triangular pieces of them in the outflow tract of the right ventricle and, in addition, a simulated valve leaflet is implanted so that it lies within the ventricle. The experiments should give information concerning not only the desirable surface characteristics of the fabrics but also the behavior of prosthetic leaflets made of each type.

At the present time, the implantation of an artificial valve, either mitral, aortic, or tricuspid in a patient is a long operative procedure and much of the time is necessitated by the placing and tying of sutures to anchor the valve. In previous reports a plastic adhesive has been described which has been found useful as a hemostatic agent. Work is continuing with this material concerning its reactions when implanted in tissue and, in collaboration with a plastics chemist, attempts are being made to improve the chemical formulation of the adhesive so that it, or a similar compound, may be used to cement artificial heart valves in place and/or to close intracardiac openings without the use of suture material. Within the heart this technique would, of course, obviate many of the present dangers in regard to complete heart-block caused by placement of sutures near the bundle of His.

The vast majority of operations carried out for the correction of heart disease now require the use

of extracorporeal circulation and several clinical and experimental projects have centered around this technique. In previously described studies it was found that animals subjected to extracorporeal circulation showed a significant loss of digitalis from the myocardium after 30 minutes of bypass. During the past year these observations were extended to the study of patients and similar conclusions were reached. Patients were given tritium labeled digoxin before operation and the radioactivity of myocardial biopsies was determined before and after bypass. An average decrease of myocardial radioactivity of 15% was observed while the radioactivity of the blood of the patient and heart-lung machine increased more than four times after the procedure. The loss of digoxin from the heart was not related to the duration of the bypass and it seems likely that the loss is accounted for by an acceleration in the normal metabolism of the compound.

At the National Heart Institute, as in most centers, homologous blood is used as the priming substance in the artificial heart and lung machine. Several undesirable sequelae have been noted after bypass such as fever electrolyte derangement, hypotension, etc. Work in other laboratories has indicated that these changes may be due to the "homologous blood syndrome." This is being evaluated experimetnally and an attempt is being made to isolate the component in homologous dog blood which is responsible for the shock-like picture which often accompanies animal perfusion. The age of donor blood, its electrolyte content, and the relative effects of plasma and red cells in producing the syndrome are being studied. Preliminary results indicate that the "toxic" factor is probably in the serum and that when washed red cells are used in the priming solution many of the undesirable accompaniments of bypass can be avoided.

Many patients with intracardiac communications have secondary pulmonary hypertension and, in the past, many of these patients were excluded as operative candidates because of the high risk attendant upon closure of the defect in this situation. In such patients the circulatory shunt is usually a bidirectional one and in periods of stress or heart failure the right heart is able to decompress itself through the intracardiac communication. Abrupt closure of the defect may abolish this safety valve mechanism and lead to acute heart failure in the postoperative period. An extensive experimental study has been carried out concerning the use of perforated patches of prosthetic material which will allow gradual closure of intracardiac communications. In dogs it was found that perforations of less than 7 mm. in diameter in Ivalon closed within 10 days, while closure of 8 mm. openings did not occur for many weeks. This technique has been applied in four patients with atrial septal defects, extreme pulmonary hypertension, and bidirectional shunts. In each of them perforated prostheses were used to close the defect and serial hemodynamic studies were carried out postoperatively. It was found that 6 mm. perforations closed within 10 days but 8 mm. perforations remained open. All the patients survived and it is felt that the decompression allowed by the perforated prosthesis was the determining factor in the success of the procedures.

Clinical assessment was also made of the changes in the pulmonary artery pressure and pulmonary vascular resistance in 29 patients who had pulmonary hypertension secondary to intra-or extracardiac communication. All of the patients were studied by means of cardiac catherization before and at intervals after operation. All had mean pulmonary artery pressure greater than 50 mm. Hg preoperatively. The pulmonary artery pessue fell significantly in all patients with extracardiac shunts and the pulmonary resistance was normal in all but one of these patients. In 17 patients with intracardiac shunts, however, the pulmonary artery pressure fell far less and elevation of both pulmonary artery pressure and pulmonary vascular resistance persisted postoperatively in virtually all of them.

Complete heart-block is an unfortunate complication of the operative treatment of various intracardiac malformations, particularly ventricular septal defect and the various forms of persistent A-V canal. Although appropriate electrical devices for maintaining the ventricular rate in such patients are now available, the chief limitation to their use has been electrode breakage. In cooperation with the Instrument Fabrication Section a device has been developed for testing various types of wire electrodes by flexing them extremely rapidly and for long periods of time. Initial observations indicate that a greatly improved myocardial electrode may be constructed from Elgiloy, an alloy used commercially for watch springs. Other

studies on complete heart-block and its physiologic manifestations have concerned the reaction of animals to shock when their heart rate has been controlled. In a normal animal, when shock has been induced by bleeding, the heart rate rises strikingly and after the shock-state has been maintained for some period of time, the reinfusion of blood is ineffective. It has been found that if the heart rate of the dog is controlled during the shock-state, by means of induced block and an electrical pacemaker, the recovery from prolonged shock is more rapid and normal levels of blood pressure and cardiac output may usually be achieved afterward.

Cardiac arrest is an all too frequent complication of the operative treatment of patients with heart disease and frequently occurs during the induction of anesthesia. To investigate the mechanism of cardiac arrest, which is usually attributable to hypoxia, the systemic and cardiac circulation were separated in animals by means of two separate extracorporeal circulations. When normal oxygenation of the heart was maintained and the body was rendered hypoxic, striking slowing of the heart rate was observed and cardiac arrest occurred in several animals. This study indicated that while the hypoxic heart is susceptible to arrest, the major cause of arrest is neurogenic stimuli which originate peripherally. The technique of separating the peripheral and cardiac circulations has made possible other investigations. When the temperature and oxygenation of the heart were kept normal and the body was cooled to 15° C. no significant changes in either the heart rate or its strength of contraction occurred. Only when the heart itself was cooled, and regardless of the general body temperature, were the usual myocardial changes which accompany hypothermia observed. The mechanism of action of angiotensin was also studied with this preparation. When the drug is given to an intact animal, striking elevations in blood pressure and contractile force occur. In the double perfusion system the effects of angiotensin on the heart and systemic circulation were separated. When the drug was given only to the heart no changes in blood pressure or heart rate occurred and there was actually a slight negative inotropic effect. When it was given into the peripheral circulation, however, a marked rise in blood pressure occurred immediately and later increases in pressure, heart rate, and contractile force were observed. These studies would indicate that angiotensin has no direct effect on the heart and that the increases in pressure and force which result from its administration occur as the result of peripheral sympathetic stimulation of the heart.

Several projects, both clinical and experimental, have been carried out by the Anesthesia Research Unit, newly established in collaboration with the Department of Anesthesia, Clinical Center. The absolute and relative refractory periods of the heart and the diastolic electrical threshold of the heart have often been determined in experimental animals but never in man. In 20 patients, undergoing cardiac operations, a stimulus-interval curve has been determined. Of greater importance have been studies of various drugs which can alter the stimulus-interval curve and the threshold of the heart to stimulation. It has been found, for example, that xylocaine has a striking effect in elevating the electrical threshold of the heart without deleterious changes in contractile force or blood pressure. This is in contrast to the reactions observed with procaine amide, the drug most often utilized for the treatment of ventricular arrhythmia. Procaine has always been noted to cause a fall in blood pressure and contractile force and an actual decrease in the electrical threshold of the heart. These observations, combined with clinical observations in postoperative patients, indicate that xylocaine is the drug of choice in the treatment of ventricular arrhythmia and furnishes the physiologic background for its mechanism of action. Further studies concern the effects of various anesthetic agents on the total volume of the venous system and the tone of the peripheral as well as the central veins of the body. These studies, when concluded, should provide information concerning some physiologic sequelae of the administration of various anesthetic agents.

GERONTOLOGY BRANCH

The research program of the Gerontology Branch is directed toward (1) identifying the biochemical, physiological and psychological changes that take place with increasing age in man, and (2) investigating the basic biological changes that influence aging in lower organisms in order to understand age-dependent alterations in the performance of humans. For the descriptive studies on age changes in the performance of humans, the

Branch has recruited a group of 500 males between the ages of 20 and 102 years who have volunteered to spend two days at the Baltimore City hospitals every 18 months to participate in a broad program which includes physiological and psychological tests as well as detailed medical examinations. These subjects live in their own homes in the community and represent a highly educated and economically successful group. It is of great importance to determine the effects of age on the health and performance of subjects of this type in view of their importance to our society. In addition, similar tests are conducted on older subjects who are admitted for custodial care in an Old People's Home. In connection with these studies, as well as those concerned with disease processes in the elderly, the Gerontology Branch staffs and operates a 40-bed ward at the Baltimore City hospitals.

The program on the basic biology of aging seeks answers to questions such as: What changes occur in the enzyme activities of cells with the passage of time? What is the biochemical basis of the increased life span of underfed animals? What are the mechanisms of cell death? Do cells lose their capacity to divide with increased differentiation and the passage of time—or is the reduction in cell division in the adult simply a reflection of alterations in the cellular environment? Is aging related to "rate of living"? What are the genetic factors influencing species differences in longevity? Can aging effects be due to alterations in genetic information? Are somatic mutations an important factor in aging? Do molecules which are important in biological processes undergo age changes? Is there formation of cross linkages in proteins with aging in cells? Investigations on these questions involve the use of a wide range of animal species such as the rat, mouse, hamster, *Drosophila*, rotifer, *Campanularia*, *Euglena*, etc., as well as cells growing and developing in tissue culture. In many instances it is necessary to extend knowledge of basic biochemical processes before age differences can be investigated and interpreted. Hence, part of the program of the Branch is devoted to basic biological problems such as studies on the mechanisms of energy transformations in living cells and the structural characterisitcs of biologically important compounds involved in these energy transformations.

Aging in the Human

The studies of age changes in physiological and psychological characteristics of a population of 500 males, aged 20–102 years, who are highly educated, successful and live in their own homes, have been extended by the completion of the second and third series of tests at 18-month intervals on part of the sample. A program for the transfer of observational data to punch cards and magnetic tape for electronic computer analysis has been worked out and the medical history and physical examinations have been coded in preparation for punching. This system has been designed with maximum flexibility to permit a wide variety of subsequent analyses.

Comparisons of Institutional and Community Residing Subjects

Although insufficient time has elapsed since the beginning of the study to assess age changes in individual subjects, it has been possible to compare average values in the successful community residing group with those obtained on similar tests in a population residing in an institution for the aged, carefully selected to exclude subjects with clinical evidence of disease. In many performance tests the average values obtained among community residing subjects are significantly higher than those obtained for the institutional residing group. Examples of such performances include maximum breathing capacity, maximum work output of manual cranking and the ability to maintain a specified low level rate of manual work. In some of these functions, such as maximum breathing capacity, the average age decrement is significantly less in the community residing subjects than those residing in an institution.

In contrast, a number of the physiological processes on which performance is based show no significant differences between the two groups in absolute values or age trends. Examples of such measurements include the strength of specific muscle groups, the total capacity of the lungs after adjustment for differences in body size, and the basal oxygen uptake based on the amount of metabolizing tissues as estimated by intracellular water.

These results lead to the conclusion that aging subjects living successfully in the community utilize their failing physiological capacities more ef-

fectively than do subjects who have resorted to congregate care. Thus, our conceptions about age changes in performance cannot be based on observations made on institutionalized subjects alone. These results also emphasize the importance of sampling problems in drawing general conclusions about age changes in human performance.

Physiological Bases of Behavior

Relationships between physiological and some behavioral characteristics were investigated in the community residing subjects. It was found that the variability of reaction time to an auditory signal increased significantly with age. This correlation between variability and age became insignificant when brain wave frequency was held constant by the use of partial correlation. It appears that brain wave frequency is the central nervous system factor concerned with age associated increases in variability as well as the mean value for response time.

Using heart rate and amplitude of spinal reflex recorded during performance of the simple reaction time task as measures of arousal or activity level of the brain stem reticular system, two hypotheses were investigated, viz, (1) that age is negatively correlated with level of arousal, and (2) that arousal and reaction time are related according to a U-shaped function. Neither hypothesis was substantiated by the data.

Evidence that older individuals are less able to maintain attention in a repetitive and monotonous task than younger people has come to light in an investigation of watchkeeping behavior. In this study the subject is required to detect certain irregularities in the movement of a clock hand which occur only infrequently in the course of the task which lasts 1 hour. While the older subjects performed as well as the younger ones in the first 15 minutes of the task, they failed to detect more of the irregular movements in the last 15 minutes of the test than the young did.

Age Changes in Psychological Performance

Previous experiments have shown that old subjects learn less effectively than young in an experimental situation, but that when more time was given to select and make the response, the old subjects improved more than the young. Another experiment has been performed in which subjects were paired on the basis of age and vocabulary scores. One member of each pair learned under conditions in which he could control the time taken to select and make each response; the other member learned at the pace set by his partner. Under these conditions, pairs of young subjects showed no significant differences in learning performance, but in older subjects the self-paced member performed better than his partner. These observations suggest that learning in older subjects is greatly favored where the individual can set his own pace.

The Army Alpha Test of intelligence has been administered to subjects in the community residing group. For young men aged 20–50 years the average age test score was 171 when the test was performed within a set time and 187 when the test was completed without time restriction. For older men aged 60–80 years the average score for timed performance was 151 and for completion without time limit was 181. Most older subjects in the sample have shown equivalent performance with their younger counterparts when time limits were removed although they perform less well in the time limited situation.

Dietary Intakes

Complete records of dietary intakes for seven consecutive days have been kept by subjects from the community residing group. These data will be used to identify age related differences in dietary intakes and patterns of food selection in human subjects and to correlate differences with changes in anthropometric, physiological and biochemical measurements. A preliminary analysis of 7-day diet records submitted by 31 subjects has been made with respect to water, fiber, calories, protein, fat (saturated fatty acids, oleic and linoleic acids) carbohydrate, ascorbic acid, Ca, Fe, vitamin A, thiamine, niacin, and riboflavin. The data suggest that caloric intake and the percent of fat in the diet decline with age. This study will provide information, not now available, on the spontaneous eating habits of normal individuals where economic considerations are not a primary factor in determining foods eaten.

Renal Physiology

In addition to studies on overall performances in humans, other investigations have been focused on age changes in specific organ systems and

physiological processes. Further evaluation of data obtained on community residing subjects during a 16-hour period of dehydration in the hospital indicates that the young subjects (age 20–39 years) show the expected diurnal decrease in urine flow, total solute excretion and the increase in urine osmolality during the hours of sleep (12 midnight to 6 a.m.) when compared to the waking hours (6 p.m. to midnight; 6 to 10 a.m.). The oldest subjects (age 70–100 years), carefully selected to exclude those with clinical signs of even mild edema or heart disease, showed a reversal of this pattern. Subjects of intermediate age (age 40–69 years) showed a gradual shift in their urine excretion pattern toward that of the oldest subjects.

The incidence of renal failure following surgical procedures increases markedly with age. To assess the mechanism of this renal failure, clearances of inulin and paraaminohippurate (PAH) have been performed in a control preoperative period and 5 hours after operation in 36 patients over 50 years of age. Operations were of major types but not limited to one anatomic area. Contrary to previous reports based primarily on observations made on young subjects, significant changes in glomerular filtration rate (Cl_I) and renal blood flow (Cl_{PAH}) were noted in the aged subjects. These findings throw new light on the relationship of both operative site and magnitude of the post-operative renal impairment. Results to date indicate: (1) following five small intraabdominal operations, glomerular filtration rate increased slightly but plasma flow remained unchanged. Thus filtrate fraction rose. (2) Five thoracic operations (lobectomies and pneumonectomies) produced about the same changes in renal function. (3) Following extensive intraabdominal operations in 15 patients glomerular filtration and plasma flow decreased proportionally so that filtration fractions were not significantly changed. (4) In four patients with abdominal aneurysim resection there was a mean decrease in glomerular filtration rate and plasma flow postoperatively. These changes were not significantly different from the changes in group 3 (extensive intraabdominal operations). Thus the nature of the changes in renal function appear to be related to the site and extent of the operative procedure.

Endocrinology

Recent observations show that acute febrile illness causes a rapidly reversible but marked increase in thyroxine degradation rate without altering the concentration of circulating thyroxine. This finding is most readily interpreted as indicating a homeostatic increase in thyroidal release of thyroxine in order to maintain a constant plasma level of the hormone. These measurements provide strong evidence that the thyroid, like the adrenal, participates rapidly in the response to at least some types of stress. Although only a few observations have thus far been made, elderly subjects seem to retain this homeostatic mechanism intact. If so, the slowed degradation seen in normal aged persons is not a fixed metabolic change and argues against an earlier proposal that the rate limiting step in thyroxine turnover is in the enzymatic degradation of the thyroid hormone.

Work has progressed on development of a method which may permit estimation of angiotension production rate in various states where the renin-angiotension-aldosterone mechanism is operative (unilateral renal ischemia with hypertension; secondary aldosteronism). The approach is to measure the renal excretion of a dipeptide which is formed in the process of *in vivo* angiotensin activation. A method for measuring the peptide in urine is near completion and will soon be applied to the problem at hand. Incidental information on the heretofore unexplored area of the renal handling of certain peptides will become available in the course of this work. If successful, the approach should have general applicability in estimations of peptide hormone production rates.

Carbohydrate Metabolism

Previous experiments have shown that carbohydrate metabolism is impaired with increasing age. This is shown by the delayed disappearance of excess glucose from the blood after the administration of either an oral or an intravenous glucose load. Furthermore, the effects of cortisone on the rate of glucose disposition is greater in aged than in younger subjects. The impairment with age has also been demonstrated by the delayed rate of fall of the blood glucose concentration after intravenous tolbutamide administration. These

tests are known to be stimuli to the release of endogenous insulin. Studies have therefore been started to test whether the age changes might be due to deficient insulin output by the pancreas in response to these stress tests or to deficient sensitivity of the tissues to insulin. Preliminary results in a group of aged subjects suggest that their peripheral tissues are indeed relatively insensitive to physiological concentrations of insulin, as tested by the forearm intra-arterial insulin tolerance technique. Yet the responses to intravenous insulin tolerance tests (0.05 units/Kg. body weight) in these same subjects were generally normal. These apparently discordant results suggest that the hepatic sensitivity to insulin might be retained with age while skeletal muscle sensitivity decreases. It is also possible that the concentration of insulin achieved in plasma in the intravenous test is so large that subtle age differences cannot be detected. Tests will therefore be repeated with smaller doses.

These "impairments" in carbohydrate metabolism with age raise challenging questions with regard to the diagnosis of diabetes mellitus. Ultimately this diagnosis rests upon the finding of blood glucose concentrations which exceed certain standards under specified experimental conditions. These standards have generally been defined by studies on young subjects. If these commonly accepted standards are applied to older subjects an unacceptably high percentage of them (generally more than 50%) would have to be classified as diabetic. Review of published reports reveals that the common solution to this problem is to raise the upper limit of normal to some level (completely arbitrarily) to achieve a more acceptable percentage of abnormal results. The issue then is whether (1) standards for these tests need to be established with age as a variable or (2) diabetes is in reality an extremely common concomitant of aging. Studies have therefore been designed on the longitudinal subjects to characterize the commonly used tests for the diagnosis of diabetes. This group unquestionably will represent the best control series available since, in other studies on aged subjects, the subjects, although generally "screened for the presence of metabolic diseases," are in reality chronically hospitalized or institutionalized individuals. As the data accumulate on the longitudinal subjects, correlation between their metabolic tests and certain diseases recognized as being concomitant or complications of the diabetic state will be examined. These analyses should provide an answer to the question of whether the high blood glucose concentrations represent a physiological alteration with age or a true disease state.

Biology of Aging

Behavioral Changes With Age

The purpose of this program is to determine the effects of age on various types of specific behavior in the rat. The rat is a useful animal for these studies because of its use in many psychological studies. Furthermore, the rat engages in a variety of activities for which quantitative indices can be devised. For example, exploratory behavior can be observed in the rat and can be distinguished from gross activity. The rat also engages in manipulative behavior which can be recorded quantitatively. Other variables, such as light vs. dark, availability of food, the presence of other animals and the effects of previous experiences, can be experimentally varied.

Preliminary experiments have shown that exploratory behavior can be reliably measured. Tests have included barriers, multiple-unit interconnected "open fields" (enclosed spaces in which the animal is placed and observed), "open fields" with and without hiding areas and small area "open field" tests of long duration. Animals were observed systematically at specific time intervals and behavior such as sniffing, grooming, or lying recorded during a short time interval. The effects of dietary restriction on animals of different ages have been studied. Exploratory responses (sniffing) increased with food deprivation while non-exploratory responses (lying or grooming) decreased. Preliminary experiments indicate that age has an influence on exploratory behavior. In senescent animals, exploratory behavior decreases with increasing trials (experience) whereas it increases in young animals. Although exploratory behavior is reduced in old animals, food deprivation results in a greater increase in exploratory behavior in old than in young rats. Other factors such as experience and conditions of rearing (such as single or multiple caging) also play a role in exploratory behavior. Future experiments will attempt to determine the relative importance of these variables and their interactions.

Effects of Nutrition and Parental Age on Longevity

Restriction of food intake, beginning early in life is one of the few conditions which is known to increase life span in the rat. The restricted animals grow very slowly and for a longer time than normal rats. Hence it has been assumed that the increased longevity resulted from a reduction in growth rate and that the retarded animals were physiologically younger than the normally *ad libitum* fed controls. Since the mechanisms of life extension in restricted rats is unknown, a research program has been instituted to try and define the biochemical basis of the phenomenon. In previously reported studies, the life expectancy of young rats was increased by reduced dietary intake and the concentrations of various enzymes determined at different times during the first year of life. Although there were demonstrable differences in selected enzymatic activities, for example, 35% increase in liver succinoxidase and 40% increase in kidney alkaline phosphatase, between these animals and normal ad libitum fed controls, they were not the same as those expected if the normal processes associated with growth had merely been retarded. Furthermore, during this year young growing animals were subjected to either 25, 50, or 75% reduction in food intake for 10 weeks. Measurements of the liver succinoxidase have shown that the increments in the concentrations of the enzymatic activities were related to the degree of restriction. On the other hand, although a decrease of 50 or 75% in food consumption increased the alkaline phosphatase of kidney 30%, a 25% reduction of food intake did not affect the concentration of the enzyme. Thus, it is apparent that quantitative differences in selected enzymes can be brought about by the degree of dietary restriction. This fact now makes it possible to determine whether longevity is related to the concentrations of these selected enzymes in rats subjected to dietary restrictions. More recent studies have indicated that the same changes in enzyme activities are brought about in adult animals subjected to reduced dietary intake. These data offer further evidence that the increased life span associated with dietary restriction is not the result of generalized retardation of normal processes but that dietary restriction induces changes in specific enzyme systems. On the basis of these findings, studies have been initiated to determine whether the life spans of adult (12 month) and even senescent (19 month) rats can be increased by dietary restriction. Although these studies are still in progress, data obtained thus far suggest that the life span of adults but not of senescent rats may be increased by reduced dietary intake. This indicates that age plays a role in the response to changes in experimental conditions which affect longevity in young growing animals.

Since it has been found that dietary restriction in growing male rats produces an increased concentration of renal alkaline phosphatase, the Gomori (cobalt sulfide) technique, modified to retain enzyme and approach zero-order kinetics during incubation, was applied to sections of kidney tissue in an attempt to localize the enzyme histochemically. Multiple incubation times were used. A "darkness index" was obtained by an objective, semi-quantitative evaluation of the slides, with the following results: (1) the alkaline phosphatase in restricted animals was confined almost entirely to the brush borders of the proximal convoluted tubules; (2) the alkaline phosphatase concentration within the brush border was increased in restricted animals; (3) the histochemical results correlated reasonably well with biochemical assays on the opposite whole kidneys in the same animals ($r=0.6053$); (4) glomerular alkaline phosphatase was detectable after 8′–16′ incubation times, but this was more pronounced in the *ad libitum* fed animals.

Glomerular localization suggested a blood borne phosphatase, with reduced serum levels in restricted animals. Serum levels were subsequently checked and a reduction of 20% was found in the restricted animals.

Studies on the rotifer (Philodina citrina) have repeated the earlier work of Lansing who showed that the life span of progeny selected from old mothers is markedly shorter than that of the parent generation. However, the total egg production of the short-lived rotifers was significantly lower than that of the controls. If the decreased life span had resulted from a uniform acceleration of normal physiological processes, it would be expected that short-lived rotifers would produce the same number of eggs but in a shorter length of time compared to the longer-lived controls. Thus, the data obtained on both the rat and the rotifer suggest that experimental conditions

which alter life span are associated with some basic change in, rather than a retardation or acceleration of, normal biochemical and physiological processes.

The replacement of H_2O by D_2O in the nutrient medium has been found to increase significantly the larval pupal and adult lifetimes of fruit flies at concentrations of 10 and 20% D_2O in the nutrient medium. However, both larval and adult mortality is increased at concentrations of 60% D_2O. Both development and reactions leading to aging thus appear to be slowed by moderate amounts of D_2O.

Effect of Environmental Temperature on Longevity

According to one theory, aging and life span of a species is related to the rates of metabolic processes taking place in the animal. The observation that life span in fruit flies can be extended by lowering the environmental temperature is offered as evidence for this theory. Experiments in our laboratory have shown that life span in the rotifer can also be extended significantly by lowering the environmental temperature. Thus rotifers maintained at 6° C. lived longer (50% mortality at 98 days) than those grown at 25° C. (50% mortality at 33 days).

These experiments were extended to determine the effect of age at which rotifers were transferred to a cold environment on extension of the life span. Although 33% of the rotifers transferred to 6° C. at 24 days of age survived the longest lived control (25° C) rotifer, the mean life spans of the two groups were not significantly different. However, the per cent survivors at 98 days of rotifers transferred to 6° C. at 6, 12, and 18 days of age were 55, 42, and 16% respectively. These results support data obtained on the rat that age does have an effect on the response of animals to experimental conditions which alter life span.

In addition, biochemical studies on the rotifer are in progress and indicate that it is possible to obtain by sonication a preparation from this organism which retains the activities of a variety of enzymes such as hexokinase, lactic dehydrogenase and glucose-6-phosphate dehydrogenase. Future experiments will be carried out to determine the effect of age on the activities of these enzymes in normal rotifers as well as those whose life span has been altered by either selection on the basis of parental age or exposure to cold.

Effect of Radiation on Longevity

The earlier observation that X-irradiation prolonged the lifetime of fruit flies has been shown to be due to sterilization and preservation of the food supply. If the medium is replaced frequently the control (non-irradiated) flies live much longer and X-irradiation has only a life-shortening effect. Under these conditions 2,000 r is approximately equivalent to one day of aging.

Aging in Cnidaria (Coelenterata)

Representative organisms from four classes of the phylum Cnidaria (Coelenterata) have now been studied to determine the time dependence of death or regression. Both *Campanularia* and *Bougainvillia* exhibit a Gompertzian mortality curve after the first few days of development while a related colonial hydrazoan, *Clytia*, shows a mortality rate independent of age. Of a group of 45 *Cyanea capillata*, an Anthazoan, no individuals were found to die in the 6-month period of observation. The wide variations in rate and mode of senescence in this related group of organisms recommends it for biochemical comparisons to reveal possible determinants of aging.

Age Pigment

While the nature and origin of the pigmented components of cardiac age pigment remain undefined, further studies of the isolated pigment indicate some similarities as well as differences from melanin and autoxidized lipid, two materials commonly believed to be components of lipofuscin. The spectral distribution of the fluorescence of the extractable fluorescent components of age pigment, the R_f values on thin layer chromatography, as well as the insensitivity of the fluorescence to pH changes, clearly differentiates age pigment from the *in vitro* oxidation products of cephalin and fatty acids, or even the *in vitro* oxidation products of the extracted age pigment lipids themselves. On the other hand, the oxidation of unsaturated fatty acid esters in an ammonia atmosphere and the alkaline condensation of diacetyl, a suspected intermediate in fatty acid oxidation, have been found to yield a variety of fluorescent products, some of which seem similar to those of age pigment.

The elemental analysis of the hydrolysis-resistant polymeric fraction of cardiac lipofuscin indicates a nitrogen and sulfur content much too high for a simple autoxidized lipid. The nitrogen content is lower and the hydrogen content much higher, however, than that of natural melanins. The elemental composition is remarkably similar to that found by Moore and Wang for a hydrolysis-resistant pigment in vitamin E deficient rat muscle lending strength to the position that age pigment formation may involve lipid autoxidation.

Analyses of the cardiac age pigment samples consistently indicate a distinctive amino acid composition similar to that of some integument proteins although the high hydroxyproline content of collagen is lacking. Sonically isolated liver pigment shows considerable differences although glycine and proline are also high. Both analyses are entirely different from that reported by Seibert, et al. for cardiac lipofuscin isolated by a different method. We hope to resolve this discrepancy in the near future through exchange of samples.

Electron micrography has confirmed that the isolated pigment particles are morphologically similar to the *in situ* pigment. There is a strong resemblance to particles described as liver lysosomes by Essner and Novikoff. Although somewhat less heterogeneous in appearance, the age pigment shows a similar osmophilia and absence of mitochondrial structural elements. A small contamination with mitochondrial fragments observed by electron microscopy may explain the cytochrome oxidase activity of the pigment preparations.

Basic Biology

In many instances investigations of the role of cellular processes in longevity and aging are hampered because of inadequate knowledge about the mechanisms involved. Hence, part of the research program of the Gerontology Branch is devoted to basic research in cellular biochemistry.

Cell Division

In many tissues, aging is associated with a gradual loss of functioning cells. This is especially true in tissues such as muscle and the nervous system where cell division is absent or minimal in the adult. Consequently, studies on the control of cell division in lower organisms may be of importance in the study of aging.

The ability to induce synchronized cell division and growth in unicellular organisms by alternating warm and cold temperature cycles has provided cells of uniform age. This homogeneity has made it possible to study certain factors regulating cell metabolism as well as the effects of antibiotics, sterols, and starvation on growth, cell division, and cell metabolism.

In *Astasia longa* the TCA-soluble "metabolic pool" formed at the end of the warm and throughout the cold period. Addition of 8-azaguanine during "pool" formation inhibits synchrony; addition after "pool" formation permits one synchronized cycle only. Thus 8-azaguanine inhibits substances which are synthesized during "pool" formation and which are necessary for normal mitosis, despite the low incorporation of this nucleic acid base analogue into cells.

Cell division can be inhibited in *Euglena gracilis* by removing the source of either carbon or nitrogen from the nutrient medium. Thus, stable colonies with relatively constant cell number can be obtained which continue to live and metabolize for extended periods of time. A model of a metazoan can be obtained experimentally which permits the study of the effects of time on metabolic processes.

A number of studies on metabolic processes in these stabilized (starved) colonies have been carried out. For example, the addition of actinomycin D to the culture medium (with refeeding) does not inhibit the lag period of growth, or protein or DNA synthesis, and has only a small effect on RNA synthesis. Growth, however, is reduced and, although protein and DNA accumulate, RNA does not. Thus, despite starvation and the antibiotic, the ribosomes evidently remain "programmed". The effect of the antibiotic on RNA is not seen unless the culture is potentially capable of active division.

Although acetate is considered to be the best carbon source for flagellate protozoa at neutral pH, both *Euglena* and *Astasia* reach population densities 10–15 times higher when grown on ethanol than on acetate.

During the period of exponential growth of *Euglena*, protein, RNA, and dry weight decline. Thus the often accepted, though rarely tested, con-

cept that in this phase of growth cells are in a uniform state, must be revised.

Oxidative Phosphorylation

The ATP-dependent reduction of nicotinamide adenine dinucleotide (NAD or DPN) by succinate discovered in submitorchondrial particles has been examined in detail in continuation of the general aims of the project. The inhibition of the reaction by uncoupling agents including 2,4-dinitrophenol and by electron transport inhibitors (Amytal and malonate) leaves little doubt that the system represents the reversal of oxidative phosphorylation in the segment between NADH and cytochrome b. The assay offers a significant advantage over the previous systems based on incorporation of phosphate into ATP since kinetic measurements can be readily carried out by following the reduction of NAD spectrophotometrically.

Since this system involves the succinic dehydrogenase and the succinic-coenzyme (CoQ) reductase in addition to the DPNH–CoQ reductase, and only the latter should be theoretically necessary for the phosphorylation, attempts were made to simplify the system further. We now have what is perhaps the simplest and most rapid assay for oxidative phosphorylation reactions. It consists of measuring NAD reduction by ascorbate in the presence of menadione (vitamin K_3) or CoQ and ATP. The menadione is reduced nonenzymically by ascorbate and resulting hydroquinone provides the reducing equivalents. ATP provides the "high energy" intermediates (via the coupling reactions of oxidative phosphorylation) that make the reaction thermodynamically possible. The system may be represented probably as follows:

$$\text{Ascorbate} + \text{quinone} \rightleftarrows \text{dehydro-ascorbate} + \text{hydroquinone} \quad (1)$$

$$\text{ATP} + \text{NAD} \rightleftarrows \sim\text{NAD} + \text{ADP} + \text{Pi} \quad (2)$$

$$\sim\text{NAD} + \text{hydroquinone} \rightleftarrows \text{NADH}_2 + \text{quinone} \quad (3)$$

Attempts at resolving the system into simpler components have been encouraging. A soluble enzyme has been extracted which is essential for the ATP-dependent endergonic reduction of NAD by succinate or ascorbate and menadione. Since the particles, without the soluble enzyme, carry out the electron transfer in the thermodynamically favorable direction (viz, the oxidation of NADH by fumarate or quinone or reaction 3 from right to left) it is highly probable that the soluble factor represents the enzyme that catalyzes reaction 2 or the synthesis, in the reverse direction, of ATP from the primary "high-energy" intermediate in oxidative phosphorylation.

Quite recently the NADH-menadione and NADH-CoQ reductases (reaction 3 from right to left) have been obtained in solution and are being purified. We thus have in solution two components of the oxidative phosphorylation system. It may be pointed out that the solubilization of DPNH-CoQ reductase transport chain has not been reported before and has proved to be the largest single difficulty in studying the mechanism of oxidative phosphorylation. Our efforts are now directed towards purifying the two components and restoring the oxidative phosphorylation system in solution using other factors if necessary.

Molecular Structure: DNA

The major effort in the field of molecular structure was devoted to the study of the various types of metal ion interaction with DNA and other polynucleotides. A large bulk of evidence has been accumulated to substantiate last year's predictions, based upon effects of the metals on the melting out of DNA, that some metal ions bind to the phosphate and others to the nucleotide bases. The importance of this dichotomy is that, whereas the phosphate-binding ions are capable of maintaining the DNA structure intact, the base-binding metals bring about its denaturation. The latter effect may have physiological implications in that an accumulation of the base-binding metals could hinder the ability of the DNA to carry out some of its intended functions.

The destabilization effect of the base-binding metals is of particular interest in the present study if the binding is base-specific, rather than random. The discovery that copper produces a bathochromic shift in the ultraviolet spectrum of polyinosinic acid, but not in the spectrum of the other polynucleotides, indicates that the binding is indeed specific for guanine in DNA. In line with this interpretation, the destabilization effect of

copper on DNA is greatest with DNA of the highest guanine-cytosine content. These experiments suggest copper ion as an important reagent for the labelling of guanine on a polynucleotide chain.

It has been demonstrated that lanthanum ion degrades all of the polyncleotides to low molecular weight end products through the scission of phosphate bonds along the chain. The use of lanthanum thus becomes another tool for the nonspecific degradation of nucleic acids. It is hoped that the degradation can be rendered specific by the use of other reagents.

Two such reagents that are specific for adenine are the nickel complex of dimethyl-sulfoxide and the copper complex of quinoline-carboxaldehyde. Methyl orange was found to react preferentially with cystosine.

An effort has been made to utilize the base contents of the DNA of different invertebrate species in order to establish their phylogenetic relationships. Data were obtained on members of the phyla Cnidaria and Platyhelminthes. The data suggested the possibility of a relation between Hydra and Platyhelminthes, and are consistent with the hypothesis that Cnidaria and Platyhelminthes have a common precursor.

Deoxyribonuclease

The effect of various metal ions upon the action of deoxyribonuclease was investigated. When the activity was plotted vs. metal ion concentration, the curves for all of the metals passed through a maximum, and the sequence of metals, listed in the order of concentrations at which they produce optimal activity, followed the sequence of metal complex stabilities. Thus our last year's generalization to explain the variability of metal enzyme activation has been confirmed in the case of deoxyribonuclease.

Age Changes in Collagen

Preliminary experiments show that solubilization techniques designed to preserve the structure of the native collagen aggregate produce maximum solubility at the young adult (12 month) level. Periodate titration studies, which measure carbohydrate crosslinking, pass through a minimum at the same level.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

BASIC RESEARCH

Introduction

Perhaps the major events in the intramural scientific life of the National Institute of Arthritis and Metabolic Diseases have been the retirement of Dr. Floyd S. Daft, who served as Director of our Institute from 1953, and the resignation of Dr. DeWitt Stetten, Jr., who has served as Scientific Director since 1954. Fortunately, Dr. Daft has been able to remain here part time and continue his work on nutrition of germ-free animals and also serve for advice and consultation. The consolation to be derived from the major loss of Dr. Stetten to our Institute is the knowledge that in his new position as Dean and founder of the Medical School of Rutgers University he can make an important contribution to medical education as well as medical research. Dr. Stetten's broad knowledge, foresight and stimulus will, however, continue as an influence in NIAMD. Further changes have been the resignation of Dr. Olaf Mickelsen from the Laboratory of Nutrition and Endocrinology and Dr. Heinz Specht from the Laboratory of Physical Biology. Their replacements are Dr. John C. Keresztesy and Dr. John Buck. The long planned Laboratory of Molecular Biology is now fully and actively functioning with Dr. Gordon Tomkins as Chief.

Honors have come to several of the scientists of this Institute. Dr. Joe H. Tjio received one of the new Kennedy Foundation awards for his work in isolation and characterization of human chromosomes, a work with an impact only now beginning to be felt. Dr. Harold Edelhoch received the Van Meter prize for his work on thyroglobulin.

The work of the NIAMD is summarized in the following pages and a further summary here is hardly possible except to note the wide range of disciplines now represented and the broad interests in this Institute. At its most theoretical end is the Laboratory of Mathematical Research, whose work is in part theoretical derivations of mathematical relationships relevant to biological and chemical problems and in part formulation of methods for the use of high speed computing devices for the solution of biomedical problems. There is then a gradation from theoretical chemistry through physical chemistry to organic chemistry where achievements have been outstanding in this Institute for many years. The discovery of new sugars of biological importance, of new methods of analysis of proteins, and of novel synthesis are reported. In molecular biology, a spectrum of disciplines are used in exploring the mechanism of enzyme synthesis, of genetic control, and of genetic transmission. Biochemistry is as usual well and productively represented in its activities. Vitamin metabolism and nutrition also continue to be a major interest of numerous of the Institute scientists. The broadly based clinical investigation program is dealt with separately in this annual report.

The Reserach and Clinical Associate programs are drawing some of the brightest and most inquiring young physicians in the country and represent not only a valuable educational service but reciprocally a yearly renewal of young talent for this Institute. It is not unreasonable to inquire about the other segment of medical investigators represented by those with the Ph.D. degree or those desirous of obtaining that degree. The young postdoctoral scientists now have a new program, the Staff Fellowship program, which permits an appointment of two or three years duration, specifically designed for both their own development and our Institute's needs.

LABORATORY OF MOLECULAR BIOLOGY

Investigations of Structure and Activity of Glutamic Dehydrogenase (Drs. G. M. Tomkins and K. L. Yielding)

Investigations have continued on the relation of the structure to the activity of crystalline glutamic dehydrogenase. Further work using ultracentrifugation and light scattering have clearly indicated that the state of aggregation of the protein molecule does indeed influence its substrate specificity. Using light scattering, the influence of various reagents on the molecular weight and enzymic activity of the enzyme has been investigated.

Mechanism of Enzyme Induction in Mammals (Drs. L. D. Garren, S. Appel, R. R. Howell and G. M. Tomkins)

It has been found that an increase in "messenger RNA" specific for several induced enzymes is produced when enzyme induction in mammals is stimulated upon the injection of steroid hormones. Indications have been obtained that there are two classes of "messenger RNA" in mammals—a stable unresponsive form and a labile form responsive to the presence of inducers.

Mechanism of Enzyme Induction in Bacteria (Drs. D. Alpers, S. H. Barondes and G. M. Tomkins)

A system of lysed protoplasts from *E. coli* which can form β-galactosidase in the presence of inducer is being investigated. The system is inhibited by actinomycin and puromycin and seems to have requirements for the various nucleotides and amino acids. The relation of this system to intact protoplasts is presently under investigation. It is hoped that the mechanism of induction of enzymes in bacteria will be elucidated by further studies using this preparation.

Properties of the DNA-Dependent RNA Polymerase From Mammalian Tissue (Drs. S. H. Barondes and G. M. Tomkins)

The DNA-dependent RNA polymerase responsible for "messenger RNA" formation in mammalian tissue is being purified from liver nuclei. Its properties are under investigation and it is hoped that a study of this enzyme will yield results pertinent to the problem of the regulation of "messenger RNA" synthesis in mammalian tissues.

Active Transport of Histidine in Salmonella (Dr. G. Ferro-Luzzi Ames)

A specific system for the transport of histidine in *Salmonella typhimurium* was shown to exist with a Km for histidine of 10^{-7} M. A second permease for which the affinity of histidine is 10^{-4} M is more general and responsible for the transport of aromatic amino acids tryptophane, phenylalanine and tyrosine, as well as histidine, although for the latter three amino acids the affinity is somewhat higher. Mutants resistant to aromatic amino acid analogs have been shown to be defective in the transport of aromatic amino acids.

Effects of Natural and Synthetic Polyribonucleotides on Amino Acid Incorporation (Dr. E. S. Maxwell)

The effects of natural and synthetic polyribonucleotides on amino acid incorporation into protein were investigated in a cell-free system from rat liver. Isolated microsomal RNA stimulated the incorporation of all the amino acids tested, suggesting that mammalian microsomes contain some "messenger RNA". Soluble RNA, on the other hand, inhibited the incorporation. Using synthetic copolymers, RNA coding units were determined for 6 amino acids. Assuming triplet codes, the following compositions were indicated: phenylalanine, 3U; leucine, 2U1G or 2U1C; valine, 2U1G; glycine, 1U2G; tryptophane, 1U2G; and serine, 2U1C. These results are in complete agreement with data reported for a system from *E. coli*. The fact that the same degeneracy is observed for leucine in the two systems seems of particular interest. The data indicate that at least a portion of the code may be universal.

Properties of Nucleic Acids and Their Components (Dr. H. T. Miles)

Structural investigations on the nucleic acids have been carried out using infrared spectroscopy and the results have been correlated with other methods such as ultraviolet spectroscopy and optical rotation. It has been shown that the infrared is very sensitive to small changes in molecular structure and therefore possibly more sensitive to changes in configuration of complex macromolecules such as the nucleic acids. Ordered structures involving a single strand of poly C with both 5′ and deoxy 5′ GMP have been studied, as well as

the interaction of poly C with poly A and poly I. Studies of this type are of great interest in chemistry of nucleic acids, as well as in understanding the possible biological role of these compounds.

Chemical and Genetic Studies on Hemoglobin (Drs. H. A. Itano and A. Gottlieb)

The genetic and chemical studies of inherited disorders of hemoglobin synthesis are being continued. The chemical modifications of the arginyl residue of proteins is being investigated with the object of simplifying the analysis of tryptic hydrolyzates.

Studies on Bacteriophage Lysozymes (Drs. W. Dreyer, T. Merigan and J. C. Bennett)

The lysozyme produced by *E. coli* on infection with bacteriophage T4 has been investigated by chemical, physical and enzymatic means. The amino acid sequence of the normal enzyme and certain mutant forms is under study; a number of mutants seem to produce a variety of alterations in amino acid sequence. It is presumed that the study of these mutants and a comparison of the enzyme produced by the wild type bacterial virus will be important in understanding the genetics of enzyme formation, amino acid interaction involved in stabilization of proteins and the nature of serologically reactive groups in the enzyme.

Investigations on the Structure of Guanylic Acid (Drs. D. R. Davies, M. Gellert, M. Lipsett, H. T. Miles and P. Sigler)

It has been found that concentrated solutions of 5′ guanylic acid, a small molecule, exhibit all the properties of regular helical polynucleotides. Under suitable conditions, fibers can be obtained which give well oriented X-ray diffraction patterns which have been interpreted in terms of a specific model for hydrogen bonding between the bases. Qualitatively similar results have been obtained for 3′ isomer of GMP as well.

Kinetics of Interactions Between Synthetic Polynucleotides (Drs. D. R. Davies, M. Gellert, M. Lipsett, H. T. Miles and P. Sigler)

Reactions involving hydrogen bonding between strands of synthetic polyribonucleotides have been investigated. It has been demonstrated that these interactions are reversible and that their kinetics can be studied in the same way as those of simpler reactions. These studies are of considerable importance in understanding the mechanisms of synthesis and the reaction of the natural nucleic acids.

X-Ray Diffraction Studies on Crystalline Chymotrypsin (Drs. H. C. Skinner, C. Coulter, D. R. Davies and P. Sigler)

In order to begin a full scale investigation on the structure of crystalline chymotrypsin, a search for a suitable heavy atom derivative of the enzyme has been made and computer programs for the analysis of collected data have been started.

A Study of the Melting Point Dispersion Method Applied to Polynucleotide Structure (Drs. G. Felsenfeld, C. Smith and C. Stevens)

This method, which allows the detection of selective melting out of AT or GC base pairs, has been applied to the problem of denatured DNA and of the structure of amino acid acceptor RNA. It has been shown that SRNA contains sequences of base pairs which are not random but consistent of runs of GC and AU base pairs. An additional feature of these studies is that an automatic data collection system has been designed which will facilitate the large numbers of experiments necessary for the application of this approach.

Studies of Micrococcal Nuclease (Drs. G. Felsenfeld and P. von Hippel)

It has been found that micrococcal nuclease is stabilized at high temperature by native but not denatured DNA. Thus, the ordered structure of the native nucleic acid interacts specifically with the protein. Since the enzyme can now be used at rather high temperatures, it is hoped that it will aid in the study of the early stages of nucleic acid degradation.

Thermodynamics of Helix-Coil Transformations (Drs. P. Ross and G. Furukawa)

It has been found that the helix-coil transition observed with DNA is accompanied by an anomalous heat capacity vs temperature plot. This permits the calculation of the standard enthalpy and entropy change accompanying the melting of DNA. This technique thus provides an approach to the determination of energy stabilizing ordered structure of nucleic acids and it could be extended to other macromolecules of biological interest.

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Biochemical Control Mechanisms in Histidine Biosynthesis (Dr. B. N. Ames)

Further work has been done on the histidine operon—the cluster of 8 genes that makes the enzymes of histidine biosynthesis in *Salmonella*. Dr. Ames, in collaboration with F. Jacob and P. Hartman has investigated an operator mutant which had previousy been shown to cause all the histidine genes to be shut off even though they were not damaged by the mutation. It has been possible to mutate the operator mutant to new strains which have recovered the histidine enzymes. These strains have been found to be no longer under histidine repression control. One class of mutants has a deletion at the end of the histidine sequence, which suggests that the histidine genes have become part of a neighboring operon. The other class was found to have a duplicate histidine cluster translocated out of the chromosome: the duplicate piece is functional and not under histidine control. A number of other experiments also indicate that the 8 histidine genes function as a unit which can be turned on and off in response to the histidine repressor acting at an operator site in the gene at one extremity of the cluster—the gene that determines the sequence of phosphoribosyl-ATP pyrophosphorylase — the first enzyme of histidine biosynthesis.

Dr. Martin has investigated feedback inhibition in the first enzyme of histidine biosynthesis. He has purified the enzyme and shown that there is a separate site on the enzyme for the histidine inhibitor distinct from the substrate sites. This histidine site could be inactivated reversibly.

LABORATORY OF BIOCHEMICAL PHARMACOLOGY

Spermine and Spermidine (Drs. H. Tabor, C. W. Tabor, U. Bachrach and L. deMeis)

These amines are present in a variety of animal, plant and bacterial materials, and our studies have been directed towards their distribution, metabolism and possible functions.

During the past year our efforts have been mainly concerned with the enzymatic oxidation of these amines. Two enzyme systems have been studied:

(1) *Serum amine oxidase*

$$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2+2O_2+2H_2O \longrightarrow OHC(CH_2)_2NH(CH_2)_4NH(CH_2)_2CHO+2NH_3+2H_2O$$

(2) *Serratia amine oxidase*

$$NH_2(CH_2)_4NH(CH_2)_3NH_2+O_2 \longrightarrow NH_2(CH_2)_3NH_2+\text{  }+H_2O_2$$

Additional studies in this area during the past year have been (1) Development of a gas chromatographic assay for these amines, (2) Studies on the inhibition of phage growth by spermine, (3) Further studies on the concentration of the various amines in the pancreas of various animals, (4) Further studies with bacteriophage on the toxicity of the aldehyde formed by the action of serum amine oxidase on spermine, (5) Further studies on the effect of spermine in the stabilization of DNA against denaturation due to heating, (6) Further studies on the effect of spermine in preventing shearing of DNA (with Dr. Dale Kaiser of Stanford University Medical School).

Sialic Acids (Drs. L. Warren and R. Blacklow)

The term sialic acid, refers to a group of compounds that are derivatives of neuraminic acid, a 9-carbon carbohydrate. They are usually found linked to various carbohydrate polymers, and, as indicated in part by the work summarized here, appear to be of importance in a variety of biologic areas.

Further work has been carried out on the biosynthesis of one representative of this group, N-acetylneuraminic acid. An enzyme has been purified 125 fold from extracts of *Neisseria meningitidis* that carries out the following reaction:

N-acetyl, D-mannosamine + phosphoenolpyruvate → N-acetylneuraminic acid + Pi

The reaction differs from that found in mammalian extracts which was described in last year's report (with Dr. H. Felsenfeld).

N-acetyl, D-mannosamine-6-phosphate + phosphoenolpyruvate → N-acetylneuraminic acid-9-phosphate + Pi

Another reaction that has been described in extracts of *N. meningitidis* is:

N-acetylneuraminic acid + CTP $\xrightarrow{Mn}$ CMP-N-acetylneuraminic acid + PP

This reaction is of considerable potential importance, since it may represent the activation of sialic acid for the synthesis of such mucopolysaccharides as capsular material, etc.

An interesting and somewhat novel phase of the studies on sialic acids has been a study of their distribution in nature. Vertebrates and other chordates have sialic acids. These are also present in echinoderms, but not in tunicates. These findings have led to the suggestion that the usually-accepted theory of the tunicate origin of vertebrates is incomplete. In addition to its evolutionary significance, these studies have led to the discovery of several new members of the sialic acid group, including a methoxy-derivative in starfish.

Biochemistry of Sulfur-Containing Compounds (Dr. S. Black)

It has been found that yeast glutathione reductase is a flavine-adenine-dinuckotide-containing flavoprotein with -SH groups sensitive to -SH reagents only in the presence of reduced pyridine nucleotide. Because of these properties the enzyme becomes an especially interesting subject for mechanism studies. To make such investigations feasible, methods have been sought to obtain purified enzyme in adequate quantity. Several new procedures have been developed based upon the enzyme's surprisingly high stability in acid solution and its adaptability to purification by ion-exchanging chromatography. The best preparations thus far have a minimum molecular weight of about 400,000 based on FAD content, and a turnover number of 8,000. It is hoped that homogeneous material will be available in the near future.

Turnover and Intracellular Distribution of Amino Acids in Bacteria (Drs. E. Levine and Loretta Leive)

The formation of certain adaptive enzymes in *Bacillus subtilis* requires the presence of trace amounts of amino acids. This is not true in *Escherichia coli* cultures. Studies have demonstrated that the rate of protein turnover in *B. subtilis* is approximately $1/10$ of that in *E. coli*, and presumably this accounts for the above observations. As part of these studies an apparatus was developed to distinguish intracellular turnover from intercellular turnover due to cell death and autolysis. This apparatus, which permits the equilibration of culture fluids of separated bacterial suspensions, will be of general use in studies of turnover in bacteria.

In other studies differences were observed in the utilization of labeled exogenous diaminopimelic acid by the bacterial cell for two different reactions; i.e., (1) incorporation of diaminopimelic acid into cell-wall precursor and (2) decarboxylation to lysine for protein synthesis. It has been shown that this is the result of heterogeneity in the distribution of the added amino acid within the cell. In addition, the data indicate spatial separation of the two enzymes of diaminopimelic acid utilization. These results are important since they show that the intracellular environment is not an undifferentiated pool of metabolites, but is inhomogeneous both with respect to enzyme location and metabolite distribution (with Dr. B. Davis of Harvard Medical School).

Control Mechanisms for Enzyme Levels in Mammalian Systems (Dr. R. Schimke)

Considerable work has been carried out in various laboratories on the factors controlling the level of enzyme in bacterial systems. Comparable control mechanisms are present in mammalian systems. These have been shown in (a) whole animal experiments and (b) tissue culture studies.

The whole animal experiments represent a continuation of the work done last year, and show that in the rat each of the enzymes involved in urea synthesis (carbamylphosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase and cleavage enzymes, and arginase) increases when protein increases and decreases when protein intake decreases. A particularly important aspect of this work is the demonstration that the enzyme level is regulated by active control of *both* enzyme synthesis and of enzyme degradation.

Tissue culture studies have also been carried out on arginine metabolism, and demonstrate phenomena comparable to repression. For example, low arginine concentrations in the medium result in a marked increase in the level of argininosuccinate synthetase and a decrease in the level of arginase. Contrariwise, high arginine concentrations in the media have opposite effects.

An important development during the work was the demonstration of the importance of using PPLO-free cells for the tissue culture studies. In

the presence of PPLO, arginine was rapidly degraded by arginine deaminase, coupled with ornithine transcarbamylase, and carbamyl phosphokinase present in the PPLO. Many cell-lines commonly used are PPLO-contaminated (with Dr. M. F. Barile of the Division of Biologic Standards).

Metabolism of Histidine, Histamine and Related Imidazoles and Amines (Drs. C. McEwen and H. Bauer)

Further studies have been carried out on the enzyme, diamine oxidase, which carries out the oxidative diamination of both histamine and a variety of diamines. We have developed a number of new assays for diamine oxidase, but the most satisfactory assay developed has been a modification of a previous method based on the colored quinolinium compound formed when the oxidation product of putrescine, Δ'-pyrroline, reacts with o-aminobenzaldehyde.

This assay has been applied to the measurement of diamine oxidase in sera from pregnant woman, and is considerably more convenient than the biological assays used for this purpose in the past. In pregnancy the diamine oxidase of the serum progressively rises, and is many times higher than the levels in non-pregnant controls.

Diamine oxidase has been further purified from hog kidney with a 500 fold overall purification. Spectrophotometric studies on the purified enzyme have demonstrated a substrate reducible peak at about 500mμ. The cofactors of this enzyme are not known yet, and further work is being carried out on this aspect of the problem. Continuing attempts are being made to synthesize imidazoleacetaldehyde, the probable product of histamine oxidation.

Studies on Burns (Drs. R. C. Millican, K. Markley and S. M. Rosenthal)

Studies were continued on the factors involved in the acute and delayed mortality following a high-temperature scald burn of the leg in mice. Septicemia was observed during the shock period, which was reduced by antibiotics. It seems likely that this septicemia is caused by invasion through the gut wall by the normal intestinal flora.

Animals which had survived a previous scald of one hind leg demonstrated a decreased acute and late mortality when scalded on the other hind leg, as compared with the controls which had not been previously scalded. These experiments show that mice can acquire resistance to repeated scalding. This resistance can also be transferred passively as reported last year when convalescent scald plasma was found to protect animals against a severe scald.

Studies have been continued in both the laboratory and in a clinical study (Lima, Peru) on the Pseudomonas infections that occur in late deaths in burns. This is a very important problem in burn therapy since the development of these infections is usually fatal. In the laboratory, antigens have been prepared from various Pseudomonas strains isolated from clinical cases for use in typing studies (with Dr. Ronald Wood of Johns Hopkins).

As part of these studies on Pseudomonas organisms, the biosynthesis of pyocyanine was studied; this is the pigment formed by *Pseudomonas aeruginosa*. Pyocyanine was shown to be derived from C^{14}-shikimic acid.

Mevalonic Acid (Dr. K. Markley)

Studies in this area have been directed towards discovering new pathways for mevalonic acid utilization. As part of these studies, *Lactobacillus acidophilus* has been grown on C^{14}-mevalonic acid, and a large incorporation of label has been demonstrated in an unidentified neutral lipid, containing a free hydroxyl group, that does not appear to be one of the common steroids. Glucose has been found closely associated with the radioactivity, and further studies are in progress on the identification of the lipid-glucose complex.

Leprosy (Dr. Y. T. Chang)

The chemotherapeutic studies on mouse leprosy that have been conducted for many years are continuing, but have now been supplemented with chemotherapeutic studies on human leprosy. For the latter studies, the technique of Dr. Charles Shepard on the growth of these organisms in mouse footpads has been used. Definite suppressive activity in mouse leprosy was observed with ethambutol, vadrine, sulfamethoxypyridazine, and sulfamonomethoxine. Streptomycin, B663, and diaminodiphenylsulfone were very effective against the human leprosy organism in the footpad experiment (with Dr. C. Shepard, CDC, Atlanta).

Continued improvements have been attained in

the culture of *Mycobacterium leprae murium*. The growth of this organism in macrophages in tissue culture represents a particularly important contribution to these studies. Only preliminary studies (with Dr. Richard Adler of NIAID) have been carried out with human *M. leprae*; healthy bacilli were observed in cultures of mouse peritoneal macrophages.

Effect of Fasting on Fatty-Changes in Mice (Dr. S. H. Webster)

Studies of the triglyceride content of liver, kidney, and heart showed that this increased after fasting. Fasting also resulted in marked increases in glutamic-oxalacetic transaminase and lactic dehydrogenase; this increase was not found in mice pretreated with dibenzyline (with Dr. B. Highman of the Laboratory of Experimental Pathology).

LABORATORY OF NUTRITION AND ENDOCRINOLOGY

Vitamin E, Antioxidants and Selenium

The metabolic relationship between vitamin E and selenium has not been clearly elucidated. Earlier studies from this laboratory showed that one probable function of selenium is to increase the antioxidant capacity of tissues as does vitamin E. Continuing studies have revealed another similar physiological effect produced by dietary vitamin E or sodium selenite. It has been known for many years that the vitamin effectively protects animals against certain types of chemical poisons. It has now been found that in chicks trace amounts of selenium have a similar action. Dietary levels of selenite which are below the usual requirement effectively prevent the toxicity and death which result when relatively high levels of certain organic compounds are fed.

Another relationship between these two nutrients, and also to cystine, has been demonstrated in their effect on coenzyme A levels in chick liver. When chicks were fed purified diets with either 15 or 25 percent of protein, minus the usual supplemental cystine, coenzyme A in liver was reduced by one-third. Addition of vitamin E or selenite to the diet resulted in normal concentrations of the coenzyme.

The fate of α-tocopherol (vitamin E) in the body has received only limited attention. In experiments with rats and chicks in which α-tocopherol labeled with C^{14} was given in a single oral dose, it was found that from 35–50 percent was excreted in the feces, with no change in the compound, in a 21 day period. The uptake by different organs varied considerably, with liver, lungs, spleen, kidney and blood having from 1–5% of the dose after 24 hours. Elimination from most of the tissues was rapid and by 21 days only lungs and small intestine had as much as 0.5 percent of the dose. All radio-activity was accounted for as unchanged α-tocopherol, with no evidence for metabolic intermediates or end-products (Drs. J. Bieri and S. Krishnamurthy).

Protein Deprivation and Energy Metabolism

This continuing study is designed to determine how variation in the protein status of animals affects the energy utilizing mechanism in liver cells. Individual enzymes, and cofactors, which participate in the transformation of energy have been measured while rats are gradually depleted of protein, and also while they are being repleted with protein. It has been found that the liver loses an appreciable number of cells during protein depletion but that cell reproduction proceeds rapidly when protein is re-fed. Methionine in the protein-free ration significantly reduced the loss of liver cells.

In protein deficient rats not receiving methionine, the efficiency of energy utilization was considerably better than that in similar rats given methionine, even though the livers from the latter animals had a much higher rate of oxidation of succinate. The livers of the protein-depleted animals had considerable losses of succinic dehydrogenase and also of cytochrome oxidase.

About 80% of the liver coenzyme Q was lost during severe protein depletion. Following the repletion of protein, liver coenzyme Q initially rebounded to above normal levels and then returned to normal after about eight weeks (Dr. J. N. Williams, Jr.).

Lipid Metabolism

This investigation is directed toward a biochemical explanation for the abnormal lipid metabolism in rats which result from feeding 1% of orotic acid, a metabolic precursor of pyrimidines which occurs in milk. The large accumulation of fat in the liver which orotic acid produces has been found to be independent of wide variations in

the dietary protein or fat levels. The liver lipid has been characterized as almost all triglyceride containing only palmitic, palmitoleic and oleic acids. Cholesterol in these livers increased 5–6 fold. Serum triglycerides fell almost to zero in orotic-fed rats, and cholesterol, phospholipid and unesterified fatty acids were reduced to 20% of normal.

By labeling the body water with tritium, it has been shown that orotic acid causes an increase in fat synthesis in all tissues, and that it is this newly synthesized fat which accumulates in liver.

Orotic acid isotopically labeled in various positions is rapidly oxidized to carbon dioxide after its injection into rats; it is converted, however, to pyrimidine nucleotides before being catabolized. Addition of adenine to the diet alters the pathway of metabolism of orotic acid (Dr. H. G. Windmueller).

Guinea Pig Nutrition

The best purified diet suitable for experimental studies with the guinea pig was previously devised by Dr. Reid. This diet, however, does not produce the optimal growth obtainable with a commercial ration made of natural feedstuffs. It has been found that if 20% of the casein in the purified diet is replaced with an isonitrogenous amount of alfalfa, growth now becomes optimal. Studies are currently attempting to determine what component of alfalfa gives this growth response.

Contrary to what occurs with other species, it has been found that the D and L-isomers of methionine are not equally effective in the guinea pig. It is not clear whether this is due entirely to an actual metabolic difference, or whether variations in the dietary protein or possible changes in intestinal flora may be involved (Dr. M. E. Reid).

Diabetes and Fat Metabolism

The effect of insulin on fatty acid synthesis in pancreatectomized rats was studied by measuring incorporation of tritium labeled body water into carcass and liver fatty acids. Pancreatectomy had no effect on fatty acid synthesis in fasting rats. The rate of synthesis in fed pancreatectomized rats, however, was 80% less than that in fed normal rats; insulin restored the rate to normal. In contrast to findings made in alloxan diabetic rats, insulin deprivation for 48 hours did not alter the responsiveness to insulin. *In vitro* studies made with liver slices and adipose tissues from the above animals, measuring conversion of glucose and fructose to fatty acids, confirmed the *in vivo* studies.

The high rate of fatty acid release by incubated adipose tissue from pancreatectomized rats deprived of insulin for 48 hours was markedly reduced by *in vitro* addition of a small amount of insulin. Glucose uptake by incubated adipose tissue of untreated diabetic rats was normal. However, conversion of glucose to CO_2, glyceride-glycerol and fatty acids was markedly depressed, while lactate formation was greatly increased. Utilization of glucose was normalized by addition of insulin.

Dexamethasone at very low concentrations, 10^{-8} to 10^{-7} M, accelerated release of fatty acid by incubated adipose tissue from normal fasted rats. Corticosterone produced similar effects if ten times as much was added. 2-α-methyl cortisol was as potent as corticosterone, whereas 2-α-methyl cortisone was ineffective. Deoxycorticosterone had little effect on fatty acid release. Dexamethasone also decreased uptake and metabolism of labeled glucose to carbon dioxide, total lipid, and fatty acid. Addition of small amounts of insulin to the media (4 mU/mill) reversed the effects of dexamethasone on adipose tissue. These findings support the hypothesis that development of ketosis, fatty liver, and hyperlipemia in the diabetic rat is the result of a direct action of the glucocorticoid on adipose tissue (Dr. R. O. Scow, Dr. S. S. Chernick and Dr. J. N. Fain).

Two aspects of fat formation were investigated in the liver: Lipogenesis, the formation of new fatty acids; and esterogenesis, the formation of new glyceryl ester bonds, as in triglycerides and phospholipids. Esterogenesis may involve esterification of free fatty acids or transfer of fatty acids from one glyceride-linkage to another. *In vitro* incorporation of glycerol, formed from the metabolism of C^{14} glucose or fructose, into triglycerides and phospholipids by liver slices was found to be extensive and independent of the rate of lipogenesis. This finding supports the previous observation that the perfused liver readily transferred fatty acids of triglycerides to phospholipids and other triglycerides without the appearance of appreciable amounts of free fatty acids in blood or tissue (Dr. S. S. Chernick).

In perfusion studies adipose tissue removed tri-

glycerides intact from the circulation. The triglycerides were then hydrolyzed, extravascularly, to glycerol and fatty acids by lipoprotein lipase. The resultant fatty acids were re-esterified, probably within the fat cells, to triglycerides or were released to the circulation.

Fat cells were isolated from adipose tissue by treatment with collagenase. All of the lipoprotein lipase in the tissue was found associated with the fat cells. The isolated fat cells appeared to be viable since they released free fatty acids when incubated with epinephrine or ACTH and utilized glucose at a faster rate when incubated with insulin (Dr. M. Rodbell and Dr. R. O. Scow).

Protein Hormones

Growth of the body and various organs were studied in rats bearing transplantable pituitary tumors (MtT). Previous studies had shown that these tumors produce relatively large amounts of prolactin, growth hormone, and adrenocorticotrophic hormone. The weights of the heart, liver, kidney, spleen, preputial, and mammary glands were greatly increased in tumor bearing rats, whereas the body weight and length were only slightly greater than that in normal rats. The weights of the pituitary, pancreas, ovary, and uterus were less than normal while the thymus and white fat were completely atrophied. Adrenalectomy greatly increased body weight and weight of the tumor, thymus, and preputial gland in tumor bearing rats. The liver, kidney, and heart, however, were normal in weight. Thyroidectomy prevented the gain in body weight, gut length, and weight of the tumor and preputial gland in rats bearing tumors but did not prevent hypertrophy of the viscera.

The changes in organ weights seen in tumor bearing rats were reproduced in normal rats by injecting a mixture of prolactin, growth hormone, and ACTH in very large doses. When the hormones were given singly, ACTH was the only one that produced hypertrophy of the heart and kidney.

The systemic bioassay for prolactin has been greatly improved by using adult instead of juvenile pigeons and injecting for seven instead of four days (Dr. R. W. Bates and S. Milkovic).

Thyroid stimulating hormone (TSH) has been isolated from several species of animal, including human. Amino acid analysis has revealed differences in their composition, principally with respect to cystine, glycine, lysine, and the amino sugar, galactosamine. Differences in electrophoretic mobility and biological and immunological properties can be related to differences in amino acid composition. Eel TSH was found to contain only half as much cystine as does the hormone from other species. The ineffectiveness of this preparation in the thyroid gland of higher vertebrates may be due to a less highly organized tertiary structure as well as differences in primary structure.

Density gradient centrifugation studies have shown that all preparations have the same sedimentation rate. TSH in blood of mice bearing TSH-producing tumors can be readily separated from the major protein fractions of plasma by density gradient centrifugation and by gel filtration. Thus, it appears that the hormone in blood is not associated with β or γ globulins, as reported by other laboratories.

Antibodies prepared against purified human TSH are highly specific for human TSH and have little cross-reactivity with bovine TSH. This casts doubt on the usefulness of anti-bovine TSH in the measurement of human TSH in blood.

Examination of freshly prepared bovine TSH by molecular sieving on sephadex has revealed that purification can be increased beyond that achieved by ion-exchange chromatography. This finding suggests that the natural form of the hormone is more potent than any isolated so far (Drs. Condliffe and Y. Fontaine).

Recent studies have established the purity of parathyroid hormone obtained as a homogeneous polypeptide. The methods used included starch gel electrophoresis, ultracentrifugal analysis of the hormone in guanidine using interference optics, and recovery of N-terminal alanine. Untracentrifugal studies have shown that pure parathyroid hormone forms high-molecular weight aggregates in certain buffers. This probably accounts for separation of pure preparations into three fractions with ammonium sulfate and for alteration of elution patterns on gel columns with changes of buffer conditions. Now that these properties have been defined, it is possible to proceed with structural analysis of parathyroid hormone (Dr. G. Aurbach and Dr. J. T. Potts, NHI).

Folic Acid

An investigation of the folic acid profile in chicken liver extracts, on DEAE-celluose columns revealed the presence of a variety of monoglutamate and polyglutamate derivatives. Using the analytical procedures developed in this laboratory the monoglutamates identified include the N^{10}-formyl-,N^5-formyl- and the N^5-methyl derivative of tetrahydrofolic acid. A spectrum of N^{10}-formyl- and N^5-methyl- polyglutamyl derivatives of reduced folic acid of varying complexity was also observed. From this it appears that no single simple method of assay for the important vitamin cofactor in tissue is available at this time.

The overall concentration of folic acid derivatives in tissue and the significance of the relative concentrations of the various forms of folic acid is a major problem and is being continued (Drs. Silverman and Noronha).

While enzymatic oxidation of 5-methyltetrahydrofolate (Prefolic A) yields 5,10-methylenetetrahydrofolate, the product of the initial chemical oxidation was a dihydro-derivative. This compound, shown to be 5-methyl-5,6-dihydrofolate, was readily reduced back to the tetrahydro- level by ascorbic acid, β-mercoptoethanol and homocysteine. However, attempts to reduce this compound enzymatically were unsuccessful. In addition, attempts to oxidize the methyl group of this derivative to the methylene level were also unsuccessful (Dr. K. O. Donaldson and Dr. J. C. Keresztesy).

Attempts to purify chemically prepared 5,10-methylenetetrahydrofolic acid by gradient chromatography on DEAE or TEAE-cellulose columns using carbonate buffer (pH 9.5) resulted in the separation of the two diasterioisomers of this derivative. One was identified as the biologically active isomer which corresponds to the l,L isomer. The other was shown to be biologically inert and corresponds to the d,L isomer. However, both forms could be reduced to the methyl level by chemical reduction. This procedure provides a simple method for the preparation of fully active derivatives at the reduced forms of folic acid (Dr. B. Kaufman, Dr. K. Donaldson and Dr. J. C. Keresztesy).

Large-Scale Laboratory

The large-scale laboratory has continued to increase the help it gives to investigators throughout the whole National Institutes of Health. The greatest number of requests have come for growing non-pathogenic organisms and bacterio-phage in the 100-gallon fermenter, to be used as sources of enzymes, DNA and RNA, in protein synthesis studied. The low temperature concentrating facilities were found to be extremely useful for processing heat-sensitive biological materials (Dr. J. C. Keresztesy and Dr. D. L. Rogerson, Jr.).

Germ-Free Program

Work in the germ-free animal area has progressed along several lines during the past year.

It has been established by comparison of germ-free and conventional animals that a "normal" bacterial flora contributes folic acid to the host animal. A portion is obtained by the rat by ingestion of feces but when coprophagy is completely prevented by affixing tail cups to the animal sufficient folic acid is obtained, undoubtedly by direct absorption, to prevent the appearance of frank deficiency (E. C. McDaniel, Dr. F. S. Daft, Dr. L. G. Herman [DRS], and R. G. Horn).

Under certain conditions (when large amounts of vitamin C or certain antibiotics are added to a deficient diet) the conventional rat requires no dietary pantothenic acid. Under these conditions the vitamin synthesized by gastrointestinal bacteria is made available to the host animal in sufficient amounts to meet the animal's needs. It has been established, however, that this phenomenon depends on coprophagy. It is still possible that the rat obtains some pantothenic acid by direct absorption without coprophagy but, if so, in amounts far below the animal's needs (E. G. McDaniel, Dr. F. S. Daft, Dr. L. G. Herman [DRS], and R. G. Horn).

Dietary liver cirrhosis due to a deficiency of choline and related compounds has been postulated by other investigators to depend on the action of bacteria on a liver weakened by dietary insult. It has been established, however, that germ-free rats and mice develop dietary liver cirrhosis with an incidence and severity probably

equal to or greater than conventional animals (Dr. S. M. Levenson [Walter Reed Army Medical Center], E. G. McDaniel, and Dr. F. S. Daft).

Dietary liver necrosis on the other hand is favorably influenced by the germ-free state. Germ-free rats grow more rapidly and develop liver necrosis more slowly and in lower incidence than their conventional or conventionalized counterparts. The basis of the deleterious influence of gastrointestinal bacteria on this condition is under investigation (E. G. McDaniel, Dr. F. S. Daft, Dr. L. C. Herman [DRS], and R. G. Horn).

It has been shown by Pearson, a grantee, that arthritis can be induced in conventional rats by the injection of Freund's adjuvant. The question naturally arose as to the possible participation of infection in the development of this pathological condition. In collaborative studies it has been shown that this phenomenon occurs in "germ-free" animals, thus excluding the participation, as far as we have been able to determine, of all bacteria. Still further studies will be carried out, however, in an attempt to exclude—or implicate—any extremely fastidious bacteria, whose presence might possibly have been missed in the tests which have been performed (Dr. C. M. Pearson [UCLA], Dr. F. D. Wood [UCLA], E. G. McDaniel, Dr. F. S. Daft, and Dr. L. G. Herman [DRS]).

LABORATORY OF PHYSICAL BIOLOGY

As the lineal descendent of the Industrial Hygiene Research Laboratory, the Laboratory of Physical Biology is one of the oldest of NIH, as well as one of the largest. As such it illustrates the diversification of scientific activities that inevitably develops over the years when independent investigators are free to explore subjects of their own choice and to follow where experiment leads. Even so, it has been surprising to find that a few major research themes encompas nearly all the work of the Laboratory. In the following eclectic summary of current LPB findings and interests in these primary areas, only the work of full time professional members of the Laboratory are mentioned. In the reporting, an effort has been made to omit technical details and to simplify the presentation so that it will be more accessible to the non-specialist.

Physiology

This not ideal heading is used to include the multifarious LPB projects in which intact organisms or cells are studied with one or more of the following objectives in mind: (a) Elucidating some normal function, (b) observing mechanisms of adaptation or compensation to stress, (c) unraveling changes occurring during growth and differentiation. For example, tolerance to altitude in rats has been found to be lower in males than in non-pregnant females, and lower at puberty and in old age than in middle age (Altland). Curiously, 18–20 hours of altitude hypoxia enables rats to avoid the edema ordinarily caused by intravenous dextran, and prior intraperitoneal hypertonic glucose has a similar protective effect, shown not to be osmotic (Marshall).

In continuing work on the complex problem of what induces somatic cells of the simple metazoan *Hydra* to differentiate into gonad tissue, it has been found that constant stirring of the culture medium inhibits sexuality in sparse populations but not in more crowded cultures (suggesting the presence of a soluble endogenous inducer) and that low temperature promotes sexuality in one species but inhibits it in another (Park). A superficially similar inverse temperature effect on the relative infectivities of *Trypanosoma cruzi* and *Leishmania enrietti* is being studied in relation to respiratory metabolism and the integrity of the mitochondrionlike kinetoplast (Greenblatt).

A literature report of a specialized CO_2-eliminating mechanism in certain insects was found to be groundless, but new data on the respiratory system was obtained as a byproduct (Keister). In another investigation on insects, involving the effects of temperature on respiratory rate during different stages of development, an interesting byproduct was the discovery of cold-inactivation of the hormone that induces pupation (Burkett).

Molecular Structure

In addition to much work on macromolecules (see below), LPB chemists and physicists have put great effort into elucidating the structure, properties and reactions of simple molecules which can form building blocks of biologically important substances or yield clues to their construc-

tion. For example, periodicities in electron micrographs of calcium stearate crystals have been studied to assess certain diffraction effects caused by molecular lattices which have to be understood in order to interpret pictures of protein crystals (Labaw). Similarly, a paracrystalline state of protein fibers has been implicated as the basis of the transparency of the lens of the eye (Trokel). Studies on the vibrational spectra of quinone derivatives (Becker, Charney) have an obvious bearing on bonding in quinoid molecules in general and in many complex natural products in particular. Hydrogen bonding, of great importance in protein structure, has also been studied intensively by Dr. Becker and collaborators.

Among physical methods of studying molecular structure, optical rotatory dispersion has certain unique capabilities. By applying this tool to nonplanar cis and trans dienes it has been possible to confirm theoretical predictions of the configurations and conformations of such molecules and to elucidate the structure of *Erythrina* alkaloids, compounds which have a curare-like action (Weiss, Ziffer, Charney).

Complexes of metal ions with organic molecules such as amino acids (chelates) have been studied from several angles. From nuclear magnetic resonance spectra the binding sites and rates of exchange of cupric and cadmium ions with several glycyl peptides have been established (Li, Sheinblatt, Becker), while ultraviolet spectroscopy has shed light on the association of mercury with amine and imide groups of nucleosides, in relation to the mercurial disruption of the nucleic acid helix (Simpson). Still other methods were applied to the study of binding of inorganic ions (Na^+, H^+, Ca^{++}) to B-lactoglobulin, ribonuclease and serum albumin (Baker, Saroff, Carroll, Wolff), the results of which are important in relation to protein structure.

On the analytical side, both cis and trans isomers of a new amino acid from sponge collagen and from the antibiotic telomycin (3-hydroxy-L-proline) and a new di-peptide from iris (α-L-glutamyl-β-alanine) have been isolated (Irreverre) and one of the bright red, photosensitizing pigments of the mold *Elsinoe* has been purified and structurally characterized (Weiss, Batterham). Progress continues on the adaptation of gas chromatography to amino acid analysis, using the methyl and trifluoroacetylmethyl esters (Nicholls, Makisumi, Saroff) and esterification has also been used successfully in the gas chromatography of the organic acids of the Krebs cycle (Sharpless).

Protein Structure, Activity and Synthesis

LPB work on macromolecular structure has centered on the proteins of muscle and of blood clotting, and on certain enzymes. The tropomyosins of larval and adult insects have been found to show only minor differences (Kominz, Maruyama, Levenbook). Configurational changes in succinylated myosin have helped elucidate the mechanism of polymerization and dissociation of this muscle enzyme and its cleavage at the disulfide bond (Kominz, Nihei). Similarly, bacterial dehydrogenases are being studied in an effort to understand their variations in molecular aggregation (Carroll).

Certain polypeptides which appear during the clotting of fibrinogen and which have the interesting property of potentiating the bradykinin-induced contraction of smooth muscle, are under investigation, as is sialic acid, a carbohydrate moiety that is lost during the clotting process (Laki, Gladner, Osbahr, Irreverre, Chandrasekhar). Bovine thrombin has been found to be an unusually small enzyme (62 residues: m.w. ca. 8000) and its polymerization and amnio acid sequence are being studied (Gladner, Osbahr, Laki, Carroll).

Biosynthesis of protein is being studied analytically in the favorable situation of insect metamorphosis (Levenbook, Shigematsu), using the enzyme aldolase as the test molecule. In the yeast *Candida utilis* various purine and pyrimidine analogs have been shown to influence both distribution and total quantity of both endogenous and exogenous amino acids in an "internal pool" and studies of the incorporation of labeled amino acids have revealed fallacies in a standard method of assessing amino acid turnover (Kempner).

Biological Energy Transduction and Coupling

A major LPB effort is going into the very interesting and promising domain which concerns the biological machinery for transforming one type of energy into another or of triggering the release of one type of energy by another. The absorption of light quanta, for example, may lead to electrical signals in nerve (which are membrane phenomena) that eventuate in vision, or to endother-

mic chemical combination (photosynthesis). Conversely, chemical changes may lead to light production (bioluminescence), with or without the mediation of triggering by nervous impulses, or may result in cellular intake or expulsion of material against concentration gradients (active transport). Similarly, bioelectric events may set off chemical reactions which lead to folding of protein chains (muscular contraction).

Insight into mechanisms by which pigments trap light was gained by the demonstration that reactivity of excited diazoacetophenone molecules can be related to the electronic configuration of the ground state (Ziffer, Sharpless). Investigation on one photosensitizing pigment ("Elsinochrome A") have already been mentioned (Weiss), and it has been shown that the ability of the chlorophyll molecule to absorb energy in the far red depends on its specific physical orientation in the chloroplast (Olson, Jennings). Also, in an attempt to determine the quantum yield of rhodopsin bleaching, retinine isomers are being separated by thin-layer chromatography (Adams).

By the use of a novel method for measuring simultaneously the absorption spectrum and electrical activity of thin slices of living retina it has been shown that a sequence of chemical reactions occurs, transforming the highly oriented pigment rhodopsin into the bleached, unoriented alkaline metarhodopsin. The onset of receptor action current coincides with the final step (Hagins, Srebro). The relation between chemical and bioelectric events has also been studied in two other neuroeffector systems, the firefly light organ and striated muscle. In the former, flashing has been shown to be invariably associated with characteristic volleys of nerve spikes and numerous other details of central and peripheral modulation and excitation have been reported (Buck). In living myofibrils activated by micro-application of Ca^{++}, the dependence of contractility on sarcomere length indicates that myofilament interdigitation, rather than peculiarities of the activation system, controls the sarcomere length at which contractility vanishes (Podolsky).

In glycerinated muscle fibers, energy transfer from ATP in relation to hydrolysis products and ionic strength were investigated, and the kinetics of ATP diffusion into muscle have been studied intensively, both empirically and theoretically (Bowen). Studies are continuing on the relationship of the actin, actin-myosin and actin-tropomyosin complexes to muscular contraction via detailed physical-chemical studies (Kominz, Maruyama, Laki).

Most, if not all, excitation and activation processes in living cells involve changes in or at biological membraness. It is therefore most relevant that some recently developed liquid membranes were shown to have an extreme degree of ionic selectivity, being readily permeable to anions and virtually impermeable to cations (or vice versa), with relative rates of the two permeation processes up to 100,000:1 or better. These membranes also show a considerable degree of ionic specificity, thus opening up interesting vistas for the problem of ionic specificity in living membranes, carrier mechanisms, and ion-binding in general (Shean and Sollner). Similarly, the action of drugs and hormones on surface films of precisely known composition is yielding quantitative data on both permeability and structural alterations of interfaces (Gershfeld).

In conclusion it may be of interest to mention that various members of LPB are beginning to explore the potentialities of computer analysis of complex data, for example spectrophotometric absorption curves of steroids (Brackett, Hahn, Sharpless), nuclear magnetic resonance spectra (Becker, Bradley) and in the computation of molecular weights, ion binding curves and amino acid sequences (Shapiro), Computation Branch, NIH.

LABORATORY OF CHEMISTRY

Rotatory Dispersion of Nucleosides

As a first step in a study of the causes of the remarkable optical rotatory anomalies shown by certain pyrimidine deoxynucleosides the rotatory behavior of a wide variety of 2-deoxy-D-ribose derivatives has been examined by Drs. Ness and Bhattacharya. Where oxygen or halogen is attached to carbon one of this sugar, rotational behavoir appears to be completely normal. Attention is now being turned to various nitrogen glycosides bearing special relationships to the nucleosides.

Cis-Nucleosides

Conventional methods for the synthesis of nucleosides (other than 2-deoxynucleosides) are suit-

able only for the preparation of nucleosides wherein the aglycon bears a *trans* relationship to the hydroxyl at carbon two of the sugar moiety. *Cis* isomers are needed for optical rotational studies as well as for studies in the chemotherapy of cancer. Through the use of 2,3,5-tri-O-benzyl-D-arabinofuranosyl chloride a *cis* nucleoside, 9-β-D-arabinofuranosyladenine ("spongoadenosine") has been synthesized in good yield by Dr. C. Glaudemans.

Octuloses and Nonuloses

The study of the higher-carbon sugars in avocado and *Sedum* species was continued. The structure of the second octulose from the avocado was proved by Drs. Sephton and Richtmyer to be D-*glycero*-L-*galacto*-octulose by degradation with lead tetraacetate and with oxygen in alkaline solution, and was confirmed by synthesis, both enzymatic and chemical. The structure of the first nonulose from the avocado, previously established by degradations as D-*erythro*-L-*gluco*-nonulose, was confirmed by chemical synthesis. Although aldoheptoses have been reported previously as constituents of bacterial polysaccharides, the isolation of D-*glycero*-D-*galacto*-heptose from the avocado marks the first known appearance of a heptose in the plant world.

Anhydroheptuloses

About five years ago a quantity of L-*galacto*-heptulose was transformed by hot dilute acid to give about a 5% yield of nonreducing anhydro sugars. Separation of the mixture on a Dowex-1 column (borate form) led to the isolation of about equal amounts of two products whose structures have now been established by Mr. Zissis as 2,7-anhydro-α-L-*galacto* heptulofuranose and 2,7-anhydro-β-L-*galacto*-heptulopyranose by the application of methods used earlier for similar compounds.

Neighboring Group Rearrangements of Cyclitols

Continuation of studies on the behavior of carbohydrates in liquid hydrogen fluoride has shown that cyclitols and 1,5-anhydroglycitols as such are unattacked but that their esters smoothly undergo Walden inversion at the central carbon atom of a *cis-trans* triacycloxy sequence. It is obvious that this experimentally simple process shows great promise for the synthesis of difficultly accessible organic structures. As an example, the work in the cyclitol series demonstrated that the hexaacetate of the common form of inositol (*myo*-inositol) is readily converted to *muco*-inositol, hitherto one of the least accessible cyclitols.

Glycosyl Cyanides

The increasing use of mercuric cyanide as an acid acceptor in the Koenigs-Knorr synthesis of glycosides and disaccharides makes the side reaction, the condensation of mercuric cyanide with acylated glycosyl halides, a matter of immediate importance. Dr. B. Coxon has shown that 2,3,4,6-tetra-*O*-acetyl-α-D-glucosyl-pyranosyl bromide condenses with mercuric cyanide to give *two* products, the expected 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl cyanide and the highly novel structure 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-cyanoethylidene)-α-D-glucopyranose. The glycosyl cyanides are readily preparable and offer interesting intermediates for the synthesis of C-glycosides related to psendouracil.

Progress on Benzomorphans

The chemistry, pharmacology and clinical studies on this new series of centrally acting agents have progressed to the point where, (1) structural limitations are fairly well defined; (2) relatively accurate predictions may be made regarding transfer of results from animal to man; (3) the β-series of 5,9-dialkyl-6,7-benzomorphans (5-alkyl quasiequatorial, 9-alkyl axial for the hydroaromatic ring, i.e. oriented toward nitrogen) corresponding to the unnatural morphine configuration at the β:C ring junction are 10–60 times more potent than the more accessible α-isomers and morphine and not commensurately more toxic or addictive; (4) it can be stated beyond reasonable doubt that a sharp, consistent separation of strong, central analgesic activity from addiction liability and other adverse effects (morphine toxiphrenia) has been achieved for the first time; (5) a parallel between analgesic and conditioned response-blocking activity is observed in animals. Of the three 2′-hydroxy-5-alkyl-2-methyl-6,7-benzomorphans studied to date, 5-ethyl and 5-propyl are much better than 5-methyl and are comparable to morphine (levo) even as the racemates. With the 5,9-dialkyl compounds maximum activity is shown in both the α- and β-series when the sum of the carbons of the two C-alkyl groups is 3. Activity

does not fall sharply until the carbons of these two groups totals 6 (dipropyl). Especial attention is directed to the following 2′-hydroxy-2-methyl-6,7-benzomorphans as candidates for further study. (a) (±)-5-Ethyl and 5-propyl, ED_{50} 2.3 and 2.1 mg./kg. (analgesic activity, mouse); physical dependence capacity (PDC, monkey) none in single-dose suppression studies up to 30 mg./kg. Morphine has ED_{50} 2.1 and is rated high in PDC with a stabilizing (abstinence suppression) does of 3 mg./kg. (b) (−)-5,9-Dimethyl, ED_{50} 1.7, analgesic activity in man comparable to morphine, PDC (monkey) low, abstinence suppression capacity in man one-eighth that of morphine, capacity for producing physical dependence in man less than that of morphine. (c) α-(±)-5,9-Diethyl, ED_{50} 4.1, nearly equivalent to morphine in relieving pain in man, PDC in monkey none to 60 mg./kg. (single-dose suppression), low in 30-day chronic administration compared with high for morphine; acute toxocity very low. (d) β-(±)-5,9-Diethyl, ED_{50} 0.28, PDC none to 12 mg./kg.; acute toxicity (mice) low.

Role of Phenolic Hydroxyl in α- and β-Benzomorphans

The analgesically favorable effect of the phenolic hydroxyl in structures with a heterocyclic tertiary nitrogen has been known for some time. However, the role of this substituent in tolerance and physical dependence has not hitherto been studied because deoxy compounds of the order of potency of morphine are not known. The high activity of the aforementioned benzomorphan β-diastereomers provided optimism that deoxy compounds of morphine-like potency were not implausible. Consequently, α- and β-5,9-diethyl-2-methyl-6,7-benzomorphans have been synthesized by two different routes and their stereochemistry confirmed by methiodide-formation-rate experiments and by conversion to known compounds. Preliminary evaluation indicates that the α-compound (devoid of oxygen) is indeed nearly as active as morphine and therefore suitable for the further research mentioned above. The β-counterpart expected to be more potent than the α, is however, much less so and constitutes the first exception in the benzomorphan series in this respect (Arthur E. Jacobson).

Approach to β-Benzomorphans, the More Potent Series

Further attempts to improve the yields of the pharmacologically more interesting β-benzomorphan diastereoisomers of the benzomorphan series have been successful. Thus, cyclization of the hydrochloride or hydrobromide salt of 2-benzyl- and 2-(p-hydroxybenzyl)-3,4-dialkyl-1-methyl-1,2,5,6-tetrahydro-pyridines with aluminum bromide or chloride has afforded 40–50% yields of the β-isomers in 3:1 and 2:1 predominance over α in contrast to 48% HBr at 140° which favors α 10:1 and 85% H_3PO_4 (180°) which gives 50% yields of α and 10–15% of β (J. H. Ager, E. M. Fry, S. E. Fullerton, E. L. May). After a complex series of rearrangements and chemical manipulations with 1,2-dihydro-pyridines it has been possible to devise a feasible synthesis of *trans*-2-benzyl-1,3,4-trimethyl-1,2,3,6-tetrahydropyridine hydrobromide (I) which can be cyclized to β-2,5,9-trimethyl-6,7-benzomorphan in 95% yield or to almost exclusively the α-congener with 48% HBr. Since it was also shown that I was readily rearranged to the corresponding Δ^3-piperidene by strong acid one may postulate that the formation of the β-compounds from the Δ^3-piperidenes may be preceded by rearrangement to I and related Δ^4-piperidenes and that α-compounds are produced principally from Δ^3-piperidenes (E. M. Fry).

Structure-Activity Studies in the Codeinone Series

Efforts to determine the effect on analgesic activity, of altering the point of attachment of nitrogen from carbon 9 to carbon 7 in dihydrocodeinone have continued. The difficulty of fission of the oxide ring during dry distillation of an intermediate quaternary bromide has been circumvented by performing the pyrolysis (for methyl bromide extrusion) in 1-heptanol. Quantities of the new tertiary base are being prepared for carbonyl reactions with organo-metallic reagents. The stereochemistry of these reactions and products as well as the pharmacological effects of such

substitutions will be emphasized (L. J. Sargent and B. C. Joshi).

Preliminary Screening of Analgesics

Preliminary evaluation (mice) for analgesic activity and toxicity of new compounds coming principally from outside sources continues. Exploratory experiments on 78 substances, complete assays on 71 compounds and 150 toxicity experiments have been conducted (E. L. Atwell and J. Goodwin), and the data analyzed by statistical (probit) analysis (W. Ness).

Morphine Tolerance Studies

Study of morphine tolerance development as a possible immune response phenomenon was conducted by comparison of the analgesic response (rat, hot plate) of animals treated with a combination of Freund's adjuvent and morphine with that of animals treated with morphine alone. Adjuvant-treated animals retained sensitivity to morphine several months longer than those treated with morphine alone (J. Cochin and J. Goodwin).

Rapid Test for Addiction

Thin-layer chromatography analysis of urine for narcotics and metabolites is rapid and sensitive, complements the Nalline test in the quick detection of addiction, and may be important to prompt treatment of barbiturate overdosage. Several new metabolites have been uncovered by this technique. Thin layer chromatography has also been developed for estimation of other toxic materials as antihistamines, tranquilizers, etc. in blood and tissues. Many clinical and criminological laboratories have adopted these procedures. (J. Cochin and John Daly).

Synthetic Antigens

Polyethyleneimine-amino acid polymers were fabricated and haptenic groups introduced. Several of these are being tested by Dr. Sehon of Canada (T. D. Perrine) for the synthesis of potential antigens containing a strictly hydrocarbon backbone with built-in, judiciously-spaced sugar moieties as determinant groups, practicable syntheses for p-vinyl-phenyl β-glucoside, maltoside and cellobioside have been worked out. These glycosides were purified and characterized extensively for the subsequent polymerization reactions (Lionel Clingman, T. D. Perrine)

Chemistry of Narcotine

A research program involving transformations of the plentiful, relatively non-toxic, little-used opium alkaloid narcotine has been initiated, with a view to exploring the pharmacologic possibilities of derivatives of this versatile chemical. $LiAlH_4$ reduction gave the known diol but the Grignard reaction gave not the reported unsaturated compound, but due to a milder isolation procedure the precursor carbinol (A. E. Jacobson).

Quinazoline Glucosides as Potential Carcinostatics

Because of its overall steric resemblance to purine, quinazoline (as an unnatural base) was selected for nucleoside and nucleotide studies more particularly for elaboration of abnormal nucleosides and ultimate incorporation into small synthetic nucleotides. These base derivatives might be expected to exert biological (carcinostatic) effect by inhibition of certain key enzymes (thymidylate synthetase) or by acting as purine antagonists. They might also be effective as general antiviral agents. Quinazolinedione-mercury, 2,4-diethoxyquinazoline and their tetrahydro counterparts were severally condensed with acetobromoglucose to give three new, acetylated glucosides. Attempts to deacetylate these substances resulted in unexpected cleavage to the initial base and glucose suggesting that O- rather than N-glucosides were formed. The good possibility of rearranging these O→N glucosides is now being considered.

Active Center of Ribonuclease

The revised sequence of ribonuclease, amino acid residues 11–18, has been published jointly by the group at the Rockefeller Institute, the Laboratory of Cellular Physiology and Metalbolism of the NHI and by the Laboratory of Chemistry, NIAMD. Dr. Erhard Gross of the latter group has extended these studies to the non-anzymatic cleavage of the methionine bond of the so-called S-peptide, the 20-unit peptide obtainable from ribonuclease by the action of Nagarse. The heptapeptide 13–20 was purified and characterized on ion exchange columns where it appeared as a double or triple peak depending on subtle differences in the pH of the citrate buffer. Simultaneously there was investigated a synthetic heptapeptide prepared in the laboratory of Prof. K. Hofmann

which also gave rise to one or two corresponding peaks on ion exchange chromatography. It is suspected that the striking sensitivity or subtle changes in pH of the "natural" as well as the synthetic heptapeptide may reflect in some degree a lability which is part of the intrinsic property of the active center of ribonuclease. It is well known that the S-peptide and the ribonuclease core recombine to form an active enzyme. It will now be possible to study the recombination of the core with the two S-peptide fragments, namely, the 13-unit peptide and the heptapeptide and to look for reappearance of enzymatic activity.

These findings have stimulated further applications. It may well be that peptide sequences that are part of the active center or are associated with the mode of action of certain enzymes will not exhibit unusual properties such as conformational, configurational or structural tautomerism or lability until they have been peeled out of their tertiary structure casing. After the loss of stabilization by hydrogen bridges the active center *in situ* or *in nuce* may then show lability phenomena accessible to investigation by physico-chemical methods. So far trypsin has been cleaved into three fragments by cyanogen bromide, whereas any fragments resulting from cleavage of chymotrypsin are apparently still held together by S-S-bridges.

Considerable preliminary work has been devoted to an investigation of the reaction with pepsin with cyanogen bromide (A. Arens). Unlike ribonuclease the stability and solubility of pepsin is unsuitable for reactions at acidic pH. At higher pH, such as pH 5 in acetate buffer, autolysis of pepsin is faster than the reaction with cyanogen bromide which reacts with the ϵ-amino groups of lysine. In addition, pepsin denatures in a manner hitherto not properly recognized by forming 4–5 definable fragments.

The reaction of cyanogen bromide in the pH range of 4–8 is now under study with model amino acids, peptides and proteins (J. Schreiber).

Probing the Tertiary Structure of Proteins by Selective Chemical Methods

The controlled oxidative cleavage of ribonuclease with limited amounts of N-bromosuccinimide has led to a partial assignment of free and buried tyrosyl residues based on a quantitative assay of liberated amino terminal residues. This work has led to an extension in which positive bromine was transferred to ribonuclease from a high-molecular carrier. Ribonuclease in contact with such N-bromosuccinimide polymers lost its enzymatic activity long before any modification of tyrosine residues was noticeable by ultraviolet spectrophotometry *in situ* (L. A. Cohen and J. G. Wilson).

Three-dimensional structures of proteins at the present time are determined only by X-ray crystallography of crystals. There is no satisfactory method for the determination of 3-dimensional structures of proteins in solution under conditions of enzymatic activity. The use of chemically reactive polymers capable of selectively and rapidly modifying functional groups only on the surface may provide an important step forward in this problem.

The principle of selective chemical modification of a protein with regard to its binding capacity has been utilized by N. M. Green in the laboratory of A. Neuberger (St. Mary's Hospital Medical School, London). He was able to adduce spectroscopic evidence for the participation of tryptophan residues in the binding of biotin by avidin. Dr. Green, who has joined the Laboratory of Chemistry, is extending these fruitful studies to the mechanism of binding involved in the complex formed between trypsin and trypsin inhibitor.

A New Dimension in Analytical Sensitivity: Configurational Tautomerism of Cyclopeptides

Thin layer chromatography of the cyclopeptide-antibiotic gramicidin A on silica gel has led to a resolution of gramicidin A into several discrete new tautomers whose concentration is dependent on the polarity of the initial solvent. This mobile equilibrium is interpreted in terms of configurational or conformational tautomers, possibly as a result of epimerization at the asymmetric carbon atoms involved as bridgeheads for the aminoethanol group which is assumed to span the cyclopeptide ring by adding to two peptide carbonyls. These results suggest further application of the powerful tool of thin layer chromatography for the resolution of other open-chain or cyclic peptides into conformational tautomers. In the light

of these findings, the criteria for homogeneity of peptides or proteins may have to be redefined (S. Ishii).

Rapid Configurational Analysis of Peptide Hydrolysates by Combination of Enzymatic Assay With Quantitative Gas Chromatography

Hydrolysates of gramicidin A were treated separately with D- and L-amino acid oxidase. The residual amino acids were converted to the methyl esters of the dinitrophenyl derivatives and then assayed by quantitative gas chromatography. This rapid method clearly showed that gramicidin A consists of a valine-gramicidin (2 D-Val, 2 L-Val) and an isoleucine-gramicidin in which L-valine is partly substituted by L-Ileu. This novel technique is generally applicable to the study of peptides and proteins consisting of L- and D-amino acids (S. Ishii and R. Sarges).

Chemistry of Gramicidin A

The complex splitting pattern of gramicidin A by N-bromoacetamide in aqueous ethanol containing lithium acetate has been investigated by E. Gross and S. Ishii. A new cleavage method has been detected in the action of lithium aluminum hydride which liberated fragments containing lysine, valine and alanine as amino terminal residues. Catalytic hydrogenation of gramicidin over rhodium on alumina in acetic acid containing perchloric acid led to the uptake of 4 moles of hydrogen with no change in the ultraviolet spectrum. The fragmentation of choice has been found in methanolysis at room temperature, a procedure which leaves the tryptophan residues fully intact.

Stereospecific Synthesis of 4-Hydroxyproline and γ-Hydroxyornithine

In collaboration with Prof. N. Izumiya, head of the Dyst. Biochemistry, Kyushu University, the stereochemical investigation of the conversion of allylglycine to *threo*-γ-hydroxyornithine and allo hydroxyproline has been completed. The intermediate *cis*-bromoaminolactone lends itself to the preparation of several other trifunctional amino acids such as the γ-hydroxy-δ-N-hydroxyornithine, the "prosthetic group" of ferrioxamines and ferrimycines from *Actinomycetes*.

Synthesis and Stereochemistry of *cis*- and *trans*-Hydroxyproline, a New Amino Acid of Widespread Occurrence

trans-3-Hydroxyproline has been prepared in 70% yield by stereoselective hydroboration of 3,4-dehydroproline. *cis*-3-Hydroxyproline, prepared from this material via sodium borohydride reduction of the 3-keto compound, was identical with the amino acid discovered in the hydrolysate of telomycin by F. Irreverre. K. Morita and F. Sakiyama have completed a one-step synthesis of 3-hydroxyproline by reacting aminomalonic acid with the addition product of acrolein to sodium bisulfite in a buffer solution. By modification of the conditions yields up to 50% have been obtained of mixtures of *cis*- and *trans*-3-hydroxyproline whose ratio depended on the nature of the buffer system and the cations present.

Selectively tritiated 3-hydroxyproline has been made accessible by K. Morita to Prof. E. Katz, Dept. of Microbiology, Georgetown University, for incorporation studies into the peptide part of actinomycin by *Streptomyces antibioticus*.

Congener of Actinomycin

In collaboration with E. Katz and H. Weissbach, F. Märki has found evidence for the existence of a congener of actinomycin in the culture medium of growing *Streptomyces antibioticus*. At least three different acidic compounds containing the yellow phenoxazine chromophore and the same number of amino acids as actinomycin have been purified by differential extraction and chromatography. These new congeners may differ from actinomycin by the opening of one or two of the lactone rings. Whether they may be precursors or metabolites of actinomycin still remains to be seen.

Studies on Dehydrobufotenine, the Major Ingredient of the Parotid Gland of *Bufo marinus*

The synthesis of dehydrobufotenine and its nor and bisnor derivative has been started in cooperation with Prof. A. Burgstahler of the Univ. of Kansas and the Regis Chemical Co. under a Psychopharmacology contract. In addition to this ring synthetic approach starting from N-tosyl-1,2,3,4-tetrahydro-8-hydroxyquinoline, Dr. John

Daly is investigating the direct conversion of serotonin to tricyclic analogs of dehydrobufotenine by selective oxidation with Fremy's salt.

A number of indolevinylamines, previously thought to represent dehydrobufotenine, have been synthesized and characterized in cooperation with the Regis Chemical Co. These indolevinylamines are not sufficiently stable to permit their evaluation as psychopharmacological agents in studies with intact animals.

The Venom of the Arrow Poison Frogs (Dendrobates) of Southern Colombia

F. Märki has participated in an expedition to the Choco Jungle region of southwestern Colombia and has succeeded, in cooperation with Explorer-Zoologist Mrs. Marte Latham, to collect for the first time a sizeable amount of Colombian arrow poison frogs. By skinning the frogs and extracting the skins immediately after collection he was able to prepare stable extracts of the venom which, even before purification, kills mice in a dosage of fractions of one gamma. Purification by column chromatography and countercurrent distribution has so far led to a 30-fold increase in the activity of the venom in 90% yield. It appears that the venom belongs to a chemical class hitherto not represented in the animal kingdom.

Specificity of Dopamine-β-Hydroxylase

Dr. Daly has made a careful investigation of the potent isosteric inhibitors of dopamine-β-oxidase, e.g., derivatives of benzylhydrazine and O-benzylhydroxylamine. None of these inhibitors has been found to be a substrate for the enzyme.

New Catecholamine Metabolites

6-Hydroxydopamine and 6-hydroxy-(nor)-epinephrine have been converted to their O-methylation products enzymatically *in vitro* and *in vivo*. 2,5-dihydroxy-4-methoxyphenethylamine and -phenethanolamine are significant metabolites of dopamine and possibly also of norepinephrine (J. Daly).

Chemistry of Adrenaline

In collaboration with Dr. R. A. Heacock, Univ. Hospital, Saskatoon, Dr. J. Daly has investigated the NMR spectra of a large number of oxidation products of (nor)epinephrine, isoproterenol and 3,4-dihydroxynorephedrine. The evaluation of these spectra has provided unambiguous proof for the position of the halogen in iodo and bromoaminochromes, which is 7 and not 2, as has been assumed for the past 25 years.

Metabolic N-Methylation of a Purine

In collaboration with Dr. Julius Axelrod, Dr. J. Daly has established the first enzymatic methylation of a normally-occurring purine. An enzyme occurring in rabbit lung converts adenine to 3-methyladenine. Certain other purines are also methylated by this enzyme, but not pyrimidines. So far 3-methyladenine could not be detected in rabbit lung, either in the free form or bound in RNA or DNA.

Oxidation Mechanisms of Tocopherol and Ubiquinones and Their Metabolic Significance

Drs. Dürckheimer and Cohen have found that the ionic oxidation of α-tocopherol leads, via a hydroxydienone intermediate, to α-tocopheroquinone. On the other hand, radical oxidation leads to dimeric products *exclusively*. Since autooxidation in tissues is probably a radical process, the failure to observe the quinone as a metabolic product of tocopherol oxidation is readily explained. However, dimeric materials, identical with those found by chemical methods, have recently been isolated with animal tissues.

Studies on the oxidation of simple quinol phosphates demonstrates that only 20% of the oxidative energy is conserved as metaphosphate, the remainder being lost by hydrolytic formation of inorganic phosphate. Such studies indicate oxidation of a ubiquinol phosphate to be the most likely pathway for synthesis of the desired dienone phosphate.

These chemical studies have a direct bearing on the fate of tocopherol in metabolism and in that respect may help to reveal its possible significance as an antioxidant or its role in fertility and (animal) muscular dystrophy.

Dienone-Phenol Tautomerism and Its Significance in Metabolic Processes

For some time Dr. Cohen and Mr. Jones have been studying the chemistry of phenols and the methods for increasing the contribution of dienones to their structures. Such knowledge has a

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direct bearing on tyrosine metabolism, thyroxine biosynthesis, aromatic hydroxylation and oxidative phosphorylation.

From a study of the effect of substituents on the pK values of hindered phenols, it has been possible to evaluate the importance of solvation at various sites on the phenolic ring. A method for correlating the ultraviolet spectra of phenols with the nature of *para* substituents has been achieved for the first time; similar correlations have been obtained for both infrared and nuclear magnetic resonance spectra. From such data, it has been possible to predict and to demonstrate experimentally that certain phenols will react exclusively as dienones or as quinonemethines.

Synthetic and Degradative Studies of Nucleic Acids

G. W. Milne, John M. Steele and L. A. Cohen have synthesized dihydrothiophene and dihydrothiopyran as potential blocking agents for the 2′-hydroxyl of ribotides. It has been shown that secondary hydroxyl groups react with the reagents and that the resulting thioacetals are cleaved easily, in aqueous solution at 25°, by the action of silver or mercury ions; the necessity for using acid or alkali for the removal of blocking groups is thus avoided. Adenosine monophosphate is converted to a fluorophosphate by the action of dinitrofluorobenzene and triethylamine in dimethylsulfoxide. The activated phosphate may then be converted to a thiophosphate by the action of hydrogen sulfide. The utility of cyclic vinyl thioethers as blocking agents is further explored and methods are being developed for the selective activation of terminal phosphates in oligonucleotides with the aim of selective sequential degradation of nucleotides by chemical methods.

Sea Cucumber Poison

Holothurin, the poisonous saponin, isolated from certain sea cucumbers, has been purified and submitted to hydrolysis. The aglycone appears to be a C–27 or 28 steroid containing two methoxy groups, one double bond, and a five-membered lactone ring. During this work it was found that dimethyl sulfoxide acted as an excellent solvent for cleavage of steroidal digitonides. A nearly quantitative recovery of the sterols is possible. This has some advantages over the conventional method of cleaving steroidal digitonides (C. H. Issidorides, G. V. Nair, I. Kitagawa and E. Mosettig).

Sapogenins

The structure of pennogenin, a steroidal sapogenin, has been partially solved by degradative and nuclear magnetic resonance studies. The controversial position of the hydroxyl has been fixed at C–17 or C–20. In these studies it became of interest to synthesize 20-isocholestane, 17-isocholestane, and 17-iso,20-isocholestane. These compounds are of practical and theoretical significance in the steroid and triterpene field (G. V. Nair and E. Mosettig).

Carcinogenicity and Structure

A six-step synthesis of 3α,12α-dihydroxy-Δ^7-cholenic acid from cholic acid has been perfected. This precursor to apocholic acid, a known carcinogen was desired for correlating carcinogenicity to structure (J. A. Waters and E. Mosettig).

Chemistry of Cortisone

A number of sulfur analogs of corticoids and androgens have been synthesized for the CCNSC program. Among them, the enol acetate of 9α-methyl thioadrenosterone was found to exhibit high antigonadotropic activity. During the course of these preparations a novel rearrangement which consists in the rupture of the -C-N- bond at C–11 of the thiazoline ring and reattachment to C–5 with formation of a thiazine ring was observed. This rearrangement occurred with 2′-methoxythiazolino(4′,11α; 5′,9α)-hydrocortisone and -dihydroadrenosterone (I. Kitagawa and E. Mosettig).

Steroids of Insects

In cooperation with the Entomology Research Division of the Department of Agriculture, the major sterol present in the house fly was identified as campesterol. It was found that the fly selectively takes up the sterol from the CSMA media. It is of some interest to speculate on its relationship to cholesterol, a sterol required for insect growth. In conjunction with the above work, pure β-sitosterol was needed for comparison purposes. It was prepared from stigmasterol by reduction of 3,5-cyclostigmasterol (i-sterol) (J. A. Waters, J. A. Steele and E. Mosettig).

Steroid Biosynthesis in Plants

In the work on the biogenesis of plant steroids (cooperating unit: U.S. Dept. of Agriculture) it was found that the leaves of *D. spiculifora* contain the sapogenin diosgenin, yamogenin and gentrogenin and the sterols β-sitosterol and cholesterol. *The presence of cholesterol in higher plants has been observed for the first time.* It was further found that radioactive mevalonic acid was incorporated into stigmasterol diosgenin and β-sitosterol in the leaves in the ratio 1:2:6. The higher specific activity of the steroids in the leaves as compared to that in the tuber suggests that the former is the active biosynthetic site and the latter a place of storage. The greater radioactivity of β-sitosterol as compared to stigmasterol indicates that stigmasterol is formed from β-sitosterol by dehydrogenation contrary to animal steroid biosynthesis. In solanum tuberosum, radioactive mevalonic acid appears to be preponderantly incorporated into stigmasterol and β-sitosterol. There is evidence that cholesterol is also present (R. D. Bennett, D. F. Johnson and E. Heftmann).

Microbial Hydroxylations of Steroid Alkaloids

The steroidal alkaloids, solasodine and tomatidine have been hydroxylated by the fungus *Helicostylum piriforme* to yield products which have been unambiguously established. They are 9α-hydroxy-, 11α-hydroxy- and 7β-hydroxy-solasodine, and 7α-hydroxy-, 9α-hydroxy- and 7α,11α-dihydroxytomatidine. The steroidal sapogenin diosgenin has also been transformed microbiologically into 7β,11α-dihydroxy- and 11α-hydroxy-7-oxodiosgenin. A third component believed to be 11α-hydroxy-7-oxotigogenin was also isolated. These are the first reported instances of the microbiological transformation of steroidal alkaloids and sapogenins. They are not only useful in the synthesis of hormone analogs but also important in the understanding of steroidal metabolism in plants and animals (Y. Sato, S. Hayakawa and A. Reine).

Role of Steroids in Cell Membranes

Some doubts have been cast on Willmer's theory that steroid hormones regulate cellular permeability by specific interaction with lipids in cell membranes. In model experiments the steroid hormones failed to form stable monomolecular films and did not penetrate lipid monolayers (E. Heftmann).

Steviol as a Growth Factor in Plants

Steviol whose structure was elucidated in this laboratory was found to produce significant growth response in the d-5 dwarf mutant of *Zea mays*. This demonstration of gibberellin activity outside of the group of natural gibberellins changes our present concept of the structural requirements necessary for gibberellin activity and also indicates a possible role of diterpenes in plants (cooperating unit: California Institute of Technology) (E. Mosettig).

Steroid Nature of Florigen, the Flowering Hormone

When inhibitors and radioactive precursors of steroid biosynthesis were applied to short day plants, floral induction was suppressed. Simultaneously, the biosynthesis of stigmasterol and β-sitosterol was inhibited. These experiments shed some light on the chemical nature of the flowering hormone (cooperating unit: California Institute of Technology). They may lead to methods for the control of crop production and in the words of Prof. James Bonner, CALTECH, may have far-reaching effects on the world's food supply (E. Heftmann).

Qualitative Analysis of Steroids

Thin-layer chromatography has been successfully adapted to sterols, steroidal sapogenins and steroidal alkaloids as well as to cortocosteroids. This is the first practical method for resolving the C–25 epimers of sapogenins and the simplest method for the qualitative analysis of aldosterone yet devised. All adrenocortical hormones, in amounts of less than 0.01μg, can be separated and identified in 10 minutes. Useful correlations between structural features and chromatographic mobility have been derived (R. D. Bennett and E. Heftmann).

Automatic Steroid Analyzer

The performance of the steroid analyzer has been tested with respect to sensitivity, precision and accuracy. The efficiency of separation of steroids has been improved by a detailed study of elution gradients. Individual steroids and other migrants can be eluted at any desired interval by

proper selection of gradient cams. The time previously required for complete automatic quantitative analysis of complex mixtures containing all 7 adrenocortical hormones has been reduced by one-half (D. F. Johnson, D. Francois and E. Heftmann).

Infrared Fine Structure of Steroids

A high-resolution infrared spectrophotometer is being used to obtain precise information for absorption band studies and to provide spectra for Lorentzian analysis of absorption band envelopes. Analyses of this kind should provide the chemist with details of molecular structure which, until now, have been hidden in complex absorption envelopes of the fingerprint region (H. K. Miller and Mrs. A. H. Wright).

Rotatory Dispersion of Sugar Lactones

The Rudolph spectropolarimeter is being improved to provide constant schedules of half-intensity band width and precision thermostatting. The instrument has been used in a study of the ORD characteristics of D-ribonolactones.

Microanalytical Services

Approximately 8,200 analytical determinations were carried out for 130 of the NIH research staff and for several scientists in other Government agencies. These included about 7,900 routine microanalyses and approximately 275 nonroutine analysis requiring varying degrees of literature and laboratory investigations.

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Carbohydrate Metabolism

A. Reactions Involving Carbohydrate Polymers (Dr. J. Preiss and Dr. G. Ashwell)

Recent studies on the metabolism of polygalacturonic acid by cell free extracts of adapted bacteria have led to the isolation and identification of a new diketouronic acid, 3-deoxy-D-glycero-2,5-hexodiulosonic acid. The initial monomeric reaction product, 4-deoxy-L-threo-5-hexoseulose uronic acid was found to be acted upon by a specific isomerase to produce the above diketo uronic acid intermediate. The latter, in turn, was reduced in the presence of a DPNH-linked dehydrogenase to form 2-keto-3-deoxy-D-gluconic acid. The subsequent metabolic pathway was shown to be identical to that previously described for glucuronic, galacturonic, and alginic acid.

(Drs. A. Ginsburg and B. M. Gesner) The intriguing hypothesis that mammalian heteropolysaccharides act as recognition surfaces for cellular interactions was investigated using the "homing" reaction of isologous P^{32}-labeled lymphocytes. The "homing phenomenon" means that injected white cells congregate in certain sites in the animal body. It was found that the "homing phenomenon was destroyed by pretreatment with small amounts of glycosidic enzymes (crude clostridial extract) under conditions which had no effect upon the vital staining or motility properties of the lymphocytes. The effect of the glycosidic action was completely abolished by the presence of five sugars (L-fucose, D-mannose, D-galactose, N-acetylglucosamine, and N-acetylgalactosamine). Strikingly, equal amounts of D-glucose exhibited *no* protective effect. The experiments suggest a hitherto unsuspected function of mammalian polysaccharides.

B. Reactions Involving Small Carbohydrate Molecules and Carbohydrate-Containing Coenzymes (Dr. J. Preiss)

A new nucleotide sugar, GDP-mannuronic acid, has been identified and shown to arise by the action of a specific pyridine-linked dehydrogenase upon GSP-mannose. The enzyme was partially purified from a cell-free extract of a bacterium which forms a mannuronic acid containing exopolysaccharide.

(Dr. P. J. O'Brien) The biosynthetic pathways for several unusual sugars which are present in the surface antigenic lipopolysaccharides of certain microorganisms are under investigation. The novel sugar nucleotide, guanosine diphosphate-D-glycero-D-mannoheptose (GDPH) has been isolated from yeast and characterized. An enzyme, GDPH pyrophosphorylase, derived from yeast, cleaves this nucleotide to GTP and heptose-1-P. The reverse reaction apparently represents an activation step in the pathway of heptose incorporation into polysaccharides. A similar study of D-fucosamine biosynthesis is also underway.

(Dr. A. Grollman) The biosynthesis of the oligosaccharides found in milk, both *in vivo* and *in vitro*, was studied. *In vivo* labeling experiments indicated that these oligosaccharides are

built by stepwise addition of various sugars to D-glucose. *In vitro*, particle preparations from lactating mammary glands were found to catalyze the transfer of L-fucose from GDP-L-fucose to lactose, forming the naturally occurring trisaccharide, fucosyl lactose. The total synthesis of this trisacchardide from D-glucose can now be written using known reactions.

(Dr. F. Eisenberg, Jr.) Sucrose is the prototype of a homologous class of non-reducing plant oligosaccharides which serve as storage nutrient for plants and fuel for animal organisms. The interglycoside linkage in these sugars has been shown in studies with transferases to be a high energy linkage, but it has not been possible, in these enzyme studies, to differentiate the glucose-O bond from the fructose-O bond with respect to their stability to cleavage. From consideration of the structure of groups surrounding the linkage, one would predict that the fructose-O bond is the more labile. The results of experiments in which sucrose was hydrolyzed in H_2O^{18} with IR–120 (H) cation exchange resin provide direct proof of this supposition.

(Drs. Y. J. Topper, L. Laster, and S. Segal) It was previously reported that two subjects who had typical symptoms of congenital galactosemia in infancy and who now have no detectable P-gal uridyl transferase enzyme in their red cells are near normal in the capacity to oxidize galactose *in vivo*. Five tissues from one of these subjects have been examined *in vitro* for their ability to convert galactose-1-C^{14} to $C^{14}O_2$. Of these, only liver compared favorably with tissue from control subjects. The finding of such phenotypic differences among several tissues from a single galactosemic patient indicates that galactosemia is a more complex disease entity than has been appreciated hitherto.

Dr. M. Stetten) There exists a mammalian particulate inorganic pyrophosphatase, which has been found to have transphosphorylation activity. It phosphorylates glucose on carbon-6 in the presence of PP. The results of kinetic studies are compatible with the assumption of a single protein on the surface of which glucose and water compete for pyrophosphate. Glucose, rather than water, seems to be the preferred substrate. The possibility exists that under suitable conditions this enzymatic reaction may afford an alternative route for the formation of glucose-6-phosphate.

Other Studies on Biosynthesis

A. Thiamine (Dr. I. G. Leder

The formation of thiamine phosphate is catalyzed by a bakers' yeast enzyme discovered in this laboratory, thiamine-phosphate pyrophosphorylase. This enzyme promotes the condensation of the two ring moieties according to the following equation:

$$\text{"pyrimidine"-PP} + \text{"thiazole"-P} \xrightleftharpoons{Mg^{++}} \text{thiamine-P} + \text{PP}$$

The enzyme has been purified 1,200-fold and crystallized. It sediments as a single peak in the ultracentrifuge (sedimentation constant approximately 11) and moves as a single enzymatically active protein band on disc electrophoresis. Studies on mechanism indicate a direct reaction of the pyrimidyl and thiazyl esters without the participation of an activated pyrimidylenzyme intermediate.

The enzyme "thiazole" kinase, which catalyzes the phosphorylation of "thiazole" to "thiazole" monophosphate, has been partially purified.

B. Fatty Acid Synthesis (Drs. B. Bloom and D. W. Foster)

As an extension of studies into the metabolic fates of the several classes of hydrogen, the incorporation of tritium, derived from tritium oxide, into fatty acids and glucose by surviving tissues has been inactivated. Isotopic palmitic and stearic acids synthesized by rat liver slices in the presence of T_2O have been sequentially degraded in order to determine the intramolecular location of the tritium. It was found that the beta and delta positions contained more isotope than the alpha and gamma positions. This was unexpected since the hydrogens at beta and gamma are derived, as determined with soluble enzyme techniques, primarily from TPNH rather than from protons of the medium. These observations suggest that an exchange reaction, probably mediated by a flavoprotein, is involved in the system which utilizes TPNH for the reduction of carbon-carbon double bonds of fatty acids. Glucose synthesized by the same liver slices was found to contain 2–3 times as much tritium at position 1 as at position 6. Whether this is due to the phosphomannose isomerase catalyzed reaction, reactions of the non-

oxidative sequence of the hexose monophosphate shunt, or some other cause will be determined.

Regulatory Mechanisms and Hormones

A. (Drs. J. Cohen, A. R. Brenneman and Y. J. Topper) In continuation of studies on the metabolic effects of oxytocin and acetylcholine on rat mammary gland *in vitro* it has been found that the stimulation of glucose oxidation effected by these hormones is depressed by puromycin. The depression of the effects is a function of the degree to which puromycin inhibits protein synthesis. Subsequently it was found that whereas the hormones do not alter the incorporation of leucine-1-C^{14} into *total* protein, they do appear to change the distribution of newly synthesized radioactive protein among the various cell constituents. Oxytocin has no such effect on the synthesis of protein by liver *in vitro*, nor does insulin have this effect on surviving mammary gland. The relationship between the effect on protein synthesis and the stimulation of glucose oxidation is under investigation.

B. (Drs. F. Tietze, H. M. Katzen and D. Stetten, Jr.) Both immunological and adipose tissue assays indicate that the oxidation of reduced insulin in the presence of oxidized glutathione and the enzyme glutathione-insulin transhydrogenase leads to the formation of insulin-like activity in excess of that formed in the absence of the enzyme. This observation constitutes the first indication that an enzyme can influence the establishment of the "correct" disulfide linkages of a protein. It suggests that enzymes might be involved in the *in vivo* establishment of the three-dimensional structure of proteins subsequent to the formation of the polypeptide backbone.

Nucleic Acids and Other Polynucleotides (Dr. M. F. Singer)

Several new investigations on polynucleotide phosphorylase were completed. In one, the arsenate-dependent breakdown of nucleoside diphosphates by polynucleotide phosphorylase was found to be stimulated 10 to 100-fold by the addition of oligonucleotides having unesterified C-3′-hydroxyl groups. The oligonucleotide acts catalytically and kinetic evidence indicates that the oligonucleotide acts as a second substrate in the reaction. A general mechanism involving the formation of a new phosphodiester bond between the nucleoside diphosphate and oligonucleotide and its subsequent arsenolysis was developed for this reaction. This mechanism also applies to other reactions catalyzed by the enzyme.

(Drs. M. F. Singer and F. N. Brenneman) Polymerization experiments were carried out with various ratios of GDP to the other nucleoside diphosphates. Under certain conditions there is an enrichment of guanylic acid residues in the polymer, along with an inhibition of reaction rate.

(Drs. M. F. Singer, O. W. Jones and M. N. Nirenberg) It has also been found that the efficiency of a synthetic copolymer containing guanylic acid, poly GU, when acting as messenger RNA, is related to the extent of secondary structure in the polymer. The data strongly suggest that messenger RNA must be single stranded and without extensive intramolecular hydrogen bonding.

(Dr. L. A. Heppel) A study of specific polymer inhibition of polynucleotide phosphorylase has shown that the enzyme is specifically inhibited if nucleoside diphosphate and polymer have bases that form a hydrogen-bonding pair. For example, poly A inhibits UDP polymerization and poly I inhibits ADP polymerization. In the latter case it is possible to show that the enzyme inhibition is sharply temperature-sensitive, as if a complex were being "melted out."

(Dr. M. N. Lipset) As mentioned above, guanylic residues in polynucleotides appear to have special properties. Guanylic polyribonucleotides, even as small as GpG, have a strong tendency to aggregate, forming a viscous, high-molecular weight complex that is almost completely inaccessible to enzymic attack. This process of aggregation makes it impossible to separate guanine oligonucleotides by ordinary methods. However, it was discovered that they can be separated by DEAE chromatography in the presence of 7 M urea. The pure guanine oligonucleotides, thus made available, are now being investigated as to their own interactions as well as complex formation with cytosine-containing polynucleotides.

(Drs. M. Gellert, M. N. Lipsett, and D. R. Davies) The monomer, 5–GMP, is able by itself to form a viscous aggregate, of highly ordered struc-

ture. These special properties of guanylic residues are probably of great significance in nucleic acid function.

(Drs. D. R. Harkness and R. J. Hilmoe) Alkaline phosphatase from *E. coli* has now been highly purified and freed of nuclease and diesterase activity. It was possible to use this enzyme as a reagent to remove specifically the 5′-terminal phosphate from s-RNA. It was found that removal of the terminal phosphate did not injure the ability of s-RNA to accept amino acid or to transfer amino acid to the ribosome.

Biochemical Studies of Lysogeny (Drs. D. Korn and A. Weissbach)

Biochemical studies of lysogeny in *E. coli* K12 have shown that the DNA polymerase present in these cells is the same as that found in lysogenically induced cells. However, lysogenic induction of *E. coli* K12 λ has been shown to cause the formation of a specific new deoxyribonuclease whose properties are being studied. This enzyme is also found after infection of K12S cells with lethal mutants of the phage λ. It has also been found that lysongenic K12 cells which require thymine (K12 λ Thy$^-$) can be induced to form mature λ if they are suspended in a thymineless media for a certain time and then are resuspended in complete media.

Enzymatic Utilization of Model Compounds (Dr. W. B. Jakoby)

Continuing studies on the enzymatic utilization of model compounds have led to the isolation of three enzymes catalyzing deamidation, from three separate microorganisms. The enzymes differ from each other in their substrate specificity reacting with acetamide, butyramide, and benzamide, respectively. Preliminary experiments indicate the possibility of a transfer reaction with hydroxylamine, resulting in the formation of the appropriate hydroxamic acid, as well as a deamidation of an amide with the formation of the respective free acid.

(Dr. A. J. Aspen) Studies on the oxidation of threonic acid have resulted in the isolation of a highly purified preparation from a pseudomonad which catalyzes the DPN-linked oxidation of threonic acid to β-keto threonic acid. The latter, in turn, is decarboxylated to form dihydroxy acetone.

LABORATORY OF EXPERIMENTAL PATHOLOGY

Anatomical Pathology

This Laboratory continues to provide consultative pathology services to the Division of Indian Health and other facilities of the Public Health Service which has stimulated interest in aspects of geographic and environmental pathology. In addition, the specimens provide a source of human material for histochemical and other investigations. There has been a substantial increase particularly in the number of surgical specimens submitted. Problems related to sarcoidosis, diabetes, dietary hemosiderosis, cholecystitis, and to atherosclerosis and its complications are of particular interest. Consultative service to two Korean Charity Hospitals has resulted in the collection of more than 50 cases of fatal *Pneumocystis carinii* infection in children.

In addition to the research projects summarized separately, certain scientists in other laboratories received advice from members of our staff, particularly in pathologic anatomy. Our histopathologic preparation unit also took part in this cooperative effort by cutting and staining sections from about 1,700 animals this year for 18 investigators not in laboratories of NIAMD.

Pathologic studies of tissues from nutritional experiments under the direction of Dr. F. Daft, NIAMD, are continuing. Subjects currently under investigation include acute hemorrhagic renal necrosis in choline-deficient rats and dietary hepatic necrosis in vitamin E and selenium deficient rats (Dr. Horn).

Altitude Studies

The effects of age and exercise on altitude tolerance in rats continue to be investigated in cooperation with Dr. Altland. It was found that young adult rats withstood exposure to 34,000 feet better than younger or older animals and that exercise during exposure reduced tolerance by 3–4,000 feet. Females fared better than males except in late pregnancy. The poor altitude tolerance of older rats did not seem to be related to myocardial degeneration, chronic nephrosis, or chronic murine pneumonia of moderate severity. More severe lung lesions, characterized by the presence of multiple large bronchiectatic cavities containing

necrotic exudate, had a pronounced effect in reducing survival to altitude exposure and were particularly frequent in older males (Dr. Highman).

Bacterial Endocarditis

Evidence was obtained that the marked susceptibility of high altitude rats to bacterial endocarditis is not due merely to hyperactivity of the adrenal or to impaired antibody formation. It required the presence of both polycythemia (increased cardiac work load) and hypoxia (additional deleterious factor). It is postulated that hypoxia causes biochemical and enzymatic changes that impairs the ability of host phagocytic cells to destroy certain bacteria. A similar explanation is proposed to account for the development of bacterial endocarditis in 82–100% of rats maintained in a cold room at 1.7°C for 1 to 36 days before and 7 days after an intravenous injection of a culture of *Streptococcus mitis*, JH 26. In control rats maintained in a normal temperature environment and in a group of cold rats returned to a normal temperature environment after the bacterial inoculation, the incidence of induced severe bacterial endocarditis was only 16–19%. In rats acclimatized to cold 35–36 days, the mortality rate was lower and the length of survival was longer than in rats placed in the cold room only one day before the bacterial inoculation (Dr. Highman).

The tissue source of the serum enzymes which increase in animals subjected to various stress is being investigated. Electrophoretic separation of the elevated serum enzymes into their isozymic components will be utilized to elucidate this problem (Dr. Garbus).

Cytogenetic Studies

(1) In studies of the karyotypes in patients with congenital disorders, the following abnormal karyotypes have been found among 50 patients referred by clinical investigators of NHI, NINDB, NCI and private physicians:

(a) Down's Syndrome with 47/trisomy 21 (6 cases)
(b) Mental retardation (?) with 47/trisomy 18 (1 case)
(c) Phenotypic female with Turner's syndrome with 45/XO (4 cases)
(d) Phenotypic female with Turner's syndrome with 45/XO and 46/mosaicism (1 case)
(e) Identical twin sisters of wnich one is a typical Turner's and the other was apparently normal and menstruating. However, both had the same chromosomal abnormality, i.e. mosaic 45/XO and 47/XXX
(f) Female with 47/XXX (1 case)
(g) Phenotypic male baby with androgenital syndrome with 46/XX (1 case)
(h) Female with testicular feminization syndrome 46/XY (1 case)
(i) Male with Klinefelter's Syndrome 47/XXY (3 cases)
(j) Male with 47/XYY (1 case)
(k) True hermaphrodite with 46/XY (1 case)

(2) In cooperation with DBS–LVR chromosome studies have been carried out on an SV 40 virus susceptible cell line designated BS–C–1 established from kidney tissue of *Cercopithecus aethiops* as part of their program on the development of tissue culture lines for use in testing of vaccines. This line is now in its 125th passage and has retained its sensitivity to infection with SV 40 virus although the chromosome complement was found to have changed from diploid (up to the 30th passage) to subdiploid (at the 41st passage) and finally to subtetraploid at the 111th passage.

(3) In cooperation with the NCI–MB, studies of the chromosomes of malignant cells in patients with leukemia and related diseases have continued.

(a) Studies on the chromosomes of a patient with lymphoblastic lymphosarcoma, leukocytosis, eosinophilia and granulocytic hyperplasia of the bone marrow revealed a consistent chromosomal abnormality in the bone marrow specimens taken at various periods, before and after treatment and also in one lymphnode aspirate. There were consistently 2 chromosomes missing, one each in group 6–12 and 13–15, respectively. Instead, two new chromosome types were present. One was very similar to pair 3 and the other was acrocentric and slightly longer than pair 21. The skin fibroblast and peripheral leukocytic cultures had a normal female karyotype, thus excluding the possibility that the patient had an abnormal chromosome constitution.

(b) Of 40 patients with chronic myelogenous leukemia studied, 34 revealed the so-called Ph^1 chromosome (a small chromosome replacing one

of group 21–22 in the marrow cells). The cells with this specific marker persisted after therapeutic treatment and also during remission.

Of 32 treated patients with chronic myelogenous leukemia, 9 had improvement in their bone narrow to the extent that erythroid elements composed 20–60% of the nucleated cells. In 6 of these 9 patients, 100% of the scored metaphases in the marrow contained the Ph^1 chromosome. Differential counts of cells in mitosis on Giemsa stained marrow smears from these 6 patients showed 30–80% of the mitoses to be of the erythroid series. Thus it may be assumed that the erythroid as well as the myeloid cells of the marrow from CML patients apparently contain the Ph^1 chromosome.

(c) Acute Leukemia. We have studied the chromosomes in bone marrow from 31 ALL and 21 AML patients. The majority of the patients with acute leukemia have a diploid modality with a small range of aneuploid cells. There is no specific pattern in the few chromosomally abnormal cases. They could be irregularities to be expected during progression of the malignant disease. But if chromosomal abnormalities were an expression of the degree of malignancy, the incidence and variety of such abnormalities might be expected to correlate roughly with the acuteness of the leukemia. However, from the information available it does not appear that the abnormal cases were more acute, either clinically or hematologically, than those found to be of normal karyotype, except perhaps for some so-called terminal cases in the blast stage where we found numerical and structural changes in 4 out of 5 cases examined. The relation between cancerogenesis and chromosomal changes in some of our cases is alternatively (1) autonomy induced by the chromosome changes, either by these changes alone or by the combined effect of the changes and factors agents such as virus or (2) autonomy induced prior to chromosome change. Since about 75% of the acute leukemias studied by us have a dipoid stemline with apparently normal karyotype, we believe that these apparently normal cells were malignant and that the second alternative seems to be more likely for our acute leukemia cases. In acute leukemia evidently the karyotype may remain normal after the change into malignancy (Dr. Tjio).

Carcinogenicity of *Cycas circinalis L.*

Interst in the nut from *Cycas circinalis L.*, originated when a search for a possible neurotoxin was undertaken which might, in part at least, contribute to the unusually high incidence of amyotrophic lateral sclerosis on the island of Guam (Drs. Kurland and Whiting of NINDB). Experiments were set up in collaboration with Dr. Mickelsen of NIAMD to investigate the effect on the central nervous system of rats of the untreated dried, powdered nut. Although no histological evidence of a neurotoxin in the nut was found, benign and malignant metastasizing neoplasms, epithelial and mesenchymal in type, were found singly or in various combinations in livers, kidneys and occasionally in the lung in rats which had been on one of the experimental diets for more than three and one-half months. The absence of similar proliferative and neoplastic lesions in appropriate control groups strongly indicated the presence of a carcinogenic factor in the untreated nut. The neoplastic and hepatotoxic effects are similar to those produced by nitrosamines. Investigations are being continued to establish the nature of the carcinogenic agent (Dr. Laqueur).

Enzyme Histochemistry

Work has continued on the characterization of the mammalian peptidase specific for the hydrolysis of N-terminal α-1-glutamyl and aspartyl residues. This enzyme has been partially purified and characterized by biochemical techniques in conjunction with Dr. J. E. Folk of NIDR. It is calcium activated and EDTA (versene) inhibited, and is demonstrable histochemically in such tissue sites as guinea pig pancreatic duct epithelium and islets of Langerhans and in human and rat glomerular epithelium and proximal tubule brush borders. No hydrolysis of N^{α}-substituted glutamyl peptides or of the d-enantiomorph was observed in the presence of high concentrations of the enzyme. The above data and the absence of hydrolysis of N(α-d-glutamyl)β-naphthylamide and N(N^{α}-benzoyl-α-d-glutamyl)β-naphthylamide in both the histochemical and biochemical systems and of N-carbobenzoxy-α-1-glutamyl-1-phenylalanine in the biochemical system indicates that the enzyme is stereospecific, exhibits primarily exo-

peptidase activity and has a relative specificity for the hydrolysis of N-terminal 1-dicarboxylic acid peptides (Drs. McMillan and Glenner).

In order to relate histochemical enzyme characteristics with those of a biochemical system, a technique has been devised for determining enzyme kinetic constants in a histochemical system. By means of this technique which utilizes a constant chromatic endpoint, a double reciprocal plot of velocity and substrate concentration (Lineweaver-Burk) can be constructed for the activity of an enzyme in a specific tissue site. From these data and using competitive inhibitors the kinetic constants K_m, V_m and K_i can be determined. In this way it has been possible to show the presence in human mast cells of an enzyme having both esterase and amidase activity and characteristics similar but not identical to trypsin. This technique is applicable to instrumental measurement and such an application is in progrses (Drs. Hopsu, McMillan and Glenner).

Eosinophilic Meningo-encephalitis

In recent collaborative studies with Drs. Rosen and Weinstein of NIAID an eosinophilic meningoencephalitis in man presumably has resulted from cerebral involvement by a metastrongylid lung worm of rats, *Angiostrongylus cantonensis*. Pathologic studies of monkey brain and spinal cords at various intervals after oral ingestion of *A. cantonensis* larvae demonstrated severe and diffuse eosinophilic meningo-encephalitis and parasites in the brain at day 17 after infection. The lesions in the experimentally infected monkeys closely resembled those seen previously in the spontaneous human infection and differed greatly from lesions produced in the rat, which is the natural host (Dr. Laqueur).

Experimental Obesity

The pathogenesis of the obesity in mice which results from the administration of goldthioglucose was restudied in cooperation with Drs. Schwartz and Cronkite of the Brookhaven National Laboratory. Using activation analysis and radioautography as well as serial sections and routine histologic methods, it could be shown that goldthioglucose damages several areas in the brain, including the hypothalamic area which contains the "satiety" center. The satiety center is often damaged only marginally by goldthioglucose. Only those animals in which this center was entirely destroyed developed obesity (Dr. Brecher).

Hematology

The concept proposed earlier that short lived macrocytes are produced after intense erythroid stimulation received further support with the demonstration of their production in iron deficient animals or human beings in response to iron therapy (Drs. Moores, Stohlman and Brecher) and in the regenerative phase of post irradiation anemia (Dr. Stohlman). Splenectomy did not affect the life span of these cells (Drs. Moores, Stohlman).

Further studies on the growth of small peripheral blood lymphocytes demonstrated that RNA synthesis begins about 24 hours prior to DNA synthesis; the morphologic changes in culture were further delineated by electron microscopy (Drs. Epstein, Stohlman, Brecher, Tanaka).

Proliferative capacities of peripheral blood cells are being studied by autoradiographic techniques which measure *in vitro* incorporation of tritiated thymidine into DNA. Blood withdrawn from patients with infectious mononucleosis during the first 2–3 weeks of illness has a marked increase in the percent of mononuclear cells in DNA synthesis as compared with normal controls. The percentage decreases steadily as the illness progresses. In contrast, the percentage of mononuclear cells in DNA synthesis in patients with leukemia varies widely and in many instances is normal. This would imply that high proliferative capacity is not a requisite for malignancy (Drs. Epstein and Brecher).

Hemoglobin

A sub-molecular mechanism of sickle cell formation was deduced from the optical rotary dispersion data and a series of experiments with a scale model building of the N-terminal segment of the β-chain where the genetic abnormality of sickle cell hemoglobin (HgbS) is located. This defect consisting of the substitution of valine for the glutamic acid present in normal hemoglobin (HbA) at position 6 permits the stabilization of the N-terminal polypeptide chain of HgbS in a ring structure. The amino terminal *Valyl* residue and the abnormal *Valyl* of Hb-S molecule can interlock by van der Walls forces of hydrophobic bonds; this allows a ring structure to form from

the N of Histidyl to the carbonyl group of Threonyl residue. This conformation appears to allow stacking of the molecule which results in a tactoid (or sickle cell) formation.

When the 6th residue is glutamyl as in Hb-A, it appears that this ring closure does not occur due to electrostatic repulsion of the carboxyl groups. In Hb-S at 38° this segment of the peptide chain is stabilized through this cyclization by hydrogen bonding, thus the freedom of rotation about bonds in rings formed is restricted. This is the condition necessary and sufficient for one of the Kautzmann and Eyring rules which states: "Those influences which tend to restrict freedom of orientation about bonds will tend to increase the order of magnitude of the optical rotation." But at 0° the hydrogen bonds under question appear to be transferred in a process of hydration (because hydrophobic bonds are weaker at 0°) and the chain appears to assume a conformation of no ring formation as in the β-chain of Hb-A; thus, the numerical value of the optical rotation should therefore be diminished at 0° for Hb–S hemolysate.

The optical rotatory dispersion studies of the Hb-A and Hb-S hemolysates showed that the amplitude of the positive Cotton effect is increased reversibly by about four times when the sickle cell hemolysate was warmed to 38° from 0°; the normal hemolysate does not have this property. Another physical property of Hgb-S that may be explainable in terms of the conformational alterations of the molecular model outlined above is the negative temperature coefficient of gelation, i.e., Hb-S hemolysate gels upon deoxygenation at 38°; this *gel* melts at 0°.

The mercapto-mercapto interactions in the normal hemoglobin have been compared with those in the sickle cell hemoglobin; there is no significant difference in the mercapto-mercapto interaction constants between these hemoglobin molecules.

The mercapto-mercapto interaction constants between the mercuric ion, methyl mercuric hydroxide, and ethyl mercuric hydroxide have been evaluated using the normal human and the horse hemoglobins. The energy barrier due to steric hindrance for the methyl group amounts to about 2 kilocalories $mole^{-1}$. This finding is consistent with the basic notion of the steric hindrance theory postulated for the concept of the mercapto-mercapto interactions. (This is analogous to the well known heme-heme interactions in the process of O_2 binding; when mercurials are in the process of binding the mercapto groups, the mercapto-mercapto interactions take place in the molecule) (Dr. Murayama).

Histochemistry of Mucopolysaccharides

Interest in differentiating and characterizing mammalian mucopolysaccharides by histochemical methods has continued. Efforts have been directed primarily toward development of specific histochemical methods for localizing the different mucopolysaccharides, secondarily toward application of these methods. Modification of a previously developed diamine method to include a mixture of diamines and ferric iron has provided a technique which is sensitive and almost completely specific for acid mucopolysaccharides and by several variations in the technique differentiates between sulfated and non-sulfated (usually sialic acid containing) entities. With or without added iron, the mixed diamines reveal further a number of as yet unexplained difference between members in both categories. It has also been found that an alcian blue-safranin sequence, in which the pH of the alcian blue step is varied, likewise distinguishes sulfo- from sialo- mucins and with alcian blue at pH 0.5 distinguishes further between types of sulfomucins. The mixed diamine procedure differentiates either sulfated or sialic acid containing mucins which possess vic glycols (and hence presumably hexose) in proximity of the acid group. Mucins losing the diamine staining as a result of prior oxidation with periodate are those identified as having vic glycols in stearic proximity to the acid groups. This property although not a recognized characteristic of any biochemically known mammalian acid mucopolysaccharide can be demonstrated by the histochemical procedure. It can also be clearly shown with a sequence of diamine followed by alcian blue or the Hale colloidal iron stain for acid mucopolysaccharides. In this instance the periodate reactive (hexose containing) acid mucosubstances lose alcian blue or colloidal iron staining after a periodate diamine step in contrast with the periodate unreactive polymers which retain such staining unimpaired by oxidation (Dr. Spicer). Application of these methods has been undertaken in attempting to characterize mucosubstances in pathologic states including

autopsied cases of cystic fibrosis, malignancies of various sites and certain inflammatory lesions (Drs. Spicer and Duvenci).

Hypersensitivity

In a previous study of alterations in circulating leukocytes during induction of the generalized Shwartzman reaction in rabbits, prominent metachromatic cytoplasmic granules were noted in circulating neutrophiles. Histochemical and autoradiographic studies have indicated that these metachromatic granules are similar to and presumably derived from the azurophile granules of the immature granulocytes of the bone marrow. The metachromatic substance of the azurophile granules, previously assumed to be ribonucleic acid, has been characterized as a sulfate-containing acid mucopolysaccharide, in combination with a strongly basic protein. A similar constituent, occurring in certain granules in immature eosinophiles, is less basophilic, and several observations indicate that strong bonding between acid groups of the polysaccharide and basic groups of the protein may account for its weaker affinity for basic dyes (Drs. Horn and Spicer).

Immunochemical Studies

A. *Glyceraldehyde-3-phosphate dehydrogenase*

Previous investigations dealing with the localization of glyceraldehyde-3-phosphate dehydrogenase (GAPD) by fluorescent antibody technique and its distribution in adult striated skeletal muscle of the cockroach have been extended to include a study of the presence and distribution of the enzyme in the developing myoblasts. The enzyme is found in higher concentration in the heart myoblast than in the skeletal myoblast and is localized in particular but not all mitochondria. In the culture of the heart myoblast the enzyme has been found in highest concentration in the small fibrocytes which adhere closely to the myoblast (Dr. Emmart in collaboration with Dr. Kominz of NIAMD and Dr. J. Miquel formerly of NINDB).

The localization of this enzyme (GAPD) has been further studied in rat kidneys. GAPD was not observed in the glomeruli and varied in concentration in different areas of the tubules. It was especially noted in adventitial cells of the medulla (Drs. Emmart and Spicer in collaboration with Dr. Schimke, NIAMD).

B. *Prolactin*

Immunochemical studies have been carried out in collaboration with Drs. Condliffe and Bates with fractions of prolactin prepared by chromatography on DEAE column and also by countercurrent distribution (Dr. Emmart). By means of fluorescein antibody, prolactin has been localized in the acidophilic cells of the anterior pituitary of the cat, rat and mouse (Drs. Emmart and Spicer in collaboration with Dr. Bates of NIAMD).

Juxtaglomerular Apparatus

In collaboration with Dr. G. Kaley of New York University, evidence has been accumulated to suggest that the renal granular juxtaglomerular cells are related to erythropoietin production in the rat. A direct relationship has been found between the secretory activity of these cells and the plasma erythropoietin levels. In rats the administration of cobalt, or exposure to hypoxia, or phenylhydrazine, or oligenic shock results in a distinct elevation of both parameters. Also, rats that have been experimentally depleted of their juxtaglomerular cell granularity show a very poor response to erythropoietic stimuli (Dr. Demopoulos).

Melanoma

The tyrosinase inhibitor, DL-β-phenyl lactic acid (PLA), decreases oxygen consumption in cells of pigmented S–91 melanomas, but not in amelanotic cells of the same tumor which are devoid of tyrosinase. This inhibition of respiration, which can be demonstrated in a tyrosine-free medium is accompanied by a simultaneous increase in aerobic glycolysis. Since the specific PLA-susceptible segment in melanotic S–91 cells is inhibited by PLA in a manner which is unique and similar to the way PLA inhibits S–91 tryosinase, it is postulated that the former segment of cell respiration is attributable to tyrosinase. Growth studies, *in vitro*, correlate fairly well with the manometric results. Phenyl lactic acid in levels of 1–1.5mM/liter selectively prevent growth of melanotic S–91 explants if present at zero time in their growth curve. Delayed addition of PLA to cultures that have incubated for more than two hours

in normal medium is without effects. The selectivity of phenyl lactic acid is not as marked in tissue culture as it is manometrically since higher concentrations (100% increase over 1 mM) of this agent can affect growth of non-melanotic control tissues as well. The de-aminated analogues of phenylalanine can inhibit protein synthesis *in vitro*, in high concentrations, by blocking tyrosine incorporation. This may offer a clue to the non-specific growth inhibiting effects of high doses of PLA. This inhibition can also prevent growth of melanotic S–91 tumors freshly transplanted into young DBA/2 mice while growth of their somatic tissues is not impaired, but it has no effects on established tumors (Dr. Demopoulos).

Metabolic Requirements for Cardiac Lesions in the Carcinoid Syndrome

In an attempt to reproduce in experimental animals the cardiac lesions seen in some human patients with the carcinoid syndrome, long and short term metabolic studies with guinea pigs and rabbits have been undertaken. These animals have been subjected to a niacin and tryptophan deficient diet for varying time periods and exposed to liver damage and exogenous 5-hydroxy-tryptamine, simulating the hepatic metastases and tryptophan deficiency of patients with the carcinoid syndrome having cardiac valvular lesions. A valvular endocarditis comparable in some respects to that seen in humans with the carcinoid syndrome has now been produced. A further evaluation of the causative mechanism involved in this unique disease process is in progress (Dr. Spatz).

Monoamine Oxidase Inhibitors

Monoamine oxidase inhibitors have been widely used in the treatment of mental depression and hypertension. In a collaborative study with Dr. Maling of the NHI, it was found that prolonged administration in dogs of two of these inhibitors, phenylisopropylhydrazine (JB 516) or phenylisobutylhydrazine (JB 835), consistently produced marked neurologic effects, including ataxia, tremors, nystagmus, and impaired motor coordination. Neuropathologic lesions involving the inferior olivary nucleus and less frequently the pyriform lobe, particularly the amygdaloid nuclei were seen in dogs but not in cats, rabbits or squirrel monkeys. The lesions are not due directly to monoamine oxidase inhibition, since elevations in serotonin levels in the pyriform lobe and brain stem due to such inhibition were produced by other monoamine oxidase inhibitors in animals showing no such lesions (Dr. Highman).

Pathology of Rheumatic Disease

The role of the blood vessels in the histogenesis of the subcutaneous nodula of rheumatoid arthritis has been demonstrated graphically by a three-dimensional reconstruction of an early lesion. It was based on more than 500 serial sections of a nodule only 3 days old. The inflammatory process was found to center about damaged segments of the terminal vascular bed, including minute arteries, arterioles, capillaries and venules. The episodic and centrifugal character of the process also were apparent from the reconstruction. The observations are believed to lend support to the thesis that inflammation of minute blood vessels is an integral part of the pathogenesis of rheumatoid arthritis.

A new interpretation of the nature of the elasticity of articular cartilage and a method for investigating its component elements have been developed in collaboration with Dr. S. M. Elmore, NHI, and members of the Instrument and Engineering Branch. The apparatus measures the deformation and recovery of articular cartilage during and following static loading under conditions of immersion. Older views of the plastic nature of cartilage were found to be based on tecchnical artefacts arising from drying of the tissues. The compressibility of the tissue was greatly affected by the ionic environment of the bath. The findings are of fundamental significance in the normal physiology of joints and are being pursued as a promising lead to understanding the pathogenesis of osteroarthritis (Dr. Sokoloff).

In view of the continued use of gold compounds in the treatment of rheumatoid arthritis, it was thought desirable to apply the activation analysis-autoradiographic method as described in the obesity study to determine the distribution of gold in the joints of mice following gold administration. The findings suggested selective uptake of gold-thioglucose as well as goldthiomalate in areas of rapid bone renewal and in portions of the synovium (Dr. Brecher).

Studies in Fine Structure

Ultrastructural detail revealed by electron microscopy and the localization of acid phosphatase activity are providing new insight into the mechanisms of thyroxin mobilization by rat thyroid glands. Thyroid stimulating hormone (TSH) stimulation of hypophysectomized animals permits the observation of this phenomenon as a function of time (Drs. Wetzel and Spicer in collaboration with Dr .Wollman, NCI).

Descriptive anatomical studies of the Turbellarian genus *Dugesia* have been extended with particular emphasis on the excretory organs of this animal. An outstanding degree of regional cell specialization along the nephric unit clearly indicates a high degree of corresponding functional specialization in this phylogenetically primitive form (Dr. Wetzel).

Collaborative studies with Dr. Mortimore on isolated, cyclically perfused rat livers indicate that over a period of one and one-half hours the fine structural integrity of hepatic parenchymal cells can be maintained while many physiological and biochemical parameters are accurately monitored and controlled. An absence of physiological concentrations of insulin from such a perfusate, for example, results in extensive disorganization of normally parallel arrays of rough surfaced endoplasmic reticulum. A marked decrease in protein synthesis and in amino acid incorporation by these experimental livers was observed concomitantly, thus providing additional support for the role of organized rough surfaced endoplasmic reticulum in the production of protein. The apparent lability of this system is of particular interest and will be further investigated (Dr. Wetzel).

A study is under way on the nuclear and nucleolar cytology of basic proteins with acid dyes and their modification by heavy metal salts with the objective of developing a differential cytochemical staining method for both light and electron microscopy (Dr. Suskind).

Factor 3—Selenium

The physiological significance of the element selenium was discovered several years ago (K. Schwarz and collaborators) when selenium was found to be the integral part of the so-called Factor 3, which prevents dietary liver necrosis. Since then, 18 species have been found in which fatal or severe deficiency diseases can be prevented by trace amounts of selenium compounds. The so-called white muscle disease of selenium deficiency in sheep and in cows, is of economic importance.

The rat bioassay has been used to determine the relative biopotencies of different selenium compounds. However, in recent studies by Dr. Schubert and collaborators (Oregon State University) γ,γ-diselenodivaleric acid, highly potent in the rat, was found to be relatively ineffective against white muscle disease in lambs. The result indicates that biopotencies of different selenium compounds may vary from species to species.

Organoselenium compounds, synthesized by Prof. Dr. Arne Fredga at the Univ. of Uppsala, Sweden, in collaboration with this laboratory, has led to a number of new findings. Fatty acids containing selenium in place of a CH_2 group at various distances from the carboxyl group show no difference in biopotency with the element at an even or uneven numbered position in the molecule (K. Schwarz and J. P. Stesney). The biopotency of most of these compounds is similar to that of selenite selenium. However, the 9-selena- penta- and hexadecanoic acids, and the 12-selena- penta- and hexadecanoic acids, are substantially less effective than selenite, suggesting their possible use as chemotherapeutic agents against protozoa. The most effective Factor 3-active selenium compound synthesized thus far is benzyl-seleno-ω-pelargonic acid, with an ED_{50} of .8 μg per 100 g of diet. This is close to .7, the value originally found for natural Factor 3.

Using Se^{75} as a marker, five fractions containing the main selenium carrying components in urine have been isolated in amounts satisfactory for Factor 3 bioassay in the rat (E. Sweeney and R. Jacobs). Application of more sensitive, new techniques of paper chromatography to the urine of rats after supplementation of radioactive selenite selenium has shown that there are about 15 different organoselenium components excreted. None of them seems to be inorganic.

Since there are large differences in the selenium contents of various soil formations, the Factor 3 potency of skim milk powders from various geographical areas of the USA, put at our disposal by the Atomic Energy Commission, has been determined by bioassay against dietary liver necrosis in the rat. While skim milk powder from some areas, notably Oklahoma and North Dakota,

afforded almost complete protection when added to the diet at 5% levels, those from some other areas were without appreciable effect even at 15% levels. The Factor 3 activity was not influenced by the method used for making the dried milk.

To establish the therapeutic index, the LD_{50} for various selenium compounds was determined in rats raised and maintained under conditions identical to those used in the Factor 3 bioassay procedure (L. Hopkins). For selenite selenium, a ratio of ca 2000:1 was found between the LD_{50} when applied orally, and the 50% effective daily dose required to prevent necrotic liver degeneration. No direct correlation exists between Factor 3 potency and toxicity. γ,γ-Diselenodivaleric acid, when compared to selenite selenium, was only 60 percent as toxic as the latter, in spite of its higher biopotency in the Factor 3 assay.

The selenium content of a variety of purified enzymes, many of them crystalline, varied from 0 to 105 μg per g of enzyme. If stoichiometric levels of selenium were present in bound form, the element could be expected to amount to ca 1 mg, assuming a molcular weight of the enzyme of ca 80000.

Respiratory Decline, Sulfhydryl Groups and the Mode of Action of Tocopherol

In studies on respiratory decline (K. Schwarz and C. Lee), the metabolic impairment seen in prenecrotic livers of animals on necrosis producing, deficient diets, it was ascertained that respiratory breakdown and the protective effect of tocopherol and other naturally occurring quinonoid substances is not related to the presence of sodium ions in the medium, or to the breakdown of ion transport mechanisms. The substrate specificity of respiratory decline and the protective effects of tocopherol were studied. The phenomenon occurs only with certain substrates of the Krebs cycle. Primarily involved are α-ketoglutarate, pyruvate plus malate, and also fumarate and malate. With these acids, O_2 consumption declines by 70–90% in the absence of tocopherol. With succinate, as well as pyruvate alone, or citrate, losses of O_2 consumption are less pronounced, and the protective supplements have no effect.

Lipid peroxidation does not seem to be a primary factor in respiratory decline, therefore, some more intricate mechanism of action, other than the antioxidant activity of tocopherol, is decisive for the understanding of the metabolic role of vitamin E in this system.

Respiratory decline of vitamin E-deficient livers occurs equally well at any pH between 6.2 and 7.6; however, the potency of tocopherol in the system is strongly pH dependent. A sharp break in the response occurs below pH 7. Below 6.6, homogenates do not respond at all. In the initiation of respiratory decline trace elements are involved, specifically by inactivating sensitive sulfhydryl sites (or similar sensitive loci). Complexing agents, such as EDTA, prevent the phenomenon. The pH dependency of the dose response to EDTA showed no significant difference from tocopherol.

Various electron transport inhibitors were used to clarify whether the effect of protecting substances on respiratory decline is correlated to specific sites of the electron transfer chain. These studies led to the conclusion that the primary site of the metabolic impairment in respiratory decline is neither in the electron transfer chain, nor in oxidative phosphorylation, but in certain dehydrogenase systems linking the Krebs cycle to the cytochromes. All substrates involved use DPN-dependent pathways.

With α-ketoglutarate as substrate, the enzyme lipoyl dehydrogenase has been identified as a target of the inactivation mechanism (K. Schwarz, C. Lee and J. A. Stesney). A close parallenism exists between the degree of respiratory failure and the loss of activity of this enzyme. Addition of tocopherol prevented the inactivation of the enzyme and at the same time prevented respiratory decline. Lipoyl dehydrogenase is very sensitive to trace metals such as Cd^{++} and Cu^{++}; it is inactivated by arsenite. The results indicate that the trace element sensitive site of lipoyl dehydrogenase is a point at which tocopherol exerts its protective effect. It is also apparent that under these dietary and experimental conditions, lipoyl dehydrogenase is rate limiting for O_2 consumption when α-ketoglutarate serves as a substrate.

The inhibitory effects of Cd^{++}, and Cu^{++} and Cu^{+} on a highly purified lipoyl dehydrogenase from heart muscle was investigated. With 2 or 4 μatoms of Cu^{++} per μmole of enzyme, ca 25% and 70% inhibition were obtained, respectively. Addition of α-tocopherol to the system afforded partial protection (ca 50%) against the Cu- inhibition. Substances such as the Simon metabolite of

tocopherol, tocopherylquinone, medadione and also DPPD, produced similar results. These investigations open up new approaches to the mode of action of vitamin E and similar factors in intermediary metabolism, with particular bearing on the question of a functional interrelationship of quinonoid compounds to active sulfhydryl groups of enzymes. These groups, and possibly similar groups, such as selenol groups, appear to be the primary sites of attack, on the molecular level, by deleterious agents producing degeneration and necrosis.

Studies on lipoic acid levels in livers of animals on various diets (in cooperation with V. Dewey, Amherst College) showed: In animals on the basal Torula diet, deficient in sulfur amino acids, lipoic acid levels amounted to only approximately 60% of those from control animals on normal diets. The supplementation of (selenium-free) sulfur amino acids raised the depressed lipoic acid levels to normal. Other agents preventing liver necrosis, namely vitamin E and Factor 3-selenium, were ineffective when used separately. However, the combination of selenium and tocopherol brought the lipoic acid level almost back to that found in controls. Hence, the delaying effect of sulfur amino acids on liver necrosis may be mediated by an effect on the tissue levels of lipoic acid, a sulfur containing cofactor synthesized within the organism.

Glucose Tolerance, Chromium (III) and Other Factors

In view of the role of trivalent chromium as a dietary factor (glucose tolerance factor) necessary for normal utilization of glucose (K. Schwarz and W. Mertz), the uptake of trivalent, radioactive chromium^{51} has been investigated in rats (L. Hopkins and C. Cisar). With various levels of supplementation, a ceiling for chromium uptake through the gastrointestinal wall seems to exist, indicating a specific mechanism for chromium (III) absorption. Similarly, a specific mechanism for chromium (III) transport in blood is found in the β-globulin fraction.

Glucose tolerance is known to be affected by a variety of dietary factors other than chromium (III). In animals maintained for extended periods on Torula yeast diets, which are deficient in methionine and tryptophan, impaired rates of glucose uptake were found which did not respond to chromium. Normal rates of glucose removal were re-established by dietary supplementation with tryptophan.

OFFICE OF MATHEMATICAL RESEARCH

Work has continued within the areas and along the lines indicated in the summary of last year. These are: Mathematical and computational methodology for mathematical models and detailed study of iodine metabolism in the thyroid system; mathematical formulation and analysis of the model for a dendritic neuron; general mathematical problems arising from the rate behavior of metabolic systems; mathematical studies of visual perception. In addition, two new areas have been included. These are: Theoretical studies of the kinetic, thermodynamic and statistical-mechanical properties of macromolecules; and studies on the numerical solution of integral equations, with special reference to convergence of iterative techniques and bounds for error, employing the methods of functional analysis.

The summaries below are in terms of "high lights" or examples.

The computer program developed for the analysis of kinetic data has been expanded to include reaction kinetics of zeroeth, first and second orders. The computational methods have been improved to accelerate convergence. Applications of the program to a variety of biomedical problems have increased at NIH. In addition, the program has also been put into operation at the data processing research centers of the Veterans Administration in Washington, D.C., and Los Angeles, Calif., The Brookhaven National Laboratories, Upton, N.Y., and the Lawrence Radiation Laboratories in Berkeley, Calif. Close collaboration with these centers is maintained. Several groups outside NIH have also established liaison with the group for the use of the program. A study in collaboration with Drs. R. Bellman of the Rand Corporation and Mr. Elkind of the NCI for the development of a mathematical-physical model for the interpretation of data on the dynamics of irradiated cell populations has been undertaken. The efforts of this past year went into the establishment of communications between the various points of view and the formulation of the problem. The theoretical testing of the first postulated model has started. It is hoped that the theoretical

model evolved will be sufficiently general to be useful in other areas of mathematical biology as well. Data analysis for the kinetics of iodine in patients of various abnormalities has continued. A working model has now been derived in terms of which most of the data has been satisfactorily analyzed. An evaluation of the results of this analysis is presently under way (Dr. Mones Berman and Mrs. Marjory Weiss).

The mathematical model of a dendritic neuron has been developed further, and computations are in progress to explore its implications for several problems of neurophysiological interest. By representing a dendritic tree as an approximately equivalent compartmental system, it has become possible to explore the theoretical consequences of various spatio-temporal patterns of synaptic activity on a dendritic tree. Computational exploration of this problem has been greatly facilitated by the use of a computer program developed by Berman, Shahn and Weiss. Considerable time and effort have been given to developing and testing computer programs for use on the Honeywell 800. These programs include (a) computations of somadendritic spread of core current and membrane potential in dendritic trees of finite extent, (b) computations of extracellular potential distributions for various sequences of dendritic arrangements. By means of these computer programs it is possible to compute extracellular action potentials for pyramidal cells which are arranged in cortical layers. Such calculations are being undertaken in collaboration with Dr. Gordon Shepherd (NINDB, Associate Member OMR), the results of such calculations will be compared with experimental results which he has obtained from the olfactory bulb of cats. A procedure has been developed (and programmed for the Honeywell 800) for the estimation of surface areas and dendritic input conductance of neurons from incomplete histological information. It is possible to demonstrate that even histological sections of 200 micron thickness, as prepared by outstanding neurohistologists, frequently display as little as 25 percent of the estimated total dendritic surface. This procedure is now being applied to a set of histological measurements made by Dr. Aitken of University College, London (Dr. Wilfrid Rall).

The theorem on a "mammillary matrix" of the form $\begin{bmatrix} a & r \\ c & D \end{bmatrix}$ where r and c are a row and column of n elements and D is $\mathrm{diag}(d_1, d_2, \ldots, d_n)$ has been extended. It has been shown that if λ is a repeated root of the matrix, then $\lambda = d_i$ for some i; that some d_i is a root if and only if for some suitable indexing $d_1 = d_2 = \ldots = d_r = d$ and then d is a root of multiplicity $r = 1$. The exponential component $\exp(dt)$, corresponding to a root of D which is a root of the matrix, is *always* absent from the central compartment and is absent from the peripheral compartments if certain initial conditions in them (including the commonly employed zero condition) are satisfied. This lumping effect depends upon the identity of some sub-set of the d_i and not, as in the classical case, upon identical compartments. The properties of the mammillary matrix have proven to be of use in areas having nothing to do with compartmental analysis. For example, they can be applied to stability studies of the linearized Hodgkin-Huxley equations. In particular it has been shown that if the quantity $H(A) = a - rD^{-1}c$ is positive, the system is unstable; if $H(A) < 0$ and $-H(A) > Q = -\Sigma(r_i - c_i)^2/d_i > 0$ the system is stable; if $Q > -H(A) > 0$, the system may be stable or unstable. Thus only the cases in which H(A) lies between zero and $-Q$ need be investigated in numerical detail. The relation between the column sums of a matrix and those of its adjoint and the applications reported last year have been shown to be a special case of the theorem: If v is an eigenvector of A, $vA = \lambda v$, then $v\,\mathrm{adj}(A - xI) = \mu v$, where $\mu = |A - \lambda I|(\lambda - x)$, is true for arbitrary x and relates the roots of A and adj(A − xI) even in the singular case. An extensive series of theorems on linear systems, most of which are new, with interpretation and applications have been collected in an article to appear in the Ann. N.Y. Acad. Sci. (Dr. John Z. Hearon).

The basic model for brightness perception, reported last year, has been refined. Studies of published experimental data on Mach bands led to the conclusion that the visual system is markedly non-linear in its spatial characteristics. This conclusion is contrary to that drawn by the authors who published the data and is based upon a simple but more sensitive test for non-linearities.

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Since Mach bands are a local effect and must, in any model, be produced by a local averager, it can now be asserted that local averaging is non-linear, consistent with the overall non-linear character of the original basic model. Extension of the basic model to the time domain has begun with a study of flicker response and fixed retinal image effects (Mrs. R. B. Marimont, NIMH, Associate Member, OMR).

Consideration has been given to the estimation of the equilibrium extent of polymerization of double stranded nucleic acids as they arise from nucleoside triphosphates as monomer sources. It has been found that the free energy of formation of a phosphodiester bond by a reaction represented by XTP+X ____ $\rightleftarrows$ XPX ____+PP is about zero. However, when there is a moderate thermodynamic driving force for strand association high degrees of polymerization may be anticipated at equilibrium. A matrix method for treating the copolymerization statistics of certain types of systems has been extended to allow for the influence of remote interactions in the chain. By use of the matrix calculus, certain average properties, (e.g. degrees of polymerization, numbers of specific sequences of units, etc.) of the copolymeric chain can be computed. The possible application of this approach to nucleotide copolymers is under investigation (Dr. Leonard Peller).

Work on a method to solve numerically the linear Fredholm integral equation, (1): $f(x)-\int_a^b K(x,y)f(y)dy=g(x)$, was initiated in collaboration with Dr. P. A. Anselone at the Mathematics Research Center, U.S. Army, University of Wisconsin. Here K(x,y) may have, on the rectangle, $a\leqq x,\ y\leqq b$, weak discontinuities in the sense of Kolmogorov and Fomin. We convert (1) into (2):

$$f(x)-\int_a^b\int_a^b K(x,t)K(t,y)f(y)dtdy=g(x)+\int_a^b K(x,y)g(y)dy$$

and approximate the kernel

$$K^{(2)}(x,y)=\int_a^b K(x,t)K(t,y)\,dt$$

by the Gauss quadrature (3):

$$K_n^{(2)}(x,y)=\sum_{k=1}^{N_n}K(x,t_k^{(n)})K(t_k^{(n)},y)w_k^{(n)}$$

thus replacing the kernel of (2) by an approximating kernel (3) of finite rank. This work has been continued here. Now it can be proved that the sequence of approximating operators $(I-K_n^{(2)})^{-1}$ converges, in the norm sense, in the Banach space of bounded linear operators to the operator $(I-K^{(2)})^{-1}$ and that the sequence of approximating solutions converges in the norm to the solution in the space of functions continuous on the interval [a,b]. A general formula for the error estimate has been developed and its practical aspects explored on the computer. This work is sufficiently general to include, with numerical feasibility, most applications and in particular those involving the Volterra type integral equation to indicator dilution techniques in hemodynamics (Dr. Jose M. Gonzalez-Fernandez).

CLINICAL INVESTIGATIONS

A total of 529 inpatients was admitted during the 12-month period from December 1, 1961 to November 30, 1962, an increase of 49 patients (10%) over the same period last year. The total patient days was 19,423, a decrease of 433 over the preceding year. The average inpatient stay at the Clinical Center was 37 days. The average census was 53 (76%). In the Admissions and Followup Department 2,041 patients were examined and studied, an increase of 160 over the past year.

Investigations conducted in the laboratories of clinical investigation of NIAMD have resulted in 109 publications in scientific journals, monographs, annual reviews and medical textbooks. Dr. Harold Edelhoch was awarded the Van Meter Prize by the American Goiter Association in recognition of his original work on the development of a spectrophotometric method (not requiring hydrolysis of the protein) for the analysis of tyrosine, monoiodotyrosine, diiodotyrosine and thyroxine in native or iodinated thyroglobulin. Dr. Paul A. di Sant'Agnese received an honorary degree of Doctor of Medicine at Justus Liebig University, Giessen/Frankfurt, West Germany for his contributions to our knowledge of cystic fibrosis of the pancreas.

ARTHRITIS AND RHEUMATISM BRANCH

Association of Sjögren's Syndrome With Malignant Lymphomas

Clinical investigation on a series of 60 patients with Sjögren's syndrome admited to NIAMD over

the past 4 years led to the observation that these patients have an unusual abundance of abnormal circulating antibodies to tissue components. For example, every patient had rheumatoid factor (whether or not they had rheumatoid arthritis). The majority had antinuclear factors, about one-half had complement-fixing antibodies to diverse organs and tissues that were not species specific and about one-fourth had thyroid antibodies. During the past year four of our patients developed lymphomas; three had reticulum cell sarcomas and one a lymphoma resembling Waldenström's macroglobulinemia. A fifth case of Sjögren's syndrome with reticulum cell sarcoma was found in the literature. We learned of a sixth case at the Mayo Clinic complicated with Hodgkin's disease (personal communication).

The unusual association in the same patient of these two uncommon diseases cannot be ascribed to mere coincidence. The first five patients share certain clinical and laboratory features which distinguish them from the usual patient with Sjögren's syndrome. Chief among these are splenomegaly, purpura, vasculitis, leukopenia, lymphopenia, the presence of rheumatoid factor, antinuclear factor, antithyroglobulin, complement-fixing antibodies and immune globulins. With the aid of serologic and immunochemical methods, particularly immunoelectrophoresis, these abnormalities in the 7S gamma globulins, β_2A and β_2M globulins are being examined more closely. Hypogammaglobulinemia is present in two patients and diffuse hypergammaglobulinemia in two. These alterations in immune globulins correlate well with other features of the disease and may reflect a basic abnormality in immunologic responsiveness.

It may be important to note that three of our four patients had received deep X-ray therapy to the enlarged parotid glands (part of Sjögren's syndrome) prior to developing lymphomas (Drs. Bunim and Talal).

Factors That Direct Organ Distribution of Lymphocytes

Preliminary studies have confirmed the fact that living, isotopically labelled (P^{32}) lymphocytes are concentrated in the spleen within 30 minutes after intravenous injection and that heat-killed lymphocytes are not. Preincubating lymphocytes with a partially purified preparation of glycosidases obtained from *Clostridium welchii* prevented the accumulation of lymphocytes in the spleen, though this treatment did not kill the cells (as determined by vital staining). The inhibition of accumulation of homologous lymphocytes in spleen by glycosidases was exponentially proportional to enzyme concentration and directly proportional to time of incubation. This enzyme effect could be prevented by the prior addition of simple sugars (galactose, mannose, fucose, N-acetyl galactose-amine, N-acetyl glucose-amine) to the reaction mixture. However, the enzyme effect could not be inhibited by glucose added in the same amounts as the other sugars. The sugars which were found to inhibit the enzyme effect were shown to be present in lymphocyte membrane preparations. One possible explanation for the concentration of lymphocytes in the spleen is that there are complementary binding sites in the spleen for the macromolecular surface structure of intact lymphocytes and that this specific surface structure of the lymphocyte is determined at least in part by surface polysaccharide (Drs. Gesner and Ginsburg).

Antigenic Composition of Glutamic Dehydrogenase

Mammalian glutamic dehydrogenase (GDH) is a high molecular weight tetramer that is able to dissoicate reversibly under the influence of diethylstilbestrol (DES) into monomeric units of different amino acid specificity. It can be further and irreversibly dissociated by the detergent sodium dodecylsulfate (SDS) into 16 subunits of molecular weight approximately 60–70,000. Rabbit antisera have been prepared against these two different forms of mammalian GDH. Electrophoretic, immunoelectrophoretic and agar diffusion studies have been performed in 1% agar employing a normal buffer at pH 8.2. Glutamic dehydrogenase activity can be identified even after immunologic precipitation in agar by means of specific biochemical reactions leading to a colored product.

The dissociation of GDH by DES or SDS can occur during electrophoresis or diffusion in agar. Therefore, electrophoretic and immunochemical

methods can be employed to study the protein, biochemical and antigenic nature of the molecular forms involved in these dissociations.

Preliminary investigations indicate that the three molecular forms studied (tetramer, monomer and SDS fragment) have different and characteristic electrophoretic mobilities and antigenic properties (Drs. Talal, Yielding, and Tomkins and Mr. Mushinski).

Effect of Steroid Hormone on Structure and Function of Enzymes (*in Vitro*)

During 1962 our conclusions concerning the relationship between physical structure and catalytic activity of GDH have been confirmed by independent means of measurements. It has now become evident that control of this enzyme is a rather specific process and apparently involves specific sites on the enzyme molecule which are concerned with control. Furthermore, it appears that rather specific SH groups are involved with these control sites in contrast with the sites concerned with catalysis of the chemical transformations. These conclusions, although derived from our previous investigation, have now been made more definite by the following confirmatory lines of evidence.

1. Correlation of steroid induced changes in the structure of GDH with changes in its function: Studies on the correlation of the physical state of the GDH molecule and its catalytic activity have been continued in collaboration with Dr. Gordon M. Tomkins. It has now been possible to study the effects of estrogenic hormones and various experimental conditions on both the physical state of the GDH molecule and its ability to catalyze the glutamic dehydrogenase and the alanine dehydrogenase reactions using precisely the same reaction mixture for the kinetic studies and the physical studies. The molecular weight in these experiments has been determined by light-scattering, and it has been possible to show a direct correlation between those two parameters—confirming the previous conclusion that the most aggregated form of the molecule catalyzes the glutamic dehydrogenase reaction and the disaggregated form catalyzes the alanine reaction. Furthermore, ability of the steroid hormones to dissociate the enzyme molecule in to subunits is exactly correlated with inhibition of glutamate and activation of alanine activities. Light-scattering experiments have also confirmed that the molecule is reaggregated by ADP, L-leucine and various amino acids, and that the substrates of the GDH reaction have profound effects on the physical state of the enzyme molecule. While making an attempt to determine the dissociation constant for the enzyme under various experimental conditions, it has become apparent that the dissociation of the tetrameric form into monomeric subunits involves one or more intermediate forms of the enzyme such as dimer or trimer. It is apparent that the various experimental circumstances which change the apparent weight average molecular weight of the molecule simply shift the dissociation constant of the overall reaction toward either the more dissociated form or the more aggregated form.

It has also been possible to show both in this laboratory and in Dr. Tomkins' laboratory that the specific activity as well as the molecular weight of the purified enzyme toward alanine and glutamate varies with enzyme concentration in a manner predicted for a dissociating system in which the aggregated form of the enzyme catalyzes the glutamic—and the dissociated form the alanine dehydrogenase reactions.

The interrelationship of the alanine and glutamic dehydrogenase activities of the GDH molecule have also been examined in tissue culture cells and it has been observed that sonicates of Hela cells have predominately alanine activity and must be activated with ADP to exhibit significant GDH activity.

2. Role of –SH groups in the control and activity of GDH: Experiments on the different sulfhydryl groups in the enzyme have also been continued, particularly in relation to control of enzymic function. The previously described ability of parachloromercuribenzoate to prevent the steroid inhibition and the ADP activation of the enzyme has been amplified by the additional finding that methylmercuric bromide and methylmercuric chloride are even more specific in preventing the control of the enzyme by steroid, leucine, ADP, detergents and DPNH while not producing any inhibition of the reactions themselves. N-ethyl maleamide, on the other hand, has no effect either on the catalytic properties or on the sensitivity of the enzyme to

control by other substances, but stabilizes the enzyme. It would appear that the SH groups in or near the control sites of the enzyme are somewhere intermediate between the most accessible SH groups and those which are remote within the structure of the enzyme molecule. In view of a recent report to the effect that parachloromercuribenzoate has a capacity to disrupt GDH into subunits without inhibiting its activity, it became of further interest to make a study of the effects of this compound on the molecular size of the enzyme. It was observed that when the enzyme was mixed with PCMB (or PCMPS) alone, the SH reagent did, indeed, disrupt the enzyme into subunits. However, when an identical experiment was run in the presence of substrates for the reaction, the disruptive effect of the mercurial was prevented except at very high concentrations, when inhibition could also be observed. Using the ultracentrifuge and light-scattering, the "shielding" effect of the mercurial was shown to be mediated by its ability to prevent changes in molecular size induced by the various reagents.

Studies on the nature and function of –SH groups in the enzyme have been continued. It has been shown that in addition to this ability of various mercurials to antagonize the effects of various "control" substances by binding to specific sites on the enzyme, they also interfere with binding of DPNH as shown by fluorescence enhancement. This permitted a reexamination of our previous conclusion that the DPNH binding shown by fluorescence enhancement did not represent the catalytically active cofactor, but other binding sites on the enzyme molecule. Accordingly, methyl mercuric bromide (or chloride) was shown to decrease the affinity for DPNH as shown by fluorescence enhancement while not affecting the Km for DPNH in the glutamic dehydrogenase reaction. It has also been shown that the shielding effect described previously can be obtained with a molar ratio of mercurial to enzyme as low as 10:1, thus indicating rather great specificity of the –SH groups involved. Recent data indicate that the ability of mercurials to stimulate GDH at pH 9 (originally described by Hellerman) are also related to an effect on the state of aggregation of the molecule (Drs. Yielding, Tomkins and Bitensky).

Demography of Rheumatic Diseases: Genetic and Environmental Influences

Several population surveys, described in 1961 report, on the prevalence of rheumatoid arthritis (R.A.), osteoarthrosis (O.A.) and rheumatoid factor (RF) in the random population of the United States and in the Blackfeet Indians, have been continued in 1962. In addition, at the suggestion and invitation of Dr. Leonard T. Kurland of the Epidemiology Section of NINDB who has been conducting surveys on several of the Marianas Islands for amyotrophic lateral sclerosis and Parkinsonism, about 500 serum samples from natives living on four of the islands were analyzed for uric acid concentration and for RF. As anticipated, the results were of unusual interest.

1. Population Survey of the Blackfeet Indian Tribe

The first phase of a two-part survey on the occurrence of arthritis and RF in North American Indians was completed in northern Montana (Blackfeet tribe). The second phase will be conducted in southern Arizona Pima tribe) in 1963. A total of 1,103 or 85.8% of the registered Blackfeet Indians residing on the reservation in northern Montana were examined clinically, serologically and radiologically in 2 mobile clinics. In addition, the ABO secretor status was determined in 822 and blood types in 99. Analysis has not yet been completed but preliminary results on the first 1,059 Indians examined reveal a prevalence of rheumatoid factor (bentonite flocculatlon test) in 5.7% and of "probable" and "definite" rheumatoid arthritis in 4.0%.

Blackfeet Family Study: In order to determine whether the occurrence of R.A. or RF was influenced by genetic factors two types of analyses were employed. The first consisted of determining the occurrence of R.A. and RF among the first-degree relatives (parents, siblings and children) above age 29 of four classes of probands selected from the Blackfeet tribe: (1) seropositive rheumatoid arthritis, (2) seronegative rheumatoid arthritis, (3) individuals who gave a positive test for rheumatoid factor but who had no arthritis

and (4) probands negative for both rheumatoid arthritis and rheumatoid factor. No family aggregation of either rheumatoid factor or arthritis was demonstrable among the 390 first-degree relatives of 131 probands examined. The failure to demonstrate family aggregation is in contrast to the results reported by a number of contemporary workers in this field, both abroad and in the United States. The discrepancy is due largely to the nature of the controls chosen in the various studies. The second type of analysis used to determine genetic linkage was by applying Penrose's formula for sibling pairs. All possible pairs of siblings in the entire population were studied and the number of pairs with both, one or neither member affected was determined and compared with the number that could be expected by chance. Again there was no evidence of significant aggregation in the kindreds.

Finally, a similar analysis of the occurrence of RF and R.A. in spouses of subjects with RF and/or R.A. was done in order to evaluate the possibility of a household environmental factor which, if operative, would result in an increased frequency of RF or R.A. However, no such influence was detected.

Our study has, therefore, failed to confirm the presence of a familial aggregation of rheumatoid arthritis or rheumatoid factor such as could be caused by a genetic factor or a household environmental factor as reported by other investigators.

2. Marianas Islands Survey

The field unit of NINDB has collected and sent frozen serum samples to us from 561 natives, age 40 and over. BFT's for rheumatoid factor have been done on all and uric acid levels have been determined on 447 individuals. The frequency of positive BFT and hyperuricemia differed markedly on the different islands and races of people. The prevalence of hyperuricemia in this population was 30.1% of 186 men tested and 31.9% of 207 women tested. A careful, well-designed survey may determine the basis for this phenomnenon—genetic or environmental. Uric acid concentrations determined on serum samples of 817 enlisted men in the U.S. Army by Dr. Stetten's laboratory several years ago revealed that 2.5% had hyperuricemia (above 6.9 mg% by uricase spectrophotometric method).

The prevalence of RF in Marianas natives was 3.5% among 254 men tested and 3.9% among 307 women tested. It will be noted that the Chamorros on Rota (both males and females) and the Carolinian women on Saipan have an unusually high frequency of positive BFT's. The values for these three groups are 13.2%, 8.7% and 10.7%.

As will be seen (vide infra) the occurrence of positive BFT's in random population of the United States (above age 17) is 3.6%. There are several possible explanations for the high prevalence in certain groups of Marianas natives. A careful survey may reveal the basis for this observation.

3. National Health Examination Survey

The field survey phase of the National Health Examination described in last year's report was completed on December 1, 1962. Nearly 7,000 adults (18 through 79 years) from 42 areas in the United States have had clinical examinations, radiographs of the hands and feet and serum collected for analysis for rheumatoid factor. Bentonite flocculation tests are done in our laboratory. Thus far (December 1, 1962) we have analyzed 6,669 serum samples collected from 41 areas. Of these, 237 or 3.6% gave a positive test. Examination and interpretation of X-ray films of hands and feet have been completed on 2,397 persons from 15 areas. Of these, 19 or 0.8% had X-ray changes compatible with rheumatoid arthritis.

Correlation of clinical findings have only been completed in 494 individuals from 3 areas. Six or 1.2% of these had a positive BFT and 12 or 2.4% met the A.R.A. criteria for probable or definite rheumatoid arthritis. Forty-four or 8.8% had moderate to severe changes of osteoarthritis on X-ray films of the hands and feet. All of these were 35 years of age or older (Drs. Burch, O'Brien and Bunim).

Clinical Trial of Antirheumatic Drugs in Rheumatoid Arthritis, Psoriatic Arthritis and Lupus Nephritis

1. Hydroxychloroquine in Rheumatoid Arthritis

Hydroxychloroquine has been evaluated by the Committee on Cooperating Clinics of the American Rheumatism Association in a 6-month double-blind study. Arthritis and Rheumatism Branch

of NIAMD participated in this project with the Rheumatology Service of the Georgetown Medical Division of the D.C. General Hospital, contributing 14 patients to the study. In all, 121 patients were admitted to this double-blind trial, of which 110 finished the study. There were 41 males and 80 females in the group with the median age of 53 years ranging from 31 to 72 years. All patients have had rheumatoid arthritis for more than 1 year. Results of the study were reported in the Bulletin on Rheumatic Diseases, October 1962. The double-blind trial indicated moderate effectiveness of hydroxychloroquine in the treatment of rheumatoid arthritis. The five parameters evaluated included morning stiffness, grip strength, sedimentation rate, total number of joints involved with the arthritic process and time required to walk a distance of 50 feet. Morning stiffness showed improvement in 66% of the drug-treated patients and 54% of the placebo-treated patients. Number of joints involved decreased in 70% of the treated patients and in 62% of the control group. Grip strength improved in 86% of the drug patients and in 70% of the placebo patients. Improvement in walking time was similar in the two groups. The sedimentation rate showed improvement in 64% of the drug patients and in 45% of those receiving placebo. In no single parameter mentioned was there a significant difference between the placebo and drug groups. When, however, scores were assigned to each parameter and the patients rated on a range of 0 to 5 according to the number of parameters that indicated improvement, there was a statistically significant difference between the patients treated with hydroxychloroquine and those treated with placebo. Seventy-five percent of the drug-treated patients had significant improvement, whereas only 54% of the placebo group showed such favorable change. No serious side effects were observed during the trial period.

2. High Dosage Corticosteroid Therapy in Lupus Nephritis

Reported studies by Robert Kark and others have indicated that the administration of high-dosage corticosteroids (equivalent to prednisone, 50 mg. daily) may be effective in prolonging the life of patients with nephritis due to lupus erythematosus. A study has been instituted in the A&R Branch of NIAMD to evaluate this form of therapy for this clinical problem. Patients admitted to this study must have definite lupus erythematosus with a positive LE preparation and at least two major manifestations of SLE. These patients are subjected to percutaneous renal biopsy. When the biopsy specimen indicates the presence of lupus nephritis, prednisone is administered in a dosage of 50 mg. daily. The patients are subjected to a second renal biopsy after three months. If the clinical state or the biopsy specimen indicates improvement, the steroid dose is then tapered to a lower maintenance level and the patient is observed at three-month intervals. Repeat biopsies are performed at intervals of 3–6 months in order to further assess the course of the disease. To date, six patients have had at least two renal biopsies. All patients had arthritis and clinical evidence of renal disease. In addition to these six, one patient was observed with clinical manifestations of nephritis without the biopsy procedure. Three of the seven had elevation of BUN prior to the steroid regimen. In each of these three the BUN levels became normal during the administration of steroids. One patient died in pulmonary edema after 1 month of therapy; the BUN immediately prior to death increased above 100 mg%. PSP excretion was also measured in these seven patients. In one case the value was abnormal prior to the steroid treatment and remained abnormal throughout the study period. In one other case a previously normal value fell to abnormal levels at the end of the study period even though the patient remained clinically well and the biopsy showed improvement. Side effects of the high dosage steroid regimen included peptic ulceration in one case, compression fracture of a vertebra in one, mild diabetes controlled with diet alone in two instances, psychic abnormalities in four, thrombophlebitis in one, mild hypertension in five, and edema formation in four. One patient developed posterior subcapsular cataracts, having had high doses of steroid prior to entrance in the study. It is planned to continue this study (Drs. Black, Bunim, O'Brien, Buchanan, Talal, Cohen and Alepa).

The Enzymatic Defect in Histidinemia, Metabolism of Aromatic Amino Acid and Homogentisic Acid in Phenylketonuria, and Ochronosis

1. Phenylketonuria

The micro method developed to measure the concentration of phenylalanine in blood has been used to determine how constantly the level is maintained during the day by a phenylketonuric patient on a diet very low in phenylalanine and how the level changes after a single oral dose (1 gm.) of L-phenylalanine. Finger-prick blood samples taken every 2 hours showed that very little variation occurred during the day on the special diet, but the level increased within 2 hours after the ingestion of phenylalanine and remained elevated for at least 8 hours. More data of this type is needed to evaluate the factors (and their duration) which alter the phenylalanine level in the blood of phenylketonurics, such as illness, fever, dietary changes, etc. Since patients on the special diet are tested rather infrequently, the interpretation of the values for dietary control purposes requires that these factors be recognized and evaluated.

The method to measure phenylalanine has been modified so that blood and urine histidine concentrations can also be determined and the modified method was used in our studies on the family with histidinemia.

We have continued to analyze blood samples for phenylalanine for groups outside NIH as a confirmatory test in cases suspected of being phenylketonurics because of a positive ferric chloride test in the urine. The latter test is not specific for phenylpyruvic acid. Each time we have found a normal phenylalanine blood level, we have suggested that the urine be retested, and if it remains positive, that we be sent both urine and blood samples. This procedure led us to the finding of two cases of histidinemia since in this new metabolic disease one of the histidine metabolites excreted in the urine also gives a positive ferric chloride test.

2. Histidinemia

Clinical and biochemical studies have been carried out on a family in which two sibs have histidinemia. It has been demonstrated that the enzymatic defect in this metabolic disease is the absence of histidase. As a consequence, blood levels of histidine are greatly elevated and histidine and imidazolepyruvic acid are excreted in the urine. The affected sibs were also found to lack urocanic acid in the skin and sweat. They both also have speech defects but normal intelligence; however, it is not certain that the speech defect is related to the metabolic disease.

3. Experimental Ochronosis and Ochronotic Arthritis

Our investigations have continued on the chemical and enzymatic steps involved in the formation of ochronotic pigmentation of connective tissues characteristic of alcaptonuria, and its relationship to ochronotic arthritis. Earlier studies dealt with the tissue distribution and binding of homogentisic acid and benzoquinoneacetic acid (the oxidation product of homogentisic acid). Homogentisic acid was found to be bound by physical forces to connective tissues, but benzoquinoneacetic acid reacts chemically with these tissues. Homogenates of skin and cartilage incubated with the latter acid yield brown products resembling the ochronotic pigmentation of alcaptonuric tissues.

The binding of benzoquinone by connective tissues has been studied in more detail. No significant binding was observed with the mucopolysaccharide fraction of connective tissues, but binding was found with highly purified acid-extracted collagen obtained from several animal sources. The optimal pH for binding to purified collagen was found to be between pH 3.5 and 4.2. The amount of benzoquinoneacetic acid bound to purified collagen is proportional to the concentration of protein, and about 1 to 2 molecules of the acid are bound per molecule of collagen. The behavior of the resulting product on carboxymethylcellulose columns suggest that binding of the acid has altered the secondary structure of the collagen molecules (α and β chains). Further experiments are in progress to identify the binding sites of collagen by separation of the collagen fibrils by column chromatography and to degrade the bound benzoquine-collagen product enzymatically with collagenase.

4. Folic Acid and Tyrosine Metalbolism

During the past few months we have gained further insight into the rather complicated mech-

anism by which folic acid and ascorbic acid interact to maintain the normal oxidation of tyrosine in mamalian liver. We previously had shown that the first oxidative step of tyrosine metabolism is the oxidation of p-hydroxyphenylpyruvic acid by a specific oxidase, and this oxidase is inhibited by its substrate in vitamin C-deficient guinea pigs fed large amounts of tyrosine, and p-hydroxyphenyl metabolities are excreted. If either ascorbic acid or folic acid is given to the C-deficient animals just before extra tyrosine is fed, inhibition of the oxidase is prevented. *In vitro* experiments phenyl metabolites are excreted. If either ascorbic acid or folic acid is given to the C-deficient anitected the enzyme from inhibition by excess substrate, but in this system, folic acid was ineffective. These results suggested that folic acid must have been converted to some active form, possibly a reduced folic acid derivative, to protect the oxidase *in vivo*.

In recent *in vitro* experiments with p-hydroxyphenylpyruvic acid oxidase preparations from guinea pig liver, it has been found that reduced folic acid (tetrahydrofolic acid) and some other reduced derivatives do protect the oxidase from inhibition. Dimethyltetrahydropteridine, dihydrofolic acid and anhydroleucovorin factor are all effective, and dimethyltetrapteridine is nearly ten times as effective as ascorbic acid. Furthermore, folic acid (not reduced) and some folic acid antagonists, such as amethopterin, inhibit the oxidase. These findings, with our earlier evidence that ascorbic acid protects the oxidase by some indirect mechanism, suggest that the oxidase has a reduced pteridine as a cofactor which can be maintained in its active (reduced) form by ascorbic acid. It is of interest that a reduced pteridine cofactor is known to be required for phenylalanine hydroxylase and the reactions catalyzed are similar in that p-hydroxyphenylpyruvic acid oxidase also catalyzes a "hydroxylation" step to form homogentisic acid.

5. Tyrosine Transaminase

The activity of liver tyrosine transaminase is increased up to 8-fold in rats after cortisone administration. In studies on the mechanism of this steroid-induced effect, the characteristics of tyrosine-α-ketoglutarate transaminase purified from liver of induced and uninduced rats have been carefully examined. The enzyme, purified 500-fold from both sources, has been found to be much less specific than had been previously reported. Other aromatic amino acids transaminated by the enzyme are: phenylalanine, tryptophan, DOPA, βthienylalanine and some ring-substituted tyrosine analogues. The ratio of tyrosine to trytophan transamination activity reaches a constant ratio during purification of the enzyme and is not changed by various treatments of the purified enzyme. These results would explain the reports that "tryptophan" transaminase is also induced by cortisone (which was assumed to be a separate enzyme). Several derivatives of phenylalanine, tyrosine, tryptophan and DOPA were found to act as competitive inhibitors of the transaminase. This new information will be of considerable value in further studies on the mechanisms which regulate the activity of this enzyme *in vivo*. (Drs. LaDu, Zannoni, Seegmiller, Howell, Malawista, Goldfinger and N. C. Brown).

Studies on Gout

1. Mechanism of Action of Colchicine

Our recent demonstration of an acute goutlike inflammatory reaction to crystalline suspensions of monosodium urate injected intra-articularly in gouty volunteers (1961 annual report) suggested the possibility that colchicine might act in terminating an acute attack of gouty arthritis by suppressing the inflammatory reaction to sodium urate crystals. Experiments both *in vitro* and *in vivo* were designed to examine this possibility.

The magnitude of the inflammatory response to the injection of a standard dose of a crystalline suspension of sodium urate was evaluated before and again after the intravenous administration of therapeutic doses of colchicine. In addition, the synovial effusion was aspirated six hours after injection and the leukocyte counts, crystal counts, and the percent of crystals undergoing phagocytosis was determined by direct count in a hemocytometer chamber. For evaluating the response to subcutaneous injection, measurements were made of the diameter of the areas of erythema and of induration, the warmth (using a thermocouple in later tests) and tenderness to pressure.

In vitro studies: Human leukocytes isolated by fibrinogen sedimentation were incubated in plasma with sodium urate crystals. $C^{14}O_2$ is evolved by the peroxidative degradation of sodium urate-6-

C^{14} crystals. The influence on $C^{14}O_2$ formation of colchicine added *in vitro* or of plasma from a patient who had received a therapeutic dose of colchicine was determined. Other studies utilized a second biochemical parameter of phagocytosis first demonstrated by Karnofsky. The increased oxidation of glucose-1-C^{14} to $C^{14}O_2$ that occurs in response to phagocytosis was determined and the effect on $C^{14}O_2$ formation of colchicine added *in vitro* or in plasma from a patient who had received a therapeutic dose of colchicine was evaluated.

In vivo studies: All studies were performed on gouty or non-gouty volunteer patients in the Arthritis and Rheumatism Branch of NIAMD. Pretreatment of asymptomatic gouty volunteers with therapeutic doses of colchicine administered intravenously resulted in a substantial suppression in the majority of patients of the clinical evidence of an inflammatory reaction to sodium urate crystals injected intraarticularly or intradermally. The suppression was particularly striking in those patients who obtained the greatest inflammatory response in the absence of colchicine.

Studies *in vitro* showed that addition of colchicine or of plasma from a gouty patient who had received a therapeutic dose of colchicine substantially reduced both biochemical paramters of phagocytosis. The peroxidative destruction of crystals of sodium urate-6-C^{14} that results from their phagocytosis by leukocytes was substantially diminished and the specific stimulation of the oxidation of glucose-1-C^{14} to $C^{14}O_2$ that accompanies phagocytosis was also suppressed by the colchicine treatment.

2. Uricolysis by Human Leukocytes

Despite the absence of uricase in the human species, one third to one fourth of the uric acid produced in the normal human each day is destroyed prior to excretion from the body. The prevailing view is that the intestinal tract is the sole site of such degradation through the action of bacterial enzymes. No convincing evidence has previously been found of a breakdown of uric acid by any human tissues. A peroxidative destruction by heme proteins and by verdoperoxidase has been demonstrated *in vitro* by other workers. The possibility that a peroxidative destruction of sodium urate crystals might accompany the phagocytosis of urate crystals that is seen in acute gouty arthritis led us to seek evidence of such a destruction by intact human leukocytes and erythrocytes.

Human leukocytes isolated by fibrinogen sedimentation from peripheral blood were incubated with plasma containing a suspension of crystalline sodium urate-6-C^{14}. In duplicate experiments the same quantity of sodium urate-6-C^{14} in supersaturated solution was used. The $C^{14}O_2$ formed by the peroxidative destruction of uric acid to allantoin was collected and counted.

In other studies the leukocytes were sonically disrupted and the disappearance of uric acid was followed spectrophotometrically by the decrease in absorbance at 292 mμ. The effect of adding a hydrogen peroxide generating system consisting of glucose oxidase and glucose was also evaluated.

Our studies showed that human leukocytes are able to break down uric acid. Phagocytosis of crystals of sodium urate-6-C^{14} results in a greater uricolysis (around 0.3 μgm/hr/10^6 leukocytes) than is observed when the same amount of sodium urate is present in solution. Erythrocytes showed no significant destruction of sodium urate. Destruction of uric acid by sonically disrupted leukocytes was dependent on a hydrogen peroxide generating system and the magnitude of the destruction was comparable to that observed in intact leukocytes (Drs. Seegmiller, Howell, Malawista and Klinenberg).

GASTROENTEROLOGY UNIT

Whipple's Disease

Both the clinical and laboratory research activities of this group are concerned with problems related to intestinal absorption. A major portion of the clinical activities has been focused on Whipple's disease, a poorly understood disorder that produces, as a primary manifestation, a broad malabsorption syndrome in association with infiltration of the small-intestine mucosa by mucoprotein-laden macrophages. We have demonstrated in four patients that relapses of this disease can be reversed rapidly and extensively by treatment with adrenocorticosteroids, together with broad-spectrum antibiotics. A fifth patient, treated with antibiotics only, also improved, but at a much slower rate. All five patients demonstrated "bacillary bodies" in their small-intestine mucosal biopsies when they were in relapse. With remission, these bodies, the nature of which is presently obscure, disappeared. After prolonged

remissions (1½ years) attributable to continued treatment with adrenocorticosteroids and antibiotics, two patients still show infiltration of the intestinal mucosa by mucoprotein-laden macrophages despite their generally satisfactory clinical conditions. All five patients presented with hypoalbuminemia when they were in relapse. In each case it was possible to demonstrate that excessive enteric leakage of albumin was present, suggesting that this process is partly, if not wholly, responsible for the hypoalbuminemia of Whipple's disease. Two biopsies of intestinal mucosa known to contain "bacillary bodies" have been cultured but no organisms were isolated. Studies of relatives of the patients have not as yet revealed a familial occurrence of this disease in the present series (Dr. Laster).

Metabolism of D-xylose

It is common clinical practice to assess intestinal absorption in patients by feeding them 25 grams of d-xylose and measuring the urinary excretion of this pentose during the ensuing five hours. Little is known, however, about the pathways of metabolism of d-xylose in mamalian tissues, and less is known about factors other than intestinal absorption which may influence the results of the xylose tolerance test. A study is in progress, therefore, to delineate the pathways of liver metabolism of d-xylose *in vitro*. We have partially purified an enzyme activity which appears to catalyze the dehydrogenation of d-xylose to d-xylonic acid using DPN preferentially, but also TPN, as the hydrogen acceptor. A number of enzyme activity's properties have been characterized.

In addition, the oxidation of d-xylose-1-C^{14} to $C^{14}O_2$ has been studied in intact normal and thyrotoxic guinea pigs. The results suggest that d-xylonic acid is an intermediate compound in the oxidation, and that thyroxin stimulates not only the renal excretion of d-xylose, as others have suggested, but also the oxidation of the pentose to CO_2 (Drs. Laster and Weser).

Amino Acid Transport in the Small Intestine

Because information on amino acid absorption in the healthy and diseased human subject is limited, we have initiated a study of this physiological process. The first phase of the study has consisted of an evaluation of active transport of monoaminomonocarboxylic acids by segments of hamster small intestine. Information so derived will now be applied to human subjects. Glycine, L-alanine, L-leucine, L-valine and alpha-amino-isobutyric acid are all actively transported in our experimental system. The kinetics of their transport systems conform to Michaelis-Menten analyses. K_t values, analogous to K_m values of enzyme kinetics, have been derived for each compound. Of interest is the finding that an amino acid such as glycine may have a greater maximal transport rate than L-leucine, but because its apparent affinity for the transport mechanism is 25-fold less than that of L-leucine, glycine is competitively inhibited in its active transport by L-leucine. Each of the five compounds studied was found to be maximally absorbed in the lower mid portion of the hamster small intestine. Water transport appeared markedly to influence amino acid transport —more rapid water movement producing more rapid amino acid transport (Drs. Matthews and Laster).

Glucose Metabolism by Small-Intestine Mucosa

The small intestine allegedly derives much of its energy for active transport from glucose oxidation; hence an understanding of this biochemical pathway is important in relation to probing mechanisms of the intestine's absorptive functions. In addition, pharmacological doses of hydrocortisone are known to improve acutely the absorptive capacity of a small intestine involved by nontropical sprue without altering the morphological abnormalities which characterize this disease. It is pertinent, therefore, to inquire whether hydrocortisone influences metabolism by the small intestine.

Slices of intestinal mucosa from animals or from humans metabolize glucose added *in vitro*. Both glucose-1- and glucose-6-C^{14} are oxidized to $C^{14}O_2$, the total conversion being equal to somewhat less than ½ percent of the glucose. Hydrocortisone, 10^{-3}M, suppresses oxidation of glucose-1-C^{14} by approximately 20% and of glucose-6-C^{14} by approximately 80%. Similar suppression is observed with intestinal mucosa from patients with nontropical sprue. Homogenates of animal intestinal mucosa have an even lower endogenous rate of glucose metabolism, a rate which is affected by hydrocortisone in the same manner as the metabolism by slices. The addition of ATP and TPN to homogenates in appropriate concentrations can increase the oxidation of glucose-1-C^{14}

by 20–40-fold but it does not stimulate the oxidation of glucose-6-C^{14} to $C^{14}O_2$ (it may increase lactate accumulation, but this remains to be studied). Hydrocortisone will no longer inhibit oxidation of glucose-1-C^{14} when its rate has been increased by ATP and TPN, but it will inhibit the oxidation of glucose-6-C^{14} in the presence of these cofactors (Drs. Laster and Fenster).

CLINICAL ENDOCRINOLOGY BRANCH

The work of the Branch has continued along several rather separate avenues of investigation. The major areas of emphasis, as in the past, have been on the biochemistry of the thyroid gland and its hormones and on carbohydrate metabolism. Problems in amino acid transport have been given additional attention, and an active program dealing with the physical chemistry of proteins has been pursued. An investigation centering on the steroid hormones has been initiated. The interdisciplinary character of the staff membership continues in evidence. The brief summaries which follow demonstrate to some degree the collaboration and cross-interests of the biochemists, physical chemists, organic chemists and clinical investigators who comprise the senior membership of the staff. This wide spectrum of training, knowledge and approach to problems has greatly enriched the research program as well as the outlook and development of the individual scientists. The mutual benefits derived from the presence of visiting workers from abroad have also been significant. In the past year, the Branch has been host to scientists from Japan, India, and Italy, and permanent members of the staff have had work assignments in France and England. Training has been provided for several postdoctoral fellows and students who have spent varying periods of time in the Branch.

I. Biochemistry of the Thyroid

A. Iodide Transport

The ability of various univalent anions to react with the iodide transport system of sheep thyroid slices has been investigated. Since the 1961 report, several other ions have been examined by measurement of their saturation characteristics. The K_M and/or K_I values ranged from 3–5 x 10^{-7} M to 2 x 10^{-2} M, giving the following series:

$$T_cO_4^- < ClO_4^- < ReO_4^- < BF_4^- < SeCN^- \cong SO_3F^- < SCN^- < I^- < NO_3^- < NO_2^- < OCN^- \cong Br^-$$

The partial molal ionic volumes at infinite dilution, ϕ_0, were determined by pycnometry. With the exception of ReO_4^-, SO_3F^- and $SeCN^-$, ϕ_0 was inversely related to the K values. A linear relation between pK and ϕ_0 was found between the values 25 and 46 cc/mole for ϕ_0. At larger volumes, pK declined, but a clear-cut maximum was not observed. Except for $T_cO_4^-$, which was not tested, all of the anions were shown to be competitive inhibitors of iodide transport (Dr. Wolff and Mr. Maurey).

The previously described finding that ouabain caused inhibition of thyroidal iodide transport was investigated in greater detail. Adenosine triphosphatase (ATPase) activity was measured in homogenates and subcellular fractions of thyroid and other iodide-transporting tissues of various species. A ouabain-sensitive portion of ATPase activity paralleled the properties of the iodide transport system in the following ways: (1) both were comparably sensitive to ouabain over a thousand-fold range of concentration and in a variety of species, (2) they were comparably sensitive to six other cardioactive compounds and quinidine over a ten thousand-fold concentration range, (3) both were half-activated by K^+ at a concentration of 0.9 to 1.4 mM, and partial activation by ouabain was reversed by K^+, (4) both were comparably stimulated by thyrotropic hormone. It was concluded, therefore, that this $Na^+ - K^+$ requiring ATPase system is indirectly involved in iodide transport (Dr. Wolff and Dr. Halmi).

The technical matter of the possible interference in *in vitro* iodide transport studies by iodide released into the medium from the tissue was studied with rabbit thyroid slices which had been "equilibrium labeled." The trichloroacetic acid soluble material was shown by chromatography and electrophoresis to be 20 percent in the form of iodide, 30 percent in peptides, and the remainder in protein. It was concluded that the iodide released from sheep thyroid slices was insufficient to interfere with iodide saturation studies (Dr. Wolff).

Since lecithin derived from the thyroid gland had been reported to bind iodide, the behavior of other anions was investigated. Partition of radioactive ions between aqueous and nonaqueous phases was measured at pH 4. Ions which were concentrated to a considerable extent by the thyroid (I^-, ReO_4^-, $T_cO_4^-$) were bound more strongly to thyroidal lecithin than those (Br^-, SCN^-,

$MoO_4^=$, $SO_4^=$) for which the thyroid had little or no affinity. Binding to the phospholipid is decreased at higher pH, and this pH sensitivity in relation to binding of the various ions is under study (Dr. Wolff and Dr. Schneider).

Thyroxine and Iodotyrosine Synthesis

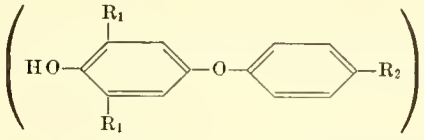
It has been generally believed that, in the iodination of tyrosine, the second iodine atom is introduced much more readily than the first. Biological iodinating system operating *in vitro*, however, usually show monoiodotyrosine (MIT) as the principal product. Studies were carried out, in collaboration with Dr. M. Berman, OMR, NIAMD, and Dr. R. Pitt-Rivers, National Institute for Medical Research, Mill Hill, England, on the rates of iodination of N-acetyltyrosine and N-acetylmonoiodotyrosine by I_2 in aqueous systems. At ph 9.0 to 10.5 the rates of introduction of the first iodine into the phenyl ring was 5 to 10 times faster than the second. The observed reaction rates varied inversely with the square of the I^- concentration, in keeping with the following scheme of reactions:

$$I_2 \rightleftarrows I^+ + I^-;\ I_2 + I^- \rightleftarrows I_3^-;\ I^+ + H_2O \rightleftarrows H_2OI^+;$$
$$H_2OI^+OH^- \rightleftarrows H_2O + HOI;$$
$$I^+ + PhenO^- \rightarrow PhenIO^- + H^+$$

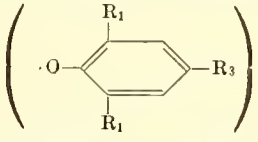
The effect of pH on this reaction is now under study (Drs. Rall and Mayberry).

Further work on model systems for the coupling reaction in thyroxine biosynthesis has been carried out. The nonenzymatic reaction in which thyroxine (T_4) is formed in 25 percent yield from 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) and diodotyrosine (DIT) was extended to related compounds. The coupling of DIHPPA with MIT gives 3,3′,5′-triiodothyronine in a yield only slightly smaller than for T_4. This method provides a practical synthesis for this compound which is of biological interest and is known as "reverse T_3." When DIHPPA is coupled with tyrosine, or when 4-hydroxy-3-iodophenylpyruvic acid (MIHPPA) is coupled with DIT, the yields are much smaller. The coupling of DIHPPA with glycyl-L-DIT-glycine resulted in the formation of glycyl-L-T_4-glycine in 7–9 percent yield. This is the first known synthesis of a thyroxyl tripeptide (Drs. Cahnmann and Shiba).

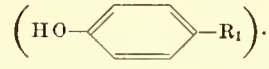
The mechanism of the reaction of various free phenoxy radicals with analogs of tyrosine, and with oxygen, were studied. Previous investigations showed that analogs of T_4



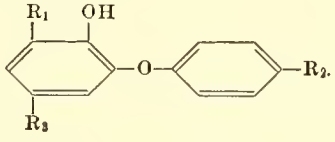
could be obtained from tri-t-butylphenoxyl



and tyrosine or desaminotyrosine



The effect of electron withdrawing groups at R_3, in place of the electron donating t-butyl group, was examined. When $R_3 = COCH_3$ or $COOC_2H_5$ or CN, the product was



It appears that these orthophenol ethers are formed via unstable ortho-quinol ether intermediates. The products of the reaction of the free radical with oxygen indicates that unstable hydroperoxides (or peroxides) are the first reaction products (Drs. Cahnmann and Shiba).

In the course of a study of *in vitro* iodination by a thyroid homogenate system, an artifact was found to be due to the contamination of commercial preparations of glucose oxidase with β-fructofuranosidase. This resulted in the production of peroxide from the sucrose used in preparing the homogenate. The enzyme also hydrolized raffinose and trehalose, but not maltose, lactose and α-fructofuranoside (Drs. Wolff and Robbins).

C. Iodoproteins

The physical chemistry of thyroglobulin has received further study. The degrees of unfolding and disaggregation of the molecule as a function of urea concentration was determined by sedimentation, viscosity, optical rotation, ultraviolet difference spectra, and tryptophan fluorescence. The molecular changes produced by urea were partially reversible.

The effects of iodination of thyroglobulin on its molecular configuration has been investigated.

Only above ~40 percent iodination of the tyrosyl groups were configurational changes observed; at higher levels, the protein became denatured. When less than 110 moles of iodine were introduced, the only important difference between the iodinated and the native molecules was the susceptibility of the former to thermal or alkaline fragmentation. The rate of tryptic hydrolysis was the same for native and lightly iodinated thyroglobulin (Dr. Edelhoch and Mr. Lippoldt).

The spectrophotometric titration method, developed last year, for the measurement of iodoaminoacids in intact thyroglobulin was applied to a study of the iodination of gamma globulin. The course of iodination resembled that of thyroglobulin in certain respects. Unfolding of the molecule with urea or guanidine resulted in a "normalization" of the iodination behavior of the tyrosine residues, but did not change the production of thyroxyl groups, which were formed only at high degrees of iodination. Gamma globulin, however, was less sensitive than thyroglobulin to urea. The similarity between iodination of native gamma globulin and thyroglobulin indicates that the latter has no special properties in this respect (Dr. Edelhoch and Mr. Schlaff).

Most preparations of thyroglobulin are contaminated with a protein having a higher sedimentation coefficient (~25 S). This substance has so far not been characterized. By repeated centrifugations, and by a sucrose gradient technique, preparations enriched up to 70 percent in this component have been obtained. The results of spectrophotometric titration indicate that the 25 S component is an iodoprotein which differs in iodoamino acid composition from 19 S thyroglobulin. The nature of these differences is under investigation (Dr. G. Salvatore and Dr. M. Salvatore).

D. Thyroxine Transport in Blood

Studies on the kinetics of the thyroxine-serum protein interactions have continued with the collaboration of Dr. Mones Berman (OMR, NIAMD). The rate of quenching of tryptophane fluorescence produced by adding thyroxine to the protein was measured by means of a rapid-filling cell and a fast recorder. A concentration-dependent quenching rate was observed, and indicated that with bovine serum albumin (5.5×10^{-7} M, pH 7.4, room temperature) the binding reaction was complete in 100 milliseconds. Although determination of the rate constants was not possible without further refinements in the instrumentation, the great rapidity of the interaction is of physiological interest (Drs. Robbins, Rall, and Andreoli).

Work was begun on the isolation of the specific thyroxine-binding proteins from human serum. This presents great difficulty in the case of the alpha globulin (TBG) because of its low concentration (about 1 mg per 100 ml). With Cohn Fraction IV–4 as starting material, and using a modified chromatographic procedure on diethylaminoethyl cellulose, it has been possible to obtain a TBG fraction contaminated only with one other alpha globulin. Efforts to improve the yield and purity are continuing. The thyroxine-binding pre albumin (TBPA) is also obtained by this procedure, and the iron-binding protein, transferrin, is also obtained in apparently pure form (Drs. Andreoli and Robbins).

The interrelationship between thyroxine-binding by proteins in blood and in extravascular fluids was investigated in mice inoculated with Ehrlich ascites tumor. Interesting differences were found. Normal serum contained mainly a "post-albumin" thyroxine-binding protein, and an alpha globulin in trace quantity. Ascites, however, contained much more of the alpha globulin. In tumor-bearing animals, the serum contained increasing amounts of the alpha globulin, depending on duration of disease. The findings suggest the possibility that some extracellular thyroxine-binding proteins may have an extravascular origin, and that some of the serum proteins may be derived from the extravascular fluid (Drs. Salvatore and Robbins).

E. The Chromatographic Separation of Iodoamino Acids

Studies of the chromatographic behavior of iodoamino acids on columns of strong base anion exchange resins have been extended. Existing methods using aqueous formic or acetic acid have been unsatisfactory for the separation of thyroxine (T_4) and triiodothyronine (T_3). Since this failure appears due to strong nonionic binding forces between the resin matrix and these compounds, a systemic study of the pH dependence of the binding in the presence of solvents with high dielectric constant and high solvating capacity for iodothyronine was undertaken. Resin cross-linking

and competing anion concentration were also varied. It was possible to obtain a high degree of differential binding between T_4 and T_3 with two resin types of low cross-linkage and with either of four solvents. Column chromatography based on these findings has resulted in two satisfactory techniques for the complete resolution of iodide, MIT, DIT, T_4 and T_3 in serum or in thyroid hydrolysates (Dr. Lewallen).

F. Action of Thyroxine on Isolated Systems

The investigation of thyroxine effects on liver mitochondria has continued in collaboration with Drs. R. Michel, O. Michel, and S. Varrone at the College de France, Paris, France. It has been shown for both T_4 and T_3 that the rapid swelling induced by these agents was not accompanied by detectable metabolism. The binding of these compounds to the mitochondria were largely concentration independent over the range 10^{-5} M to 10^{-9} M. The results indicate an absence of obligatory coupling between metabolism or binding of the hormone and its effect on mitochondria.

Iodine (I_2) (5×10^{-6} M) and ICN (10^{-6} M) have been shown to swell rat liver mitochondria. This swelling was reversed by ATP, and prevented by albumin, hypertonic sucrose, amytal, antimycin A, cyanide, EDTA, and dinitrophenol, irrespective of the oxidation-reduction state of the respiratory carriers. In all respects, the effects of I_2 and ICN mimic that of thyroxine, and the possibility is raised that I^+ may be the effective agent in each. I_2 at 4×10^{-5} M also caused an increase in oxygen consumption of liver mitochondria and a slight decrease in P/O ratio with succinate as substrate. At 4×10^{-4} M, both respiration and phosphorylation were suppressed. ICN likewise depressed the P/O ratio at 4×10^{-5} M, but with a decrease in oxygen consumption attributable to the cyanide (Dr. Rall).

G. Studies on Congenital Goiter

Patients with congenital goiter have been under study because they present the opportunity to identify specific defects in thyroid function. A number of such defects, similar to those described by others, have been observed. In the past year, however, an unusual defect was found, there being one other case in the world literature. In this 7-year-old girl with goitrous hypothyroidism, the thyroid gland, salivary glands and gastric mucosa all failed to concentrate iodide in the normal manner. After a brief period of treatment with 12 mg of KI per day, many of the signs of hypothyroidism were reversed. Thus, by supplying sufficient iodine through simple diffusion, it was demonstrated that the subsequent steps in hormone synthesis were intact. The role of the iodide trapping mechanism in regulation of hormone synthesis will be clarified by further studies (Drs. Robbins and Wolff).

II. Physical Chemistry of Proteins

Emphasis on fluorescence of proteins has continued as an extremely sensitive means for detecting alterations in molecular configuration. This work has been done in collaboration with Dr. R. Steiner, Naval Medical Research Institute. The tryptophan fluorescence has been used in conjunction with optical rotation difference spectra, and polarization of fluorescence, to study the influence of high pH and urea on the structure of soy bean trypsin inhibitor. It was demonstrated that the kinetics of the transitions could be followed by fluorescence measurements. The trypsin inhibitor has an extremely stable structure, and is influenced only at very high urea concentration (9 M) at room temperature (Dr. Edelhoch).

The effect of the solvent on tryptophan fluorescence of protein was investigated, and model tryptophanyl peptides were employed to evaluate the system. The ability of solvents with low dielectric constants to alter fluorescence intensity appeared to depend upon the accessibility of the tryptophanyl residues to the solvent molecules. The accessibility could be varied by various denaturing solvents such as urea, guanidine HCl and detergents (Dr. Edelhoch and Mr. Lippoldt).

The effect of iodination on the structure and function of rabbit antibody gamma globulin was examined. Detectable changes in protein configuration occurred only when more than 50 moles of iodine were introduced. At these levels, aggregation occurred. Formation of only 2 moles of MIT and 2–3 moles of DIT (12.5 moles of iodine), however, were sufficient to reduce the precipitin activity of rabbit antithyroglobulin antibody. Activity was further reduced at 25 moles of iodine, but progressively greater incorporation of iodine had no effect. It appears that tryosyl groups which are important for antibody activity are preferentially iodinated. This is in sharp contrast to the

lack of effect of iodination on the precipitin activity of the antigen, thyroglobulin (Dr. Edelhoch and Mr. Schlaff).

Studies on the genetic coding ratio for protein synthesis were carried out in collaboration with Dr. M. Nirenberg, LNE, NIAMD, and LCB, NHI. Molecular weights were determined for polyuridylic acid polymers used as "template" RNA in an *in vitro* E. coli ribosomal system, and on the C^{14}-polyphenylalanine polymers produced. The measurements were extremely difficult because of solubility problems which required that new techniques be devised. The preliminary results indicate that the molecular weight ratios are consistent with a coding ratio near three (Drs. Pfuderer and Edelhoch).

III. Carbohydrate Metabolism

A. Glucose

Further progress has been made in elucidating the effects of the thyroid stimulating hormone (TSH) on glucose oxidation by thyroid tissue. By injecting as little as .01 unit of TSH into the carotid artery of the dog, stimulation was detected within 15 minutes. The effect *in vitro* on thyroid slices was independent of glucose concentration in the medium, and so was not primarily due to a change in glucose transport. It was not abolished by puromycin, which inhibited incorporation of C^{14}-leucine into protein. Prior incubation with nicotinic acid increased glucose-1-C^{14} oxidation and augmented TSH action. A good correlation was found between TPN levels in thyroid slices and $C^{14}O_2$ production from glucose-1-C^{14}, indicating the importance of this cofactor in glucose oxidation by the hexose monophosphate pathway. Direct measurement of TPN and TPNH showed that TSH, acetylcholine, and menadione increase the total TPN nucleotides. Epinephrine and serotonin also increase glucose oxidation by changing the ratio of TPN/TPNH, but do not increase the amount of TPN nucleotides (Drs. Field and Pastan).

In a study of the effect of ACTH on different zones of the adrenal cortex, it was shown to increase phosphorylase activity in the zona fasciculata but not in the zona glomerulosa. The latter was not influenced by angiotensin, which is thought to stimulate aldosterone secretion. Differences in glucose uptake and oxidation were also demonstrated in the two zones (Drs. Field and Williams).

The mechanism of the hypoglycemia induced by ethanol has been investigated further. Poor nutrition was essential for the hypoglycemia, and the effect required 44 hours of fasting. Ethanol did not inhibit the hyperglycemic effect of glucagon on the conversion of fructose to glucose, but did impair glycogen synthesis from fructose and glucose. Ethanol was found, in perfused liver experiments, to inhibit gluconeogenesis apparently at the step of amino acid deamination. Although both the decrease in glycogen synthesis and the decrease in gluconeogenesis could lead to hypoglycemia, the latter effect appeared the more important (Dr. Field).

B. Glycogen Storage Disease

An assay for debranching enzyme in leukocytes was developed and applied to patients with various types of glycogen storage disease. Low values were found in one patient who was known to have a deficiency of this enzyme in the liver. A study of this patient's family revealed values intermediate between the normal level and that found in the patient, in the mother, father and one sibling. Another sibling had a normal value. The debranching enzyme was normal in the leukocytes of patients with glycogen storage disease due to glucose-6-phosphatase and phosphorylase deficiency (Drs. Field and Williams).

C. The mechanism of action of insulin on the liver

The effect of insulin on the ultrastructure of the rat liver has been studied in collaboration with Dr. B. Wetzel, LPH, NIAMD, in an attempt to localize the sites of intracellular water accumulation that may accompany the insulin-induced accumulation of potassium. Preliminary electron-microscopic observations on perfused rat livers failed to reveal any volume changes. After 90 minutes of perfusion with insulin, however, there was a decrease in the number of dense bodies (lysosomes). There was also a striking change in the appearance of the rough endoplasmic reticulum, similar to changes usually associated with increased rates of protein synthesis (Dr. Mortimore).

Further work has been done with the improved

cyclic perfusion technique which permits the *in situ* establishment of an isolated rat liver circulation with virtually no interruption of blood flow. Insulin concentrations of the order of 10^{-10} M (6,000 MW) have been shown to have a variety of effects: (1) Insulin strongly inhibited the net hepatic release of glucose, appearing within 30 minutes and reaching a maximum of 6–7 mg/g of liver in 90–120 minutes. This effect was independent of glucose concentration. (2) Insulin inhibited the increase of lactate which normally occurs during perfusion. Since lactate is presumably derived from glycogen, this effect is consistent with a direct inhibitory action of insulin or glycogenolysis, and suggests that the glucose effect is not via insulin action on glucose penetration, glucose phosphorylation or glucose-6-P hydrolysis, none of which would affect lactate formation. (3) Insulin decreased hepatic K^+ loss, the maximum effect occuring by 60 minutes. Since the production of an increase of liver K^+ by the isosmotic exchange of NaCl by KCl in the medium also reduced glucose and lactate accumulation, it is possible that the carbohydrate effects may be secondary to the action of insulin on intracellular K^+. (4) Insulin inhibited the hepatic release of amino acids similar in time course and degree to the previously shown effect on urea. Insulin also enhanced the net increase in uptake of amino acids which resulted from their addition to the medium. Studies are planned to evaluate the possibility that this effect results from an influence of insulin on protein synthesis or degradation (Drs. Mortimore and Mondon).

D. Galactose and Galactosemia

The oxidation of C^{14} galactose by patients with the congenital disease, galactosemia, has been the subject of continuing investigation. Three out of a total of 10 patients studied were found to metabolize 1 gm of intravenously administered galactose to a normal extent although they fulfilled all the diagnostic criteria of the disease, including a demonstrated enzyme deficiency in their erythrocytes. In one subject, several tissues were biopsied, and only the liver had the capacity to metabolize galactose.

Further studies were done, in collaboration with Dr. Y. Topper, LBM, NIAMD, on the effect of progesterone and menthol on galactose oxidation. Although previous experiments had shown that metabolism of a trace quantity of galactose was increased in galactosemics, in the present work no effect of the drugs was found when four patients were given 1 gm amounts of galactose.

Experiments designed to study the effect on the offspring of galactose intake by pregnant individuals were carried out in collaboration with Dr. H. Bernstein, O, NINDB. Pregnant rats were fed a diet containing 40 percent galactose. Galactose was found to pass rapidly across the placenta, and fetal blood galactose levels were similar to those of the mother. The newborn were shown to have cataracts, as well as a decrease in liver glycogen and phosphorylase, and histological changes in renal tubular cells. These studies indicate that galactosemic infants may suffer damage *in utero*.

A sensitive and specific spectrophotometric assay for blood galactose was devised, making use of the recently discovered enzyme, galactose oxidase. This method will be useful as a clinical test in newborn infants (Drs. Segal and Roth).

IV. Amino Acid Transport

Amino acid transport in kidney cortex slices has been examined under various conditions, in continuation of the investigation reported last year. Dr. L. Rosenberg, MB, NCI, and Dr. M. Fox, MDB, NIAMD, have collaborated in portions of this work.

In human cystinuria, the basic amino acids lysine, ornithine and arginine also appear in the urine. In rat kidney slices, the amino acids exhibited mutual competitive inhibition but did not affect cystine transport. The same was true in monkey kidney, but in dog kidney, competition between cystine and the dibasic amino acids was observed.

A number of sugars were tested for their effect on amino acid transport by kidney slices, but only glucose, galactose and fructose produced inhibition. In human subjects, urinary excretion of the same sugars—in diabetes, galactosemia, and fructosuria, respectively—was associated with amino aciduria. The intravenous infusion of glucose and galactose in normal human subjects has been shown to produce an increase in amino acid clearance by the kidney.

The movement of sugar into cells is known to alter potassium flux, and the dependence of amino acid transport on potassium has been postulated.

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Ion effects on amino acid transport were, therefore, studied. Although affected by potassium, amino acid transport demonstrated an absolute dependence on sodium. Cardiac glycosides have been shown to inhibit amino acid transport.

Salicylate intoxication causes aminoaciduria in man. In the *in vitro* system, as little as 10 mg percent of the drug is an effective inhibitor of amino acid transport. The mechanism of this effect is under investigation.

Amino acid transport has also been studied in thyroid gland slices. There appeared to be two different transport phenomena, one for alpha-aminoisobutyric acid (AIB) and glycine, the second for other naturally occurring amino acids. Pituitary thyrotropic hormone stimulated AIB transport, but did not affect the other amino acids. No effect on amino acid transport has been observed from glucose, or drugs such as thiocyanate and perchlorate, which influence thyroidal iodide transport (Drs. Segal, Roth and Thier).

CLINICAL HEMATOLOGY BRANCH

I. Immunologic Studies

A. Leukocyte Isoantigen Systems

During the past 10 years many investigators have attempted to measure antileukocyte isoantibodies, but none of the techniques used, which were primarily variations of agglutination procedures, have been adequate for establishing the specificity of leukocyte antigen groups. Complement fixation techniques which were developed in the Clinical Hematology Branch to measure platelet isoantigens and antiplatelet antibodies of clinical significance (see reports of previous 2 years) were found to be applicable in measuring antileukocyte isoantibodies. Use of these complement fixation techniques lead not only to identification of specific leukocyte antigens, but also to differentiation of specific cell lines which contain the antigens. Some antigens were found to be shared by granulocytes and lymphocytes, some limited to lymphocytes only, and some shared by granulocytes, lymphocytes and platelets. We have found that antibodies against these antigens can be used to identify specific cell lines through different stages of maturity and will no doubt have applications in determining developmental origin of different cell types. These new antigen systems, which are as clearly defined as erythrocyte antigen systems, are of considerable interest as genetic markers and no doubt of importance in transplantation immunity despite their obscurity up to now.

The following are some of the clearly defined antigen groups: Pl is the symbol for platelet antigen, Gr for granulocyte and Ly for lymphocyte. The superscript letter defines the locus and the number the identified allele. All antigens named in the chart have been confirmed by identical antibodies found in different individuals sensitized by transfusion or pregnancy, as indicated.

Antigen	Antibody		Panel	
	Post transfusion	Post pregnancy	Number	Percent Positive
1. Pl^{A1}	3	3	306	98
2. $PlGrLy^{B1}$	5	4	230	37
3. $PlGrLy^{C1}$	1	1	80	30
4. Ly^{D1}	2	1	96	31
5. Pl	------	1	127	7
6. Pl	1	------	165	99. 5
7. PlGrLy	1	------	150	70
8. Ly	1	------	40	65

(Drs. Shulman, Marder, Aledort)

B. Significance of Maternal Antibodies Against Isoantigens on Leukocytes and Platelets—Effects of Infusing These Antibodies

The fact that platelets and leukocytes have some shared antigens has not been known. By and large those groups of investigators who have been working on leukocyte antigens have not tested platelets and vice versa. One reason for previous conclusions that platelets and leukocytes have different antigens is the fact that maternal antibodies produce thrombocytopenia in newborn infants not infrequently (see last year's report) but neonatal leukopenia is very rare. Nevertheless, some of the same antibodies which were found to react with the infants' platelets also reacted with leukocytes in complement fixation tests, despite the fact that the infants were not leukopenic. This discrepancy was puzzling until explained by effects of infusing maternal antibodies into adults whose cells contained reactive antigens. Three different isoantibodies against three different isoantigens shared by platelets and leukocytes were used in these tests.

In all instances there was an immediate fall in both platelets and leukocytes, but whereas platelets continued to fall as antibody was administered at the same rate, granulocytes, after reaching a level of 500 to 1,000 per mm^3, increased rapidly, sometimes to higher than control levels. The transient leukopenia and persistent thrombocytopenia in the face of continued administration of antibody produced the deceptive picture of specific action against platelets. It therefore could be postulated that maternal isoantibodies cause neonatal thrombocytopenia more often than leukopenia because compensatory mechanisms for maintaining cell levels are more effective for leukocytes than for platelets (Drs. Shulman, Marder, Aledort).

C. Clinical Significance of Isoantibodies Against Leukocytes

During the past five years, various transfusion reactions, ranging in severity from mild chills and fever to severe and fatal shock, have been blamed on isoantibodies against leukocytes. It is generally accepted that feibrile transfusion reactions which cannot be accounted for by incompatible erythrocytes are due to incompatible leukocytes given unknowingly. With the identification of specific anti-leukocyte antibodies, it was possible to evaluate the effects of intentionally mismatching leukocytes in transfusions. Such mismatched leukocytes, whether given to an individual previously sensitized or mismatched by passive infusion of antibody into an individual with reactive cells, produced a variable clinical response, ranging from no reaction whatsoever, to chills and high fever. The dose of antigen given and the rate of antigen antibody reaction were found to be important parameters in determining the severity of clinical response. In a number of instances large amounts of mismatched leukocytes could be given with no effect whatsoever. Further observations of this type will help to determine the significance of so-called "buffy coat" transfusion reactions (Drs. Shulman, Marder, Aledort).

D. A New Type of Blocking Reaction Used in Determining the Presence of Obscure Isoantibodies

In a number of instances clinically, either as the result of a transfusion reaction or failure of transfused platelets to circulate, isoimmunization against platelets or white cells is suspected, but cannot be proved because it is impossible to detect antibody activity by agglutination techniques or even by more sensitive direct complement fixation techniques. We have found that some apparently nonreactive sera from individuals suspected of being immunized are capable of inhibiting complement fixation by previously defined antibodies. Antiplatelet and antileukocyte antibodies which "block" the complement-fixing activity of other antibodies against the same antigen are similar to the so-called "incomplete" antierythrocyte antibodies which block the agglutinating activity of other antibodies. The newly recognized type of blocking antibody can be measured only by their ability to interfere with reactions of complement-fixing isoantibodies of the same specificity; and in order to test for a blocking antibody of this type, the corresponding complement-fixing antibody has to be available. Blocking antibodies accounted for at least half of the antibodies found in mothers sensitized by fetal cells and were observed to develop in transfused individuals. Evidence for mixtures of blocking and complement-fixing antibodies of the same specificity were obtained in sera of sensitized mothers and in individuals sensitized by transfusion. Blocking antibodies of this type probably account for instances when isoimmunization is suspected clinically but antibody cannot be detected by agglutination or direct complement fixation techniques.

It has been assumed generally that most antibodies which cause rapid destruction of cells require participation of complement or have strong agglutinating capacity. The ability of "blocking" antibodies (which cause neither agglutination nor complement fixation) to affect survival of cells *in vivo* was therefore questioned. Blocking antibodies when infused into reactive recipients were found to produce as rapid and marked thrombocytopenia as complement-fixing antibodies, indicating that blocking antibodies can be as effective as complement-fixing antibodies in depressing some types of cells *in vivo*. It is apparent that when more complement-fixing antibodies are recognized and available, more blocking antibodies will be found to account for isoimmunization (Drs. Shulman, Marder, Aledort).

E. Neonatal Thrombocytopenic Purpura

Last year's report described the techniques developed for measuring antibodies in mothers sen-

sitized by fetal platelets and the clinical course of neonatal thrombocytopenic purpura in six children born of four normal mothers. During the past year we have had an opportunity to study four additional families, as well as three of the same mothers studied last year who became pregnant again. In the latter instances we were able to predict the occurrence of neonatal thrombocytopenia by a rise in antibody and to prepare for post partum treatment of the infant. In all these cases that were treated, purpura was much less marked than it had been in untreated siblings born about 1 year before. This was true despite the fact that the antibodies involved were the same and were present in higher, if not equal titer. The forms of treatment which were found to be effective were administration of steroids to the mother prior to the birth of the child, and exchange transfusion of infants. These studies which will have to be continued over the years are firmly establishing the clinical picture of immune neonatal thrombocytopenic purpura, the types of antigens and antibodies involved and the best approaches to effective therapy (Drs. Shulman and Marder).

F. Effects of Isoantibodies on Leukemic Cells

In determining whether isoantibodies against specific cell lines would react with cells at different stages of maturity, cells from patients with acute and chronic leukemia were compared with normal cells. All cells, regardless of their state of maturity, contained the antigens found on normal cells. Because of this finding, isoantibodies were infused into leukemic individuals to measure the ability of these antibodies to depress circulating cells. Leukemic cells were found to be unusually sensitive to the action of these antibodies. It remains to be determined whether this effect was due to the greater affinity of leukemic cells for antibody, their higher antigen content or their limited reserve. The selective action of these isoantibodies against leukemic cells provides an approach to the immunologic therapy of leukemia and related disorders (Drs. Shulman, Marder, Aledort).

II. Coagulation Studies

A. A New Method for Measuring Minimum in Vivo Concentrations of Factor VIII Applied in Distribution and Survival Studies

Small amounts of plasma (0.5 to 25.0 ml.) containing normal Factor VIII concentrations were injected into severe hemophiliacs. After a mixing period, blood was drawn for 2-stage prothrombin consumption determinations. *In vivo* Factor VIII concentrations from 0.02 to 0.8% of normal, produced by dilution of Factor VIII in the hemophiliac's plasma, were correlated with percent prothrombin consumed (15 to 85%). Standard curves prepared in this way were identical in five different hemophiliacs, and could be used to measure unknown *in vivo* Factor VIII concentrations with approximately ±20% error. This sensitive technique permitted measurement of Factor VIII survival *in vivo* for 12 half-lives over a 5-day period. Previously available methods were useful for, at most, 6 half-lives, covering less than 2 days. Prolonged survival measurements gave decay curves with two exponential components which were not previously discernible. The initial component had a $T_{1/2}$ of 4 to 5 hours (primarily extravascular diffusion); the second component had a $T_{1/2}$ of 9 to 11 hours (degradation and back diffusion). These two components, which did not vary with the amount of Factor VIII administered, indicated an extravascular compartment approximately 1.5 times the plasma volume.

These findings are of special importance because up to now no distinction has been made between the initial phase of extravascular diffusion of Factor VIII and the subsequent phase of degradation and back diffusion. The ability to distinguish these two phases by more sensitive measurements and the constancy of these two phases over an extreme dose range permitted description of all survival curves by the same two exponents as determined by a least square's fit using a digital computer program developed by Dr. Mones Berman. Knowledge of the size of the extravascular compartment and the rates of different decay phases permit better estimation of Factor VIII requirements in therapy. This is particularly important now that concentrates of Factor VIII are available for treating hemophiliacs (Drs. Shulman and Marder).

B. Standardization of Methods of Measuring Factor VIII

In assaying commercial Factor VIII preparations, it became evident that agreement between laboratories on a standard of potency and on a standard method of measuring antihemophiliac

factor was hard to obtain. Because of differences in methodology and in standards used by Merck Sharp and Dohme, the Protein Foundation which licenses Fraction I, and our laboratory, an interest developed on the part of the Division of Biologics Standards in establishing a standard for measuring Factor VIII. This cooperative study between the four groups is continuing in an attempt to prevent unnecessary confusion which will arise as Factor VIII concentrates become more readily available for clinical use (Drs. Shulman and Marder).

C. Evaluation of an Unusual Form of Painful Purpura

About 7 years ago Gardner and Diamond described the syndrome of autoerythrocyte sensitization which essentially is the occurrence of spontaneous painful bruising in females. The diagnostic test suggested for this condition was injection of the patient's own red cells intradermally, a positive reaction being development of a painful ecchymosis at the injection site. The disease was considered to be caused by sensitization against autologous red cells.

In studying nine patients who developed spontaneous painful bruising, it was found that two of these gave no response and one variable response to autologous red cells despite the clinical similarity of hemorrhagic manifestations in all patients. Four of the patients responded to injections of histamine in trace amounts (2 micrograms) to form large painful ecchymoses, and two responded to injections of other basic amines and enzymes known to release histamine. Others gave no response to histamine or histamine releasers. Study of the histamine metabolism of positive reactors gave no evidence of abnormality, nor was there evidence of abnormality in catecholamine metabolism.

Three of the nine patients responded to blood transfusions, but it was found in one of these cases that an equivalent response was obtained with the patient's own blood used in an autologous transfusion. Only one case has been tested by autologous transfusion but there is evidence from other procedures used that it is possible to cause fluctuations and even total regressions of lesions in this disease by procedures which contain much in the way of psychological suggestion but make little sense physiologically. For this reason, a large psychiatric component appeared to be present in this disorder and Dr. Hart of NIMH is cooperating in an attempt to find some psychiatric common denominator among these patients (Drs. Shulman and Aledort).

D. Continued Studies of a Form of Acquired Hemophilia Due to an Abnormality in Gammaglobulin

In the well-recognized disorder of acquired hemophilia, perfectly normal elderly individuals suddenly become hemophiliacs and the gammoglobulin fraction of their plasma has the ability to inactivate Factor VIII of normal blood. Recent articles have appeared in several journals suggesting that this acquired activity is enzymatic in nature, but our studies have shown that the kinetic characteristics of the reaction are consistent only with a bimolecular combination characteristic of antigen-antibody reactions. The stoichiometric nature of the reaction has been demonstrated both *in vitro* and *in vivo*. By infusing plasma from a patient with acquired hemophilia, transient hemophilia (for periods of several hours) was induced in animals and in man. The quantitative relationships between the amount of gamma globulin and in the amount of Factor VIII inactivated gave some indication of the molar concentration of Factor VIII in blood and the rate of Factor VIII production *in vivo*. This indirect approach to measuring Factor VIII is the only one available at present because Factor VIII has not been separated sufficiently from other plasma proteins to permit its physical or chemical characterization. The reactions studied in acquired hemophilia have direct bearing on the problem of autoimmunity. Since the apparent antigen is a blood constituent, the possible immunologic basis of the disorder is of special interest, for current concepts of autoimmunity preclude autologous circulating blood constituents as possible agents of sensitization (Drs. Shulman and Marder).

E. Continued Studies of the Significance of Changes in Fibrinogen Levels in Familial Mediterranean Fever

In association with Dr. Wolff of NIAID, we have continued measuring the fluctuations in fibrinogen which occur in patients with familial Mediterranean fever. These patients have peculiar recurrent episodes of fever associated with a vari-

ety of abdominal and other systemic symptoms. The patterns of periodic change in fibrinogen which have been measured are comparable to changes in fibrinogen associated with pyrogenic reactions or the generalized Schwartzman reaction. Since the etiology of the disorder is still quite obscure, it is hoped that continued studies on more individuals will document changes in fibrinogen as an integral part of the disease and thus provide a more definite lead in unravelling the pathogenesis of the disorder (Drs. Shulman and Wolff).

PEDIATRIC METABOLISM BRANCH

Cystic fibrosis of the pancreas and diseases of glycogen storage in their various biochemical, physiologic and clinical manifestations have been the object of continued investigation in the year 1962 in the Pediatric Metabolism Branch. The program of diagnostic studies on the celiac syndrome and other malabsorptive states initiated in the past years was brought to a conclusion.

Cystic Fibrosis of the Pancreas

This generalized disorder of children, adolescents and young adults is thought to be caused by an inborn error of metabolism as yet undefined. The basic defect, whatever its nature, causes a widespread disturbance in exocrine gland function and leads to a variety of manifestations due to abnormal physicochemical behavior of mucous secretions and a unique electrolyte abnormality of sweat. The link between the electrolyte and mucus abnormality is unknown.

Investigations have been directed to elucidation of the pathogenesis of this disease.

Biochemical and Immunological Studies of Macromolecules in Normal Controls and Patients With Cystic Fibrosis of the Pancreas

In an attempt to define the basic defect in cystic fibrosis of the pancreas, a coordinated effort was planned using both biochemical and immunologic methods. In the first phase of the investigation, the location of an abnormal constituent common to all organs and tissues in fibrocystic patients is being sought by immunoelectrophoresis as a screening technique. Immune sera have been produced in rabbits by immunization with macromolecular antigens recovered from urine of patients with cystic fibrosis and of normal controls (Drs. Gabriel, Pallavicini, Raunio, Talamo, di Sant'Agnese, Lietman, and with the cooperation of Dr. Seymour P. Halbert, Columbia University, New York).

Chemical Investigation of Glycoproteins in Sweat of Patients With Cystic Fibrosis and Normal Controls

The finding of elevated sweat electrolyte levels in patients with cystic fibrosis prompted an investigation for carbohydrate-containing macromolecules in this secretion.

Sweat was collected from normal controls as well as from patients with cystic fibrosis by thermal stimulation in a constant-temperature room and frozen immediately. The analysis revealed the presence of precipitable, nondialyzable, polysaccharide-protein complexes containing galactose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid.

Paper chromatography as well as analytical data suggested that regardless of age the subjects tested could be divided into three different groups, according to the following ratios of total hexose to fucose: 30, 12 and 4. All patients with cystic fibrosis were in the last group. As a similar abnormality with an increase in fucose and a decrease in sialic acid was found in previous studies in duodenal contents of fibrocystic patients, this appears to be a general property of individuals with this disease and thus gives rise to important pathogenetic consideration.

This investigation has demonstrated for the first time the presence of neutral heteropolysaccharides in normal and pathologic human sweat. It has also reaffirmed the inverse relationship of fucose to sialic acid under normal and pathologic conditions which appears to be a general biologic finding (Drs. Pallavicini, Gabriel, di Sant'Agnese and with the cooperation of Drs. Whedon and Buskirk).

Biosynthesis of Mucoproteins

The first phase of the investigation in collaboration with Dr. G. Ashwell was completed with the isolation of a new precursor in the biosynthetic pathway of mucopolysacchrides. This compound isolated from hen oviduct was identified as being uridine-diphosphate-N-acetylglucosamine-6-phospho-1-galactose (Dr. Gabriel).

Glycogen Storage Disease

Systematic clinical and chemical studies were continued of the diseases of glycogen storage, a group of disorders due to errors of carbohydrate metabolism leading to accumulation of this polysaccharide in various organs and tissues of the body.

Work is now in progress to make available to us all chemical systems necessary to describe fully the various disorders in terms of function of enzymes involved in glycogen metabolism under physiologic and pathologic conditions.

In the past year study of the phosphorylated intermediary compounds of carbohydrate metabolism was continued and extended to most of the known types of glycogen storage disease. Contrary to accepted opinion it was conclusively shown that such compounds are not increased in the various blood fractions of patients with this disorder.

Investigations have been performed on tissues obtained on surgical biopsy, as well as at necropsy, in three patients with Pompe's disease, a fatal form of generalized glycogen storage. The absence of maltase in this type of disorder was confirmed by means of a sensitive assay developed in this laboratory. It was shown that this enzyme, formerly not thought to play an important role in glycogen metabolism, resides in the soluble fraction of tissue homogenates, thus allowing its further purification and characterization. Experimental evidence has been obtained that there are several proteins exhibiting maltase activity and it is planned to study this further by the application of immunologic techniques (Drs. Gabriel, Lietman, di Sant'Agnese and Powell).

Intestinal Malabsorption in Children

Two diagnostic tests for malabsorption have been refined and simplified and their reliability has been evaluated. They will be reported in the literature shortly. It is expected that they will be of practical value to pediatricians confronted with patients with malabsorption.

Deficient fat absorption is common denominator to the many diseases which cause intestinal malabsorption, but its detection has been hampered by the lack of simple and reliable tests for its presence. During the past year, we have revised and simplified a method in which lipiodol is fed to the patient and the subsequent urinary excretion of the iodine released from the absorbed lipiodol is measured by a semiquantitative tube dilution method. The results obtained with this test were compared with the results of determinations of 4-day fat balances in patients with steatorrhea due to various causes and in control subjects. The lipiodol test was positive in those children with steatorrhea and negative in those without steatorrhea, indicating that the revised lipiodol test is a simple and reliable screening test for steatorrhea.

Although the assessment of a patient's ability to absorb the pentose, d-xylose (xylose tolerance test) has been found to be extremely useful in screening adults for malabsorption and to surpass in clinical value the glucose tolerance test, the xylose tolerance test has not yet been applied to the pediatric age group. Accurate data have, therefore, been collected for normal children and for children with various pathologic conditions. Evidence has been gathered to show that the xylose tolerance test is more useful than the older glucose tolerance test. Xylose absorption appears to be impaired primarily in diseases leading to altered fat and carbohydrate absorption from the small intestine (Drs. Jones and di Sant'Agnese).

METABOLIC DISEASES BRANCH

Mineral Metabolism Studies

I. Significance of Various Nutritional Factors Including Dietary Calcium Intake in Osteoporosis

Continuing metabolic studies from this Branch further substantiate the significant relationship between dietary calcium intake and the pathogenesis and possible therapy of postmenopausal and senile osteoporosis, a disorder resulting in thinning bones more susceptible to fracture. Until the work of this Branch and that of two British laboratories was reported, the accepted concept of the pathogenesis of this disorder held that it was the result solely of impaired bone matrix formation due to hormonal imbalance. The hypothesis of multiple etiologic factors affecting mineral utilization has now become more widely accepted.

A. Effect of Increasing Dietary Intake of Calcium on Calcium Balance. To the present time, fourteen patients with postmenopausal, senile, or idiopathic osteoporosis have been studied

at several different calcium intake levels from 150 mgm. to 2.4 grams per day, with balance studies of calcium, phosphorus, and nitrogen carried out for at least 30 days at each intake level. Three types of response have been noted: (a) one group had low customary intakes of calcium (less than 300 mgm. per day) on which they were in negative calcium balance; with increasing dietary calcium, increased amounts of calcium were retained, net storage of mineral appearing at about 800 to 1000 mgm. per day of intake (four patients); (b) a second group had relatively normal or even high customary intakes of calcium (over 600 mgm. per day) on which they were in negative calcium balance; as dietary intake was increased further, calcium balances became less negative, with net storage of mineral appearing at intakes over 1,600 mgm. per day (four patients); (c) the third group was similar to the first in that customary calcium intake was low. Increasing the dietary calcium to 800–1600 mgm. per day resulted in net retention of mineral; at higher intakes, however, negative balance again resulted (three patients), a significant finding which requires further study. The data obtained with two additional patients have not as yet been evaluated. Two patients who demonstrated storage of calcium at high intake levels have been reevaluated after more than a a year of high dietary calcium and continue to demonstrate positive balances, although to a lesser extent, indicating development of some degree of adaptation to elevated intakes.

B. Effect of Dietary Phosphate on Mineral Retention. The previously noted relationship between dietary calcium and phosphate for optimal absorption and utilization of these two elements is being investigated further by manipulation of both absolute amounts and relative proportions of these nutrients in the diets of two patients with osteoporosis. In addition, the role of dietary phosphate has been explored in a patient with "phosphate diabetes" (de Toni-Fanconi syndrome) and in a patient with Vitamin D resistant rickets. In both patients, evidence for mineral retention and bone formation has been obtained in the absence of therapeutic dosages of vitamin D, by increasing dietary phosphate alone. These observations suggest that dietary phosphate and mechanisms for phosphate absorption are of significance in the retention of bone mineral.

C. Effect of Dietary Fat on Mineral Retention. Studies by this Branch had previously noted that occult chemical steatorrhea was present in the patients with osteoporosis who failed to store calcium on high intakes, with no other evidence (intestinal biopsy, xylose tolerance) for malabsorption. Dietary fat has been varied at two dietary levels of calcium in three patients with osteoporosis. Preliminary results indicate that at high calcium intakes, 100 gm. of dietary fat per day results in chemical steatorrhea and decreased calcium absorption as compared with 20 gm. of dietary fat per day. At moderate calcium intakes, calcium absorption is greater at 100 gm. of fat per day than at the lower intake of fat, and no steatorrhea is seen (Drs. Whedon, Lutwak, Laster, Gitelman, Fox, and Shapiro).

II. *Studies of Mechanisms of Nutritional and Hormonal Influence on Bone Metabolism*

Radioisotopic measurements of calcium kinetics in association with metabolic balance studies continue to be made for estimations of pool size and rates of deposition of mineral into bone via intravenous administration of the isotope. In addition, isotope has been administered orally to measure directly the degree of absorption of calcium from the diet. Tracer studies have also been carried out with *in vitro* systems in attempts to arrive at mechanisms of calcium transport and retention.

A. Intravenous Isotope Studies. Results of 34 tracer studies in patients with osteoporosis confirm the finding first reported by this group that the rate of calcium incorporation into bone is normal in this disorder. The increased retention of calcium observed by balance measurements with increasing dietary intake of this element is not associated with any increase in the bone incorporation of calcium, suggesting that the mechanism of retention is by decreased bone resorption.

Administration of corticosteroids results in decrease in bone formation, as well as in bone resorption. Formation is decreased to a greater extent than is resorption in patients who develop negative calcium balance.

The conclusions regarding bone formation and resorption arrived at from isotopic tracer studies have been confirmed by microradiographic examination (by Dr. Jenifer Jowsey of Albert Ein-

stein Hospital, Philadelphia, Pa.) of bone biopsy specimens from these patients.

B. Oral isotope Studies. Both the rate and amount of calcium absorbed from the gastrointestinal tract have been estimated from absorption and excretion of orally administered calcium–45 in two patients with osteoporosis and two patients with idiopathic sprue. These studies will be repeated in both groups after appropriate treatment. Preliminary observation of less than 1% absorption of isotope in the sprue patients explains the finding of hypocalcemia in this frank malabsorption syndrome (Drs. Lutwak, Whedon, Gitelman, Fox, and Shapiro).

C. Phosphoethanolamine Metabolism in Bone Disease. It has been suggested that phosphoethanolamine (PET), a naturally occurring phosphorylated amine, may act as an inhibitor of mineralization of bone. A new sensitive, automatic technique for analysis of this amine was developed. Previous results from other laboratories had indicated that this substance is absent from the urine and serum of normal individuals, and that its presence signified the diagnosis of hypophosphatasia. By the use of the devised techniques it has been shown that PET is present in the serum and urine of all individuals. The rate of disappearance of infused PET from the serum was found to be directly related to the serum alkaline phosphatase level. Preliminary compartmental analysis of the disappearance curves indicate the presence of a renal threshold for this amine (Drs. Gitelman and Lutwak).

D. The Role of Dermal Losses in Calcium Balance. A new technique for continuous collection of insensible perspiration losses of mineral was developed and applied to patients with osteoporosis, as well as to young normal females. Under conditions of moderate, nonsweating activity, loss of calcium, phosphorus, and magnesium from the skin was found to be less than 10 mgm. per day of each, an amount which therefore may be disregarded in calculations of metabolic balances conducted under these conditions (Drs. Gitelman, Lutwak, and Whedon).

E. Factors Affecting Renal Excretion of Phosphate, Glucose, and Amino Acids. A patient with severe osteomalacia and excessive renal losses of phosphate, amino acids, and glucose (de Toni-Fanconi syndrome) has been studied on metabolic balances at varying levels of calcium and phosphate intake. Prolonged induced hypercalcemia reduced the rate of phosphate excretion, but not to normal levels, without affecting the glycosuria or amino aciduria. In normal control subjects, infusions of glucose or galactose resulted in diminished reabsorption of phosphate and amino acids. Explanation for these findings is being sought via related *in vitro* studies (Drs. Fox, Gitelman, Lutwak and Whedon, in collaboration with Drs. Segal and Thier of the Clinical Endocrinology Branch, NIAMD, and Dr. Rosenberg of NCI).

F. In Vitro Studies of Amino Acid and Phosphate Renal Transport. Glucose, galactose and fructose diminished amino acid transport in rat kidney cortex slices studied *in vitro*. The ionic composition of the medium for optimum transport has been defined. In similar systems, the transport of inorganic phosphate has been studied, demonstrating an accumulation of P–32 against a concentration gradient. These studies are being correlated with the *in vivo* observations described above (Dr. Fox, in collaboration with Drs. Segal and Thier of CEB, NIAMD, and Dr. Rosenberg of NCI).

G. In Vitro Studies of Calcium Uptake by Skin. An *in vitro* system has been developed, demonstrating for the first time uptake of calcium-45 by surviving slices of rat epidermis. This uptake is inhibited by dinitrophenol, and is, presumably, under metabolic control. It is planned to extend these studies to slices of human skin obtained from patients with different diseases of calcium metabolism (Drs. Shapiro and Lutwak).

H. Magnesium Metabolism in Bone Disease. Using a new automatic technique for the estimation of magnesium, metabolic balances of this cation are being determined in patients with osteoporosis and related bone diseases, in an attempt to define the interrelationships of magnesium with calcium and phosphate metabolism. Preliminary results demonstrate that the dietary requirement for balance of magnesium is quite variable from patient to patient and increases with increasing dietary calcium (Drs. Lutwak and Gitelman).

I. Calcium-Binding by Serum Proteins. It was observed repeatedly during the first 24 to 48 hours after intravenous Ca–45 administration that the specific activity of the serum calcium was consistently higher than that of the urinary calcium. *In vitro* studies using ultracentrifugation and ul-

trafiltration have shown that the specific activity of the protein-bound calcium is higher than that of free calcium in serum incubated with tracer. These results suggest that kinetic studies of calcium metabolism based on isotopically derived data may be in modest but significant error due to mass differences of the radioactive and stable isotopes (Dr. Lutwak).

J. Interrelationships of Dietary and Fecal Calcium and Fat in Rats. Rats were placed on diets containing no, moderate, or high amounts of calcium, labelled with Ca–45 and no fat, fatty acid, or triglyceride fat of the myristic or palmitic family, labelled with C–14. The feces were collected for 6 days and analyzed for calcium, free fatty acids, soaps, and triglycerides, as well as for their specific activities. Evidence has been obtained for a recycling mechanism for fat excretion. In addition, the interrelationships of fat and calcium excretion were found to be dependent not only upon the total amount of fat in the diet, but also upon the chemical nature of the fat (i.e., free or glyceride) and the length of the carbon chain (Drs. Werner and Lutwak).

K. Iodine Balance in Normal Subjects. Iodine balance studies were carried out at several levels of dietary iodine intake in six normal subjects, comparing the excretion of iodide in fractional collections of urine and stools with total 24 hour excretion, as well as with serum inorganic iodide and protein-bound iodide levels and with salivary iodide concentrations. It was concluded that urinary iodide/creatinine and fecal iodide/nitrogen ratios provide adequate bases for estimates of 24-hour excretions for large scale surveys of populations. Salivary and serum iodide levels were approximately related to dietary intakes (Drs. Lutwak and Whedon, in collaboration with Drs. Vought, London, and Dublin of the Epidemiology Branch, NIAMD).

Energy Metabolism Studies

Studies of human energy metabolism involving use of the Metabolic Chamber for continuous, long-term analysis of respiratory oxygen and carbon dioxide exchange are concerned with a number of physiological and metabolic problems. These investigations deal with disordered metabolism in obesity, effects of various agents on fat mobilization and oxidation, mechanisms of temperature regulation as affected by hormonal action and in various diseases, and the action of digitalis. Collaboration with increasing numbers of groups in this and in other institutes is now a prominent characteristic of the activities of the Metabolic Chamber staff.

I. Physiological Studies of Obesity

Although no one has seriously challenged the principle that obesity develops as the result of a continuing intake of calories which is in excess of those needed to provide energy for the various processes and activities of the body, for some time questions have been raised concerning possible abnormalities in obese individuals in the rate of caloric expenditure under various conditions and in the processes of synthesis, mobilization and oxidation of fat. In essence to date, varying abnormalities have been noted in these studies and by other workers which suggest that extensive further work will be necessary under carefully controlled conditions to sort out what appear to be numerous different ways in which fat metabolism may be disturbed in the obese—at various stages and degrees of obesity, at various ages, and under the influence of numerous environmental (and possibly genetic) factors. To date the biophysical factors thus far noted (by this group) which favor conservation of calories in the obese as compared to normal individuals (diminished physical activity and less caloric expenditure on exposure to cold) have been much more consistent than the metabolic, and with respect to the latter certain observations reported by others have not been confirmed.

A. Paired Studies of Energy Expenditure. Since the Metabolic Chamber analytic apparatus presents an unique system for precise measurement of energy expenditure under a full range of physical activities, an effort was made to determine the presence or absence of quantitative differences between obese and lean in energy expenditure over an extended period. Two young men were studied who were matched in age, height and weight, the only evident difference being that one had been formerly obese and the other never obese in his lifetime; for a 3 months' period they lived together and followed an identical caloric intake and physical activity protocol. Following a 1 month period of weight maintenance, a 6 week period of over-nutrition was instituted followed by a 2½-week period of undernutrition to bring the subjects back to starting weight. During over-

nutrition the formerly obese subject clearly outgained the lean subject, the difference being accounted for by a lesser increase in energy expenditure as determined from periodic Chamber experiments lasting many hours. Differences in body composition were obscured by the experimental error of these measurements, although it appeared that approximately 75% of the weight gain by each subject was fat. The meaningfulness of such difficult paired experiments is uncertain, but it seems important to make further effort by similar careful measurements to detect, if present, differences in caloric turnover in relation to development of obesity in man.

B. Metabolic Studies of Complete Fasting. Only preliminary data are available on the effects of an 18-day complete fast in a 24-year-old 150 kg. obese male (45% fat). Unusual features were no change in resting energy expenditure for first 5 days of the fast (in previous caloric reduction studies reduction in resting energy expenditure began promptly) and development of definite prolonged ketosis (other investigators have reported "resistance to ketosis" in obese subjects during periods of fasting). Preliminary blood lipid data suggested ready mobilization of fat in this subject during the initial five days of fasting, then diminished mobilization of lipid stores (Drs. Buskirk, Thompson, Lutwak and Whedon).

II. Effects of Various Pharmacologic Agents on Fat Metabolism and Metabolic Rate

Various agents known to alter either the rate of mobilization or oxidation of free fatty acids (FFA) were given to either normal or hyperthyroid patients in an attempt to alter FFA turnover and determine the relationship between metabolic rate and FFA turnover change. Infusion of glucose was found to depress FFA but elevate oxygen consumption presumably through the specific dynamic effect of glucose. Hexamethonium and arfonad (ganglionic blocking agents) were then tried because these two drugs are known to depress the mobilization of FFA; initial experimentation was unsuccessful because increased heat loss from the body surface promoted shivering and a resultant, elevated metabolic rate. When the subjects were covered with blankets to curtail heat loss, a parallelism between the decrease in FFA turnover and metabolic rate was observed. In more recent studies, FFA turnover was increased with nor-epinephrine infusion and metabolic rate was simultaneously elevated. When the increased mobilization of FFA after nor-epinephrine was blocked with pronethelon, the usually observed increase in metabolic rate after nor-epinephrine was also blocked. Although minor interindividual variation in these several responses has been observed, it appears that resting metabolism can be measurably altered by induced changes in free fatty acid turnover (Drs. Steinberg and Nestel, NHI, and Buskirk and Thompson, NIAND).

III. Physiologic Studies of Temperature Regulation

A. Effect of Triiodothyronine Administration. As a means of obtaining fundamental physiological information on the interplay of central and peripheral temperature regulation mechanisms, experiments before and after triiodothyronine administration were undertaken to study temperature regulation in the cold under conditions of elevated heat production and heat loss as produced by an induced hyperthyroid state.

Three subjects initially were exposed to cold air 10° C. (50°F) for 2 hours to secure control observations. Triiodothyronine (T_3) was then given orally for 2 weeks in gradually increased dosage until a level of 350–400 μgm per day was attained, and cold exposures were repeated. Compared to control cold exposures, the elevation in body core, tympanic membrane, esophageal and rectal temperatures which took place in the control state failed to take place after T_3. Since vasoconstriction was less marked and mean weighted skin temperature remained at a higher value when on T_3, heat loss was greater; these differences in response to cold in body temperatures and heat exchange were consistent in all three subjects. The heat production pattern (relative change in oxygen consumption) in the cold was not remarkably altered by T_3 from control observations. Resting heat production prior to cold exposure was elevated by T_3 in two and unchanged in one of the three subjects. Time to onset of shivering was de- the same following T_3 as it was in tests before the layed in one but unchanged in two subjects. After shivering started, heat production response was the same following T_3 as it was in tests before the hormone was given; heat production during the

final hour in the cold was unchanged in one and elevated in two subjects (approximately 10 and 40 kcal/(m^2.hr)).

If a heat maintenance center is located in the posterior hypothalamus, and it is sensitive to direct temperature stimulation but relays cold receptor initiated impulses from the periphery (scheme of Benzinger), there should be a delay in shivering and a diminution in heat production in the cold during the period of administration of T_3. If the anterior hypothalamus functions as a direct sensor and inhibits the posterior center less and less as it is cooled, then heat production in the cold should be elevated more after T_3 than during control cold exposures. Actually, the experimental data suggest that both schemes of temperature regulation are operative. As measured, the relative increase in heat production in the cold was not dramatically altered after T_3, which may indicate, if the regulation schemes are correct, that in the hyperthyroid state a relatively smaller stimulus is initiated from the body surface and a lesser inhibition occurs of the posterior by the anterior hypothalamus. The net heat production following T_3 would therefore be no different from normal, as observed, because of compensation of one by the other of these mechanisms (Drs. Buskirk and Thompson, NIAMD, and Nestel and Steinberg, NHI).

B. Thermoregulation Against Cold in Normal Subjects and in Patients with Recurrent Fever or Experimental Malaria. Investigations of thermoregulatory responses to cold air are being continued to extend background information for similar studies on patients with various diseases. As described in earlier reports, the insulating effect of subcutaneous body fat and total body fat in moderating the impact of cold on the body has been partially but not completely characterized. It is apparent, however, that body fatness must be taken into account to assess properly the thermoregulatory responses in patients. Eleven patients with recurrent fever and four with experimentally induced malaria have been exposed to cold air (50°F) each on two or more occasions. Although the thermoregulatory responses frequently fell within the normal range established by earlier Metabolic Chamber work, the following findings suggest possible abnormalities in the reaction to cold in patients with recurrent fever:

(1) Heat production was frequently greater than would have been predicted from studies on normal subjects of comparable body fatness.

(2) Deep body temperatures (ear, esophageal or rectal) frequently fell immediately and more rapidly following exposure to cold in contrast to the early elevation (0.1–0.3°C) in core temperature usually seen in normals (indicating a defect in heat conservation physiological mechanisms).

(3) Intraindividual variation in heat production and heat loss appeared to be more marked in patients with recurrent fever than in normals. The final response appeared to be at least partially geared to the febrile-afebrile periodicity.

(4) Cold exposure following experimental malaria induced, in one patient, an elevated heat production, greater peripheral heat loss, and more rapid core cooling than seen in control experiments performed in this man after administration of malarial parasites but before malarial chills and fever became evident.

These studies indicate that long-standing recurrent fever and experimental malaria may, in some patients, disturb temperature regulation against cold to a measurable extent and that further exploration of the nature and extent of these abnormalities would be worthwhile, as potentially applicable to improved therapeutic management of febrile illnesses (Drs. Thompson and Buskirk, NIAMID, and S. M. Wolff, NIAID).

C. Response to Heat and Cold Exposure in Patients With Hansen's Disease (Leprosy). Studies of thermoregulatory responses which may be ultimately significant for various dermatologic diseases involving extensive areas of the skin have been initiated in Hansen's disease in collaboration with NIAID. Three patients with skin and subcutaneous lesions involving approximately one-fourth to one-third of their body surface were exposed to: (a) 15.5°C (60°F) air for 2 hours, and (b) 37.7°C (100°F) (40–50% R.H.) air for 1 hour. The patients rested in the supine position in the cold and sat or exercised on the treadmill (3 mph. zero grade) in the heat.

Heat production, as measured by oxygen consumption, in the cold was elevated in all three subjects over values commonly observed for normal subjects. In two patients body core cooling started

immediately following exposure to cold (as seen in patients with recurrent fever), but a normal elevation within the first 30 minutes occurred in the third. Skin temperatures in all three subjects, and consequently losses of heat from the body surface, were elevated, particularly on the extremities. Whereas normal subjects can easily tolerate 50°F air for two hours, the patients with Hansen's disease had considerable difficulty remaining at 60°F air for the full two hour exposure.

When exposed to 100°F air and asked to perform 15 minutes of exercise one of the patients failed to sweat to a sufficient extent, rectal temperature rose and symptoms of insipient circulatory collapse occurred; when heat exposure was cautiously attempted a second time several days later, similar failure to adjust to heat recurred. The two other patients completed a full hour in the heat, which included two periods of exercise, rather well; temperature regulation was sufficient in these two patients although sweat losses were reduced over affected skin areas.

Radiometric measurements of skin temperature were made at 9 bilateral sites (total 18) immediately after the patients entered the hot Chamber, again 12 to 15 minutes after the first exercise period, and finally 12 to 15 minutes following the last exercise period. At sites where affected/non-affected areas could be compared, skin temperature was higher (up to 1°C) at sites on affected areas; this elevation of skin temperature corresponded to diminution in sweating over the lesion areas.

It is concluded to date that disease involvement (Hansen's disease) of approximately one-fourth of the body surface area reduces vasomotor adaptability to moderate cold, with consequent high rates of heat loss which are not fully compensated for by elevation in heat production so that body core temperatures continue to fall. In one patient regulation against moderate heat was seriously impaired, but heat seemed to be adequately tolerated by the other two patients; more severe heat stress may reveal inadequate regulation in the latter patients as well (Drs. Thompson and Buskirk, NIAMD, and Dr. Adler, NIAID.

IV. Studies of Exercise or Work Physiology

Previously reported studies by this group on exercise physiology in normal subjects have been extended (by collaboration) to patients with impaired cardiopulmonary function. In this study on the action of digitalis, a significant reduction has been shown in postexercise oxygen debt by digoxin in patients with cardiac disease but without clinical congestive heart failure. Three patients with acquired valvular heart disease and cardiac enlargement who were able to perform normal everyday activity without difficulty in the absence of digitalis therapy were exercised while receiving placebo "medication" over an extended period and again while receiving digoxin. Digoxin administration did not produce a significant change in body weight. Varying degrees of exercise (7 to 15 minutes walking) were performed on a treadmill in the Metabolic Chamber, and oxygen consumption was measured continuously before, during, and after the period of exercise. The oxygen debt after exercise which developed during digoxin administration was compared to that observed during placebo administration.

Inpatients oxygen debt was significantly smaller during the period of digoxin administration, although the treadmill work performed was identical. The mean reduction in oxygen debt following 10 minutes walking brought about by digoxin was 35%. Work capacity to tolerance with motivation controlled as closely as possible was also increased significantly in all three patients when they were on digoxin. Similar studies of oxygen debt following exercise were performed with these same patients while being given a diuretic (chlorothiazide) alone and again while on digoxin and the diuretic together. The effect of the diuretic alone was similar to but not as great as that of digoxin, and the effect of the two agents together was not greater than that of digoxin alone.

The accumulation of a smaller oxygen debt when these subjects were receiving digoxin indicates that the functional status of their circulatory system was improved by the drug. The results of these studies are significant for the long discussed question in clinical management of cardiac patients on the value of administration of digitalis in such patients in the absence of frank signs of congestive failure. From these studies digitalis administration would appear to be beneficial to at least some patients who have cardiac disease with enlargement and who also have some decrease in cardiac reserve but without signs symptoms of heart failure (Drs. Kahler, NHI, Thompson and Buskirk, NIAMD, Frye and Braunwald, NHI).

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

INTRAMURAL RESEARCH PROGRAM

The intramural program in 1962 achieved a balance and a maturity that have been sought for the last 7 years. With the appointment of Dr. Maurice Landy as Chief of the Laboratory of Immunology, succeeding the late Jules Freund, all ten laboratories now possess experienced leadership. With few exceptions, all section head positions within the laboratories are occupied. In addition to these more senior places, the Institute intramural staff consists of younger scientists of accomplishment as well as a group of carefully selected junior members. A highly skilled technical staff supports the increasingly complex research procedures.

Coincident with the fulfillment of staffing plans, the direction of research programs is now well established in a course designed to lead to original and influential scientific information. NIAID clinical investigations have reached a stage of high productivity. Projects concern subjects of special interest to the Laboratory of Clinical Investigations, and also cooperative endeavors with scientists in other laboratories whose studies have a clinical component. This combination of research information derived in the laboratory and from patient study has resulted in new knowledge of precision and usefulness.

The Middle America Research Unit of the Laboratory of Tropical Virology located in the Panama Canal Zone is now, in its fifth year, a well-functioning organization of 38 NIH staff members and 8 U.S. Army members. Its laboratory facilities and technical staff are well developed and able to handle a variety of viral and other microbial agents. The building in which MARU is located has undergone recent renovations which provide additional laboratory space, a small clinical area, library area, and a more efficient office working area.

After several years, laboratory renovations in Bethesda providing new research facilities are, in effect, complete. Each laboratory has well-equipped space including special transfer or instrument rooms and animal rooms designed for the special needs of each project. The last group to accomplish this will be the Laboratory of Immunology, which is now completing the renovation necessary to support its activities. Necessary instrumentation is also readily available.

Deserving of special mention is the new experimental animal building and insectary now being constructed at the Rocky Mountain Laboratory, Hamilton, Montana. This structure will provide rooms designed to house highly infectious animals safely. It also will provide especially designed rooms for rearing arthropods and for studying transmission of microbial agents by arthropods. These modern facilities, of the most advanced design, will make it feasible for the Rocky Mountain Laboratory investigators to conduct many studies which have been difficult or impossible under previous conditions. Much needed expansion of the RML library is also under way.

The Board of Scientific Counselors met in July at the Rocky Mountain Laboratory. It had met there 3 years earlier and several Counselors attended both sessions. The Counselors found the research program of the Rocky Mountain Laboratory better focused, more mature and with a more clearly defined mission than formerly. The Counselors' advice was helpful, as usual, in clarifying overall objectives.

The Institute has continued its interest in maintaining organized research projects, as well as individual scientists, in overseas locations where investigations can profitably be done. These have comprised the Middle America Research Unit in the Panama Canal Zone and the field project on simian malaria in Kuala Lumpur, Malaya. On July 1, 1962, a new Section entitled the Pacific

Research Section of the Laboratory of Infectious Diseases was established in Honolulu, Hawaii. Four NIAID staff members are investigating eosinophilic meningitis in the Pacific Islands and will initiate studies on certain virus diseases which appear to be locally important. The Section occupies space at the Queen's Hospital, which furnishes, under contract, technical and maintenance personnel and research services. At the end of 3 years, an evaluation of the need for further continuation of this Section will be made. These activities, as well as those at Rocky Mountain Laboratory, and at Atlanta and Columbia, South Carolina, provide the Institute with a number of satellite laboratories which broaden its opportunities for research and ensure close touch with problems of importance.

Intramural scientists, as in previous years, have worked in a variety of other laboratories around the world. Dr. Jacob Brody of the Laboratory of Tropical Virology worked for six months at the Institute of Poliomyelitis, Academy of Medical Sciences, U.S.S.R., Moscow under the Director, Professor M. P. Chumakov. This was arranged under the international agreement for exchange scientists between the U.S.S.R. and this country. Dr. Karl Habel, Chief, Laboratory of Biology of Viruses accepted an appointment as Visiting Professor at the University of Glasgow for three months in the spring. He later worked at the Karolinska Institutet, Stockholm, Sweden with Dr. George Klein on basic virologic problems. Dr. Paul Weinstein is conducting collaborative research in filariasis at the National Institute for Medical Research in Mill Hill, London, England. Dr. Sanford Stone after spending some months with Dr. Halpern, Hôpital Broussais, Paris, is now working also at the National Institute for Medical Research, Mill Hill, London. Dr. Louis Olivier is detailed for 2 years to the World Health Organization Headquarters in Geneva to serve as leader of the WHO bilharziasis advisory team. Dr. John Utz is conducting research on fungus diseases in the laboratories of Drs. Segretain and Martin at the Pasteur Institute, Paris. Dr. Bill Hoyer of the Rocky Mountain Laboratory is assigned to the Carnegie Institution of Washington for special studies on the chemical components of viruses and virus surfaces.

Overseas research programs have been supported in part through the use of counterpart funds available under Public Law 480. Dr. Kenneth Warren, Laboratory of Parasitic Diseases, is currently investigating clinical-laboratory problems on schistosomiasis at the Universidade de Bahia, Brazil, where he holds a position as Visiting Professor of Medicine. Other studies under the Laboratory of Parasitic Diseases which were begun this last year include one on toxoplasmosis with the Ministry of Health, Israel and one on therapy of schistosomiasis with the High Institute of Public Health, Alexandria, Egypt. These arrangements permit NIAID investigators to conduct studies related to their specific programs in favorable locations. In addition, they provide opportunities for contact with investigators in other countries and an avenue for continuing exchange of scientific information.

Once again a significant proportion of the Institute resources has been devoted to offering opportunities for research experience to recent doctoral graduates. Clinical associates appointed in the Laboratory of Clinical Investigations have participated in laboratory programs not only in LCI but to some extent in collaboration with preceptors in other laboratories. This collaboration was particularly evident with the Laboratory of Immunology which has many similar interests and occupies space adjacent to the patient care area. As in the past, four research associates were assigned to other laboratories for full-time laboratory or fieldwork. Under the new provision for appointment of the NIH Staff Fellows, one was appointed in the Institute for a limited period of time.

The Institute has appointed its largest number of Visiting Scientists this year, a total of seven. These scientists represent a wide variety of scientific disciplines and geographic origin.

The earlier hope that an Office of Medical Biometry would prove useful to the staff has been realized. The Institute's biometrician has offered valuable consultation and advice to laboratory and field investigators, and has originated a number of statistical projects.

Virologists in the Laboratory of Infectious Diseases have worked closely with the Institute's Collaborative Program in support of the Vaccine Development and Viral Reference Reagents activi-

ties. The Vaccine Development Program is an actual outgrowth and logical goal of much of the work which goes on in virologic and field investigations. The Reference Reagents Program with the aid of Institute virologists and others eventually will provide investigators with reference and working antisera and antigens to accelerate and simplify the normal conduct of virus disease research.

Seventy-five years ago research in infectious disease within the Public Health Service began under the direction of Dr. J. J. Kinyoun at the Marine Hospital, Staten Island, New York. This event was celebrated at the NIH on November 29, 1962, with a day and evening meeting attended by staff, friends, and former Institute researchers. The current activities of this Institute are in many ways a direct descendant of that early work. The following review reflects the diverse interests of NIAID investigators and indicates the maturation of a broad research endeavor aimed at carefully selected key questions confronting microbiology and immunology today.

ROCKY MOUNTAIN LABORATORY

An assessment of current and future projects was made last spring, first by the scientific staff of RML, and then by the Director's Office at RML and NIAID. Later the Scientific Counselors evaluated the research activities in light of facilities, opportunities and needs. The deliberations resulted in a clarification of the RML mission as two interdependent areas of research: (1) the biology of animal and arthropod-borne microbiota and (2) immunologic, chemical, and physical phenomena associated with antigens and microbial constituents.

Rocky Mountain Spotted Fever

Case surveillance for Rocky Mountain spotted fever has now been intensively resumed through various public health agencies. Some explanation will be sought for the apparent 65% drop in reported cases during the decade beginning in 1949, but it appears unlikely that natural causes are the major explanation. It would be useful to obtain confirmatory evidence that wide use of broad-spectrum antibiotics played a part in the sudden reduction in both morbidity and mortality.

A study of virulence of strains of the etiologic agent, *Rickettsia rickettsi*, revealed that 42 of 43 isolates from ticks in eastern Montana were M strains. These, like U strains, do not produce signs of infection in guinea pigs or rhesus monkeys, but unlike U strains, they lack capacity to induce the formation of antitoxin against virulent strains. Both forms, however, induce high CF antibody titers in guinea pigs. The M strains are capable of intranuclear parasitism, but can be differentiated biologically from other rickettsias of the spotted fever group. Thus, the M isolates are considered to constitute a fifth group of *R. rickettsi*, additional to the R, S, T, and U strains previously characterized. The implications relative to mild human infection in the area remain to be assessed.

In addition to the finding, in western Montana, of complement-fixing antibodies in 46 to 71% of ground squirrels and chipmunks, and in 43% of snowshoe hares, 6 virulent strains were isolated from spleens of young chipmunks and a golden-mantled ground squirrel and, quite unexpectedly, from blood of a young snowshoe hare. These are the first isolations of *R. rickettsi* west of the Mississippi from naturally infected animals other than man.

Tick Paralysis

Certain female ticks of the species, *Dermacentor andersoni* in the Rocky Mountain region are capable of producing paralysis in hamsters when fed on them. Attempts to inbreed the factors which governs this is difficult because males must be selected at random. But, genetic determinants are possibly involved because this capacity was acquired by some females in the first to third filial generations from inbred males of a paralysis producing stock with females unable to produce paralysis.

Panama Mite Studies

This project was conducted by two RML staff members working in Panama at the Middle America Research Unit of the Laboratory of Tropical Virology. During the year, 406 pools of mites and a few ticks from 639 wild vertebrates, mostly rodents, were airmailed to RML for testing *Coxiella burneti* was recovered from one chigger pool from the common spiny rat and inapparent Q fever infections occurred in guinea pigs inoculated

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with two other mite pools, one from bats, the other from rodents. Spontaneous infection in test animals was ruled out.

Although Rocky Mountain spotted fever has occurred in the Republic of Panama, the first strain of *Rickettsia rickettsi* ever isolated from within the Canala Zone was recovered from three pools of immature *Amblyomma* and *Haemaphysalis*. In addition, two viruses were isolated but they had such low virulence that characterization has not yet been accomplished.

A significant augmentation of knowledge of the acarine fauna of Central America resulted from this project.

Colorado Tick Fever

Substantial progress has been made in an understanding of the ecology of this rather widespread disaese in the Rocky Mountain area. It was a surprise to discover serological evidence of past infection in 5 of 49 snowshoe hares in the environs of Ottawa, Canada, which is well beyond the known endemic area. However, virus could not be isolated from 38 pools of 2 species of indigenous ticks and further studies are planned to recover the agent responsible for antibodies in this area. Hares experimentally infected by bites of nymphal *Dermacentor andersoni* ticks developed significant viremias for 4 days, but only transiently when inoculated with large doses of virus.

Two doses of a refined formalinized vaccine, prepared from brains of infected suckling mice, produced antibodies in 90% of human volunteers without causing ill effects or sensitization to brain-tissue antigen. Such a vaccine could be useful in areas of high exposure in the field or laboratory.

Phlyctenulosis

The study of widespread disability among Alaskan Eskimos due to phlyctenulosis with resultant corneal scarring has been completed. The findings are consistent with the hypothesis that the disease is primarily a manifestation of hypersensitivity to infection with *Mycobacterium tuberculosis*. The scarring is reversible to a greater degree than previously suspected, and protection is afforded during isoniazid medication, but disappointingly, not after discontinuance of the drug.

The Encephalitides

Results of field investigations on Powassan virus and California encephalitis virus indicate that a variety of small mammals may serve as natural hosts. Serologic evidence of infection with California virus was found in snowshoe hares, golden-mantled ground squirrels, raccoons, porcupines and marmots. *Microtus* and *Peromyscus* appeared to be the more important hosts of Powassan virus. Three more isolates of California virus were made from *Dermacentor andersoni* but its role as a vector has not been determined. As yet, the natural cycle of these agents remains obscure.

From our own and others' observations on the ecology of western equine encephalitis virus (WEE) it appears that the *Culex tarsalis*-bird cycle may be relatively unimportant in the maintenance of this virus. For the third consecutive year, virus could not be recovered from *C. tarsalis* taken in the endemic area in North Dakota, and this year *C. tarsalis* from the Vale, Oregon, study area were free of virus whereas this species had high infection rates the two previous years. Furthermore, the biology of *C. tarsalis* in the spring and fall is not conducive to the spread or maintenance of virus.

Q Fever

The successful separation of cesium chloride gradients of Phases I and II in mixtures of *Coxiella burneti* has provided, for the first time, a tool for the closer control of refined preparations of vaccines and diagnostic reagents. This finding also provides a possible clue to past discrepancies in results formerly thought to have been due to strain differences. The radioisotope precipitation test developed at RML also has provided a rapid, highly sensitive serologic test for measurement of antibodies in animal and human serums. This test should have broad application in research and in epidemiologic investigations.

Q fever vaccines made from Phase I rickettsias have been shown to be 100 to 300 times more potent than Phase II in protecting guinea pigs against Q fever. As demonstrated by studies in human volunteers, a dose of 10 complement-fixing units of a refined Phase I vaccine can be given safely

to persons who are not sensitive to *C. burneti.* Efforts to extract a protective antigen that can be used safely in sensitized persons have been unsuccessful, although information about the chemical composition of the organism was obtained.

Selected Zoonoses of Regional Importance

The persistence of *Pasteurella tularensis* in natural waters for long periods is still a subject of interest. One naturally contaminated stream in the valley has been positive during all months of the year except August and September. Experimentally, it was determined that the organism survives longer at temperatures above freezing and remains viable for longer periods in creek water than in any of seven other media. At present, scientists in this country and those abroad have agreed that two types of *P. tularensis* do exist. All efforts to enhance the virulence of the mild type have failed.

To date, 25 isolates of rabies virus have been recovered from bats since studies were initiated in 1954. In studies on the pathogenesis of rabies in mice, virus was found to persist in the brain for 15 days and antibodies coexisted with virus during the late stages of the disease. Most significant was the finding that intraperitoneally injected street virus will protect against the more invasive fixed virus, or against virus injected at the same time or later by a more lethal route.

Viruses and Chronic Disease

In this new project in comparative pathology, the phenomenon of chronic viral infection, as exemplified by three natural diseases of animals, is being investigated to provide some insight into pathogenic mechanisms which may be involved in vertain chronic diseases of man. To date, the study has been limited to exploratory work on scrapie in mice, although studies on Aleutian disease of mink and chronic pneumonitis of sheep are planned. Mice became infected with scrapie virus after inoculation by intraperitoneal, intracerebral, subcutaneous, or intranasal routes. Mice, 21 days old, inoculated intracerebrally with a 10^{-1} dilution of scrapie-infected mouse brain suspension, began showing clinical signs of scrapie 111 to 135 days later. From time of onset, the course of disease till death varied from 40 to 50 days. Virus was found in the brain in at least the 10^{-6} dilution, in spleen in a 10^{-6} dilution, in thymus in a 10^{-5} dilution, and in liver in a 10^{-1} dilution. In general, the neuropathologic changes in mice resemble changes characteristic of scrapie in sheep and goats. Neither inclusion bodies nor micro-organisms were seen in in strained impressions of infected brain. Also, bacteria, including PPLO, were not cultured from infected brain.

Allergy, Its Mechanisms and Role in Disease

Studies in allergy were directed toward further explanation of the relationship between delayed hypersensitivity and the immune process, particularly the specificity and physiology of mechanisms involved in certain immunologic phenomena. While thyroxin is reported to enhance antibody production, this hormone does not affect the formation of delayed hypersensitivity and, contrary to previous reports, it did not have a stimulatory effect on antibody production in guinea pigs inoculated with diphtheria toxoid. However, both antibody production and delayed hypersensitivity were depressed in pyridoxine-deficient guinea pigs.

In further studies on the specificity of tolerance, delayed hypersensitivity, and antibody production, tolerance was first induced in guinea pigs by "gastric feeding" of contact haptens, such as dinitrofluorobenzene (DFB), dissolved in corn oil. These tolerant animals did not develop antibody or delayed hypersensitivity to the hapten when sensitized with the hapten alone. However, when subsequently inoculated with a conjugate of DFB such as dinitrophenyl-hen egg albumin (DNP-HEA), guinea pigs did develop delayed hypersensitivity to HEA and circulating antibody to the hapten. However, they remained unresponsive to contact testing with the hapten DFB. When animals sensitized with DNP-HEA are subsequently "gastric-fed" DFB, they do become tolerant to the hapten by contact testing, even though antibody to DNP is present. These findings indicate that tolerance and delayed hypersensitivity have similar specificities directed toward the basic protein whereas the specificity of circulating antibody is directed toward terminal groupings of the antigen (haptens).

Fine Structure of Microorganisms

As in the past, studies in this area are made in collaboration with numerous guest workers who are interested in using RML-developed biophysical methods of studying the fine structure of mi-

croorganisms varying in size from Protozoa to trachoma virus. The major emphasis this year was on a study of endotoxin derived from cell walls of *Bordetella pertussis* and the development and application of a practical KBr linear gradient. By phenol treatment of purified cell walls of *B. pertussis*, an extract was obtained which, in regard to chemical composition and host reactive properties, resembled a typical endotoxin.

A simple KBr gradient was developed whereby microorganisms and fractions thereof could readily be purified from crude material such as yolk-sac suspensions. Organisms contaminated with extraneous material are merely dispersed in a KBr solution of appropriate density. As the dispersed materials seek their own relative densities during centrifugation, sharp bands form in regions where the density of each material is equal to that of the KBr gradient. This method has been used successfully for separating cell walls from intact cells, and for isolating trachoma virus and rickettsias from yolk-sac suspensions. About 25 times as much material can be processed in a single 50-ml tube as is possible in other linear gradients.

Structure and Biologic Activity of Endotoxins

A more definite characterization of endotoxin is necessary before protective antigen can be separated from the endotoxin complex. Our previous findings indicated that biologic activity was dependent upon a macromolecular complex of critical size. In further studies on the inactivation of endotoxin by human plasma enzymes, the results substantiate the previous findings. Thus endotoxin can exist in aggregates ranging from macroscopic particles to those of a minimum size of 10 Svedberg units. Upon degradation of endotoxin with acid or enzymes, only particles the size of polysaccharide hapten (1 to 2 Svedberg units) are produced.

On the basis of our own studies on cellulose, chitin, and peptides and on others reported in the literature, it appeared that endotoxin may consist of a micellar system. This arrangement would explain the existence of particles having sedimentation constants ranging from 190 S or higher (filaments) to 10 S (micelles, i.e., bundles of chain molecules) but no units having S values between 10 S and 1.4 S (free chain molecules). Accordingly, the 10 S units, the smallest active particles, then would exist as fringed micelles which readily aggregate to form filaments. These filaments, independent of the chemical nature of the chain molecules, consistently are 50–100 A. thick. Indeed, electron microscopic studies of endotoxin revealed the existence of fibrils 50–100 A. thick and varying lengths. In addition to the fibrils, rodlets 100–200 A. in length were observed which correspond to the 10 S units and which were morphologically identical with the micelles we described for regenerated cellulose. If the 10 S particles actually were units of the filamentous endotoxin and had a fine structure similar to that of the cellulose micelles, the effect of acid or enzymes on endotoxin would be analogous to that produced on cellulose micelles after nitration wherein the micelles are split into their polysaccharide elements, i.e., the chain molecules of which they are composed.

Biologic Properties of *Bordetella Pertussis* Antigens

Progress has been made in the purification of the protective antigen of *Bordetella pertussis*. Heretofore, small quantities of this antigen could be isolated by certain electrophoresis and column chromatography but much larger quantities can now be isolated by starch block electrophoresis. As shown by agar gel diffusion tests, the purified product contained one main antigen whereas the starting material contained 12 antigens. All efforts to separate this protective antigen from the histamine sensitizing factor have failed. Both are found in identical electrophoretic fractions and they are precipitated by similar concentrations of acetone and at the same pH; both activities are located in the cell wall and the protective activity of vaccines correlates with their ability to sensitize mice to histamine. For these reasons, both activities are believed to be associated with the same substance in the cell walls.

Biologic Behavior of Microbial Proteins and Nucleic Acids

Results of further studies on radioisotope precipitation (RIP) tests indicate that this is an extremely useful tool for investigations concerning surface characteristics of viruses and their protein constituents. Because of the extreme sensivity and specificity of the test, minor alterations in poliovirus protein induced by heat, ultraviolet light, or lyophilization could be detected. With

this test, it was also determined that antibody directed toward whole tobacco mosaic virus would not readily combine with virus protein obtained by acetic acid dissociation.

The sensitivity of the RIP test was compared with that of the tissue culture neutralization (TCN) test and the plaque-forming-unit-precipitated (PFUP) test. In these comparisons, known numbers of antibody molecules and virus particles were used and the ratio of the first to the second at antibody endpoint could be determined. In the TCN test this ratio was 100,000 to 1, in the RIP test it was 5 to 1, and in the PFUP test it was 700 to 1.

Although type II poliovirus was originally used for development and standardization of the test, equally reliable results have been obtained with types I and III. The test is now being used in studies concerning the serology of measles, psittacosis, and trachoma.

LABORATORY OF GERM-FREE ANIMAL RESEARCH

The studies in this laboratory have the general objectives of establishing the role of the flora (or of a particular organism) in some broader disease problem or biological process, and evaluating the latter in the absence of microbial influence. Several aspects of these studies are at or nearing completion.

Penicillin Not Toxic for Germ-Free Guinea Pigs

One interesting finding during the year emphasizes the unique value of the germ-free animal as an experimental tool. Among the poorly understood reactions to penicillin is the extreme sensitivity of guinea pigs to this antibiotic. They may succumb following the injection of dosages which are small fractions or those required to kill other animal species and are at levels not generally considered to be toxic. One explanation for this anomalous circumstance has been that the antibiotic may upset some microbial balance in the animal (since such small dosages can hardly eliminate all the organisms) and a particularly virulent species may overgrow others and kill the animal. However, there has been little evidence to support this or other possibilities. We have found, in collaboration with LCI, that germ-free guinea pigs show no apparent ill effects following injections of 32,000–64,000 units of penicillin per 300-gram animal. Not a single death has occurred among several litters inoculated. However, such dosages generally result in the death of some or often the majority of conventional guinea pigs a few days after injection. These findings point strongly to the likelihood that the untoward effect of the antibiotic is exerted indirectly through some effect on the flora rather than an expression of a direct toxic action.

Death of Germ-Free Guinea Pigs

In other studies with germ-free guinea pigs it has been observed that these animals will invariably die when removed from the isolator and placed in an unsterile environment such as an animal room. This is not true of other germ-free animals. Death occurs within 2 days and, no particular contaminating organism has been incriminated consistently, as yet. In efforts to ascertain factors involved in this unusually high susceptibility, various protective measures were attempted including previous mono-infections, antibiotics, sulfa drugs, etc. Most have been unsuccessful in reducing mortality after removal from the isolator. However, some prolongation of survival time and occasional survival have recently been obtained with prior treatment with staphylococcal endotoxin. Further results in this direction can provide some insight into the role this substance plays in stimulating "nonspecific" resistance.

Other studies on the immune response in guinea pigs without a flora have indicated that they do not produce antibody to an antigen such as BGG as early, or as in high titers, as do their conventional counterparts.

Pulmonary Tumors

The first part of the study of the comparative incidence of methylcholanthrene-induced pulmonary tumors in germ-free and conventional mice, (i.e., those living in a contaminated environment) were concluded. The results indicated the same incidence in germ-free mice, their genetic controls maintained under usual animal room conditions, and NIH General Purpose mice. The mean nodule count was at least as high in the germ-free animals and in some instances higher than in the General Purpose mice. In addition to these tumors, subcutaneous sarcomas and lymphocytic leukemia were also produced in the germ-free mice

at about the same rate as in the General Purpose. Our information on the viral status of the germ-free mice is incomplete, in view of the large number of viruses reported to occur in mice. Serologic and other tests conducted in collaboration with LID for polyoma, K virus, mouse adenovirus, Theiler's GD VII, PVM, Reovirus and MHV have been negative with one possible exception, Reovirus type 3. It has not been possible to recover this virus from the germ-free colony. Mice from the General Purpose colony have shown a high incidence of antibodies to at least three of these viruses. Of course, little can be said of the role, if any, that viruses may have played in these experiments. However, it is of interest that pulmonary tumors were induced in these germ-free mice at least as well as in mice exposed to a varied microbial flora including several viruses. A difference, bordering on significance, was noted in the incidence of one of the other tumors among the groups. However, the overall incidence was so low that further studies are indicated.

Death in Conventional and Germ-Free Mice

A continuing study of the longevity and postmortem findings in germ-free and conventional mice of the same genetic stock maintained on the same diet has provided data on over 100 animals. It appears, thus far, that the causes of death, where established, are essentially similar in both groups, with tumors apparently accounting for the majority of deaths. Most of these were pulmonary neoplasms ranging from benign adenomas to widely disseminated carcinomas. A nephropathy observed in some of the conventional animals, and considered by some to be associated with response to infection, was also noted in similar incidence in germ-free animals. It should be pointed out that the conventional controls for our germ-free mice are probably similar to the so-called specific-pathogen-free animal. They are not exposed to as many pathogenic bacterial or parasitic infections as other colonies might encounter. However, they do harbor most of the usual bacterial contaminants found in mice.

Tissue Reactions to Mouse Nematode

Histopathologic comparisons of infections with the mouse nematode, *Nematospiroides dubius*, in germ-free and conventional mice appear to have resolved a question that has been raised by several investigators. Parasite larvae penetrating host tissues from a contaminated site, either from the intestinal lumen (as in the case of *N. dubius*) or from the external environment, can carry bacteria and other organisms along with them. It has been the feeling of some that the host's tissue reaction around the invading larvae is, in these instances, primarily a response to the bacterial contaminants. Some have even fed the host antibiotics to demonstrate this point. A study of the lesions and tissue reactions around *N. dubius* in the intestinal wall, from the initial penetration of the larvae throughout their development, has shown no significant difference between the germ-free host-fed sterile larvae and a bacterially-contaminated host-fed contaminated larvae.

Thyroiditis

Autoimmune thyroiditis has been found to persist at least 18 months, and in some cases as long as 2 years after a single immunizing dose of thyroid adjuvant. This is in contradistinction to reports of others, who have found that the disease disappeared a few months after its production in guinea pigs, and may be a function of the immunizing procedure.

At 6 months and later periods, some decrease in the delayed hypersensitivity to thyroid antigen, as determined by skin test, has been found, with a modified type of reaction best described at present as an "intermediate" type. This appears later than the immediate Arthus reaction and disappears earlier than the classical delayed tuberculin reaction. This new type of skin reaction is being investigated further.

LABORATORY OF BIOLOGY OF VIRUSES

The scientific work of the laboratory during the past year has been most productive and can readily be divided into five areas of basic research related to viruses: immunology, genetics, cell metabolism, biochemistry of viral replication, and biophysical studies.

Immunologic Studies

Investigations aimed at evaluation of factors responsible for recovery of animals and man from primary, acute virus infections have raised questions concerning the role and importance of specific antibodies and hypersensitivity in this situation. Other non-specific factors including

elevated body temperature and interferon have been shown to be more effective than antibodies in recovery of guinea pigs from local vaccinia virus infection and the fatal encephalitis produced in mice by encephalomyocarditis virus.

Studies of the synthesis of viral proteins in the poliovirus-HeLa cell system have led to the discovery of a new antigen in polio-infected cells which is characteristic of dissociated viral protein subunits, but is not a part of the mature virus particle. This new antigen appears early in the virus multiplication cycle. This same project is accumulating evidence on the physicochemical characterization of the subunit of the virus coat which determines the immunological specificity of the virus and therefore is directly involved in the antigenicity of poliovirus vaccines.

That cells transformed to tumor cells by polyoma virus contain a new foreign cellular antigen and immunological reaction to that antigen can limit the oncogenic potential of this virus was first demonstrated in this laboratory. It has now been shown that this phenomenon is also true in the SV40 and Moloney leukemia tumor virus systems. Furthermore, other laboratories have found it to be true with Rous sarcoma, Gross leukemia and rabbit papilloma so there is good reason to expect that it may be characteristic of all virus-induced tumors. One important recent contribution is the demonstration that these new cell antigens appear specific for the tumors produced by each virus. This means that a method is now available to prove etiological relationship between a given virus and a given tumor, provided the tumor is transplantable in adult animals.

Genetics

A continuation of studies on sulfated polysaccharide virus inhibitors has shown that growth of certain virus mutants is not only uninhibited by these materials, but is actually enhanced. Thus the current live attenuated poliovirus vaccine strains were found to contain three plaque types of particles and the use of dextran sulfate makes their demonstration easy. The possibility that these mutants may vary in their neurovirulence suggests interesting practical implications in the vaccine field.

The use of viral susceptibility as a genetic marker for cells in tissue culture has led to some pioneering results in the newly developing field of human cellular genetics. Mutant cells from cultured human amnion have been found completely resistant to infection with polio-virus, although derived from completely susceptible parent cells. The basis of this resistance is lack of adsorption of virus to the cell, and the mutation rate for this character has been determined.

Cell Metabolism

Work has continued on the definition of the enzymatic basis for resistance of mutant HeLa cells to the toxic action of 2-desoxyglucose. It has been shown that certain steroids will increase the tolerance of susceptible cells to this toxic glucose analogue.

A possible new type of RNA has been found in mammalian cells in tissue culture. This is an acid-soluble, nondialyzable RNA which is labeled much more rapidly by radioactive precursors than are the other known types of RNA. Further investigations are in progress to determine the function of this interesting cell material. In parallel studies there has been evidence that the effect of interferon may be that of inhibiting the synthesis of viral RNA.

Biochemistry of Viral Replication

The work in this area is in the forefront of the field. Although considered here in respect to poliovirus and vaccinia virus replication the real significance of the results is much broader than just its relation to a particular virus. New information obtained this year on the replication of the RNA polio-virus in human cells has shown that normal cellular DNA-dependent RNA synthesis in the nucleus stops on infection but cytoplasmic RNA synthesis continues.

A uracil analogue which inhibits virus formation when applied early after infection has been found not to inhibit synthesis of viral RNA as such, but appears to affect production of an earlier type of RNA which is only associated with virus infection.

Work with the DNA vaccinia virus indicated that cellular and viral DNA are probably synthesized by different enzymatic systems. Viral DNA replication is not necessary for formation of viral proteins and the rate of viral protein synthesis is independent of the multiplicity of infection.

Biophysical Studies

Work in this area during the past year has been aimed chiefly at the development of new physical methods to study biochemical and biological events at the molecular level with greatly increased resolution.

Refinements in the electron microscopic technics used for examination of ultrathin sections of cells have been developed and are now being used for seeking the physical nature of virus precursor materials.

Preliminary results have been very encouraging on the development of a technic whereby uranium can be combined with nucleic acid, caused to fission by exposure in a nuclear reactor and fission tracks demonstrated at the molecular level by electron microscopic technics.

LABORATORY OF TROPICAL VIROLOGY

This laboratory comprises a satellite laboratory, the Middle America Research Unit (MARU), located in the Panama Canal Zone and a base facility in Bethesda. Investigations are integrated into an attack on problems of virus diseases within North, Middle and South America. This includes ecologic, clinical, diagnostic, and serologic aspects of selected disease problems. The Walter Reed Army Institute of Research jointly supports MARU and has established investigations in mycotic and bacterial diseases.

Vesicular Stomatitis Virus

Repeated isolation of vesicular stomatitis virus, Indiana type, from Panamanian phlebotomus sandflies and the existence of nearby endemic areas of human infection, supported by findings of other laboratories, establish it as a new arbovirus of medical and not only veterinary importance. Human disease in one of its forms was illustrated by a MARU staff member hospitalized with a febrile illness (laboratory infection).

Sandfly Fever in the New World

Although the phlebotomus sandfly virus isolates are not related to either Sicilian or Naples types of sandfly fever, a virus (JW–10), isolated from the blood of a Nicaraguan patient with febrile illness in Panama, is a member of the New World Naples-like complex of viruses. Besides the Naples virus prototype (Sabin) and our agent, the complex includes a virus active in the Belem, Brazil, area. With the demonstration of common antibodies against JW–10, these findings assume potential public health importance. A new phlebotomus virus repeatedly recovered during the 3 years of the Bocas del Toro project (four strains from MARU and four from GML), was found to be related to a Brazilian (Belem) animal virus isolate, but not to Naples or Sicilian sandfly fever viruses. Its public health significance is as yet unknown.

Hemorrhagic Fever in Bolivia

Field studies of an acute febrile illness with hemorrhagic manifestations indicate that over 500 endemic cases with high mortality have occurred in an isolated area of Northeast Bolivia during the past 3 years. The clinical picture resembles Argentinian hemorrhagic fever. Extensive laboratory studies to clarify the suspected viral nature of the etiologic agent are in progress.

Encephalitis Viruses in Panama

Pioneer studies on the occurrence of EEE and VEE in Panama are beginning to clarify the local epidemiology and epizootiology of these virus infections. While studying an outbreak of EEE among horses, the virus of VEE was isolated from the blood of 5 to 10 febrile people residing in the epizootic area. The December 1962 outbreaks of encephalitis, both human and equine, in nearby Caribbean countries (Colombia, Venezuela, Cuba and Jamaica) add particular urgency and importance to the field and laboratory studies.

Bunyamwera Group of Viruses

Specific neutralization of Cache Valley virus by normal calf serum was demonstrated in cell cultures maintained with media containing serum from Illinois, Indiana, Pennsylvania, New York, New Jersey and Maryland calves. Significance of the finding was corroborated by recent isolations of Cache Valley virus from U.S. and Panamanian mosquitoes. Apparently this member of the Bunyamwera group has been active over a large portion of the Western hemisphere. The eight prototype members of this group produced CPE in tubes and plaques under agar; hopefully this will lead to plaque reduction test as a practical serologic tool to investigate the role of these viruses in human and animal disease.

Pathogenic Agents From Panamanian Acarina

Although no viruses were isolated from mites in a 2-year collaborative project with RML, two virus strains of low titer (still unidentified) were isolated from ticks; rickettsiae were isolated from both ticks and intradermal chiggers taken off Panamian animals. Of major significance was the finding of 80 species of chiggers, of which only 17 were known to exist in Panama and many were new to science.

VEE Vaccination

Attenuated VEE virus produced by the Biological Laboratories at Fort Detrick for experimental human immunization was evaluated at MARU under field conditions. The frozen virus (rather than the lyophilized material of lower potency) produced reasonable serologic responses, presumably indicative of some immunologic protection, and only mild side reactions occurred. Its use for the protection of potentially exposed personnel will be continued.

Serologic Reagents From Ascitic Fluid

Hyperimmune ascitic fluid production in mice by virus-adjuvant inoculation has yielded an excellent serologic reagent at MARU and ABVS with a variety of arboviruses; the procedure is being adopted as a basis for commercial contract production of the standard antibody reagent for national distribution (under the auspices of the NIAID Panel for Arboviruses). Polyvalent grouping reagents constitute a major need of arbovirologists throughout the world. Polyvalent hyperimmune ascitic fluids produced by repeated inoculation of mice with antigens and also combined antiserum pools, made by mixing two or more specific antisera, have been successfully made and used with several arbovirus groups. This may serve as a model for standard reagent production.

Leptospirosis in Panama

For the first time in Panama, leptospirae have been isolated from humans, small mammals and from the contaminated river water which served to infect exposed troops. New serological groups and types are being designated for these organisms.

Bats and Histoplasmosis

Recovery of histoplasma capsulatum from livers and spleens of bats belonging to five genera and collected from widely separated harborages, reemphasized the importance of the current study on the role of the bat in the ecology of histoplasmosis. Histoplasmin skin test continues to be a major epidemiologic tool: conversion to positive of 3/77 men in Panama for only 67 days (as part of Operation Swampfox II) was thus demonstrated; the first survey of the relatively isolated San Blas Archipelago Indians disclosed overall positivity of 50% among 1,038 persons tested (most reactors between 11 and 20 years of age).

LABORATORY OF IMMUNOLOGY

It is evident in the individual reports that the original programs developed in the Laboratory of Immunology, as they have widened in scope, have generated intensive collaboration among the sections of this Laboratory, with other laboratories of this Institute, and with units of other Institutes of the NIH. When collaboration with investigators outside the NIH offers special advantages for prosecution of the work, these, too, have been fostered and extended.

Cross Reactions in Human Malaria Disclosed by Fluorescent Antibody

With the discovery that by immunofluorescent methods antibody can be detected and titrated in the sera of malaria patients, it has been possible to study cross reactions in this disease. Sera from volunteers infected with the B strain of *Plasmodium cynomolgi* were allowed to react with the homologous parasite, and the heterologous parasites of two strains of *Plasmodium vivax*. Although there was considerable cross reaction between the two species the maximum antibody titers with the homologous parasite tended to be higher than with the heterologous parasites of either strain of *P. vivax*. This suggested the possibility that perhaps not only certain antigens are shared by the two species but that *P. cynomolgi* parasites may possess a different antigen or antigens. The cross reactions between various species of the malaria plasmodia may contribute to a better understanding of the mechanisms involved in immunity to malaria.

Allergy Reagins Identified as Beta $_2$A-Globulins

The reagins or skin sensitizing antibodies found in individuals with atopic allergies are believed to be responsible for the specific allergic reactions such as hay fever, asthma or various skin reactions. The specific removal of the beta $_2$A-globulin fraction of sera from ragweed sensitive individuals by absorption with a unispecific anti-beta $_2$A-globulin serum resulted in the loss of all detectable skin sensitizing activity. The beta $_2$A-globulins have been thought to possess antibody activity but there has been little direct evidence to support this hypothesis. The data indicate that the skin sensitizing antibodies in these sera were beta $_2$A-globulins. This approach is promising for the investigation of other antibody activities which may be associated with the beta $_2$A-globulins.

Physiological Basis for Susceptibility to Anaphylactic Shock

The pronounced difference in susceptibility of Strain 2 and Hartley guinea pigs to acute anaphylactic shock was not related to their reactivity to histamine, but did correspond to differences in the histamine content of their lungs. It was found that both strains had the same content of fixed (lung) histamine; the genetic difference was expressed in the amount of histamine which was liberated in the course of anaphylactic reaction. In the susceptible Hartley strain, this was approximately seven times as much as in the refractory Strain 2 guinea pigs. It may be especially significant that the ratio of liberated histamine corresponded to differences in the number of mast cells present in the lung tissue of these two strains.

Relationship of Delayed-Type Hypersensitivity to Allergic Thyroiditis

The various facets of the immune response to experimental allergic thyroiditis in the guinea pig have been studied to ascertain which, if any, are directly related to this disease. No correlation was evident between levels of circulating antibody or immediate skin reactivity and the progression of the disease. Delayed skin reactivity, on the other hand, was found consistently to closely parallel the disease during its developmental and declining phases; it may, therefore, be a causal factor in allergic thyroiditis. This approach to the study of the pathogenesis of allergic thyroiditis may help clarify the nature of the disease in man.

Implications of Allotypy for Experimental Biology

Antigenic differences in serum protein allotypes among individuals of the same species have been investigated further by chemical, immunological and genetic methods. The basic work on allotypes which originated with studies in the rabbit, have now been extended to mice and to primates, including man. The studies in mice are of unusual interest since in this species the allotypes serve directly as markers for the study of protein genetics, tumor immunity, and transplantation immunity. The findings in primates are significant inasmuch as they have direct implications for man especially with respect to transfusion reactions and to maternal-fetal interactions from allotype incompatibility in a manner analagous to the Rh situation.

Maternal-Fetal Interactions Lead to Long-Lasting Inhibition of Protein Synthesis in Offspring

In female rabbits immunized to the allotype of the father, the antiallotype antibody in the maternal circulation specifically inhibited the genetically determined synthesis of the paternally derived allotype of the young rabbits for at least one year and was associated with a compensatory increase in the allelic allotype derived genetically from the mother. This phenomenon of "allotype suppression and associated compensation" is novel and may prove to be of fundamental significance comparable to the induction of "immunological tolerance" of the newborn. In the former situation (allotypy) synthesis of a normal protein is suppressed, whereas in the latter situation there is interference with the synthesis of a new protein antibody. The basic mechanism may nevertheless be similar in both situations. The phenomenon of "allotype suppression," involving as it does genetically defined parameters, offers special advantages for delving deeper into understanding these mechanisms at the molecular level.

Cellular Production of Gamma Globulin Allotypes Shown by Fluorescent Antibody

The histological sites and the cellular production of the allotypes have been investigated. Rabbit antisera to allotypes A4 and A5 controlled by allelic genes were fractionated on a DEAE cellulose column to isolate the 7S gamma globulin for coupling with fluorescein isothiocyanate and lissamine rhodamine B (RB 200) so as to produce specific conjugates of contrasting colors. Fluorescent antibody studies of lymph nodes, with mapping of adjacent sections by conjugates to either of the two allotypes, and with double staining of the same section with the conjugates of the same or contrasting color to either of the two allotypes, yielded information on how the allotypes are produced in the heterozygote adult. No evidence was found of separate groups or clones of lymphoid cells producing only one of the two allotypes. Rather, the evidence indicates that the two allelic allotypes are produced by the same cell. In rabbits with one allotype suppressed by passively-transferred antibody, the lymph nodes did not exhibit the presence of the suppressed allotype indicating that suppression involves inhibition of allotype synthesis. Production of both allelic allotypes by one cell indicates that the mechanism of suppression cannot be explained as clonal destruction by antibody. Rather this occurs by a more subtle action of antibody on the cell which forces a shift of synthesis from the paternally derived allotype to that of the maternally derived allotype.

Allotypes Shown To Be Genetic Markers in Cell Transfer Studies

An issue of special importance in basic work on the transfer of homologous lymph node and splenic cells is the cellular source of the antibody which subsequently appears in the recipient animal. Rabbits homozygous for each of an allelic pair of allotypes of gamma globulin (A4 and A5) were used as donors and as recipients of transferred *Shigella* antigen-incubated lymph node cells. Following the appearance of agglutinins to *Shigella* in the sera of recipient animals, the allotype of the agglutinin was determined by absorbing it to *Shigella*, treating these agglutinates with fluorescein-conjugated rabbit anti-A5-gamma-globulin and the anti-A4-gamma-globulin. The reactions of the recipients' sera were consistently positive for the donors' gamma globulin allotype but not for their own. With decline of the agglutinin level in the recipients, these animals were actively immunized with *Shigella;* the antibody produced then reacted with the fluorescent antibody against their own allotype. The findings show that in such transfer experiments. the antibody which initially appears in the recipient serum is synthesized by donor cells, thus illustrating how allotypes can serve as genetic markers. In a further use of the allotype as a marker, prenatally derived thymus and spleen cells have been transferred to prenatal recipients. The appearance in the recipient's serum of the donor gamma globulin allotype indicated successful transfer of lymphoid cells to a tolerant host. With this experimental method it becomes feasible to study the role of the thymus in early life, and the onset of immunological competence.

Genetic Control of Allotypes Related to Chemical Structure

Chemical studies have shown that allelic allotypic specificities do not appear on the same molecule. On the other hand, non-allelic specificities may appear on the same molecule or separately. Genes in coupling or repulsion in double heterozygotes may contribute allotypic specificities to the same molecule. Thus far results are analagous to the well-known chemical and genetic studies of hemoglobin. The chemical studies of the gamma globulin allotypes are likewise directed toward relating the structural difference in the protein to that of the genetic control. Since the gamma globulin molecule is complex, sub-units have been studied with the objective of obtaining the smallest fragment exhibiting the genetic marker. It was established that such a small molecular weight subunit is obtainable, which would be suitable for fingerprinting studies to elucidate amino acid differences attributable to allelic genes.

New Mouse Allotypes Discovered by Ascitic Fluid Technique

Mice of several inbred strains were immunized with mouse anti-Salmonella agglutinates. The mice developed ascitic fluid containing precipitating antibodies to three allotypes. Two of these are gamma globulins under the control of allelic genes. Coisogenic lines of mice are being estab-

lished which will be useful for studies in tumor and transplantation immunity. The availability of many known genes in the mouse and the numerous inbred lines make it feasible to establish the genetic linkage group for each allotype locus as discovered.

Allotypes Found in Man

The first allotypes characterized in man resulted from the testing of sera from multitransfused patients. These allotypes were found to be the low density beta lipoproteins. The experimental approach toward finding allotypes in man has been via the use of the monkey and the chimpanzee as surrogates. This has led to the production of primate antisera which have revealed new gamma globulins and individual differences among these gamma globulins in the sera of normal individuals.

New Methodology for Characterization of Serum and Tissue Enzymes

Methods have been developed for characterization of amidase and esterase activities of trypsin-like, chymotrypsin-like and carboxytrypsin-like enzymes in serum as well as in tissue extracts. These methods allow the enzyme studies to be made after agar gel electrophoresis and even after immunoelectrophoresis, because the enzyme-anti-enzyme precipitin bands continue to exhibit enzyme activity. It has thus become feasible to identify the electrophoretic mobility, immunochemical specificity, and enzymatic activity of serum and tissue enzymes in a single laboratory procedure. Initial clinical studies with previously developed techniques of this kind have shown significant decreases in beta-naphthyl esterase activity in sera from patients with Wilson's disease and several other liver disease states. These novel methods afford new opportunities and applications in the increasingly important area of characterization of tissue antigens in autoimmune diseases and in tissue transplantation studies.

Action of Immunosuppressive Drugs on Normal Serum Globulins

The suppressive effect of antimetabolites on immune responses has been the subject of intensive study in recent years, but there have been no indications of the effect on antibody proteins in non-immunized subjects. In collaborative studies between LI and Laboratory of Clinical Investigations, the administration of the purine antimetabolite 6-thioguanine was found to have therapeutic effects in patients with the nephrotic syndrome, and to produce decreases in serum gamma globulin levels in patients receiving this antimetabolite. In rabbits both 6-thioguanine and 6-mercaptopurine were found to produce decreases in the level of serum gamma globulins, but other serum proteins were increased. The hypogammaglobulinemia produced in both humans and rabbits indicates that the effect of the antimetabolites on antibody producing cells is not limited to suppression of primary response to antigenic stimuli, but is a continuing effect on the mechanisms for maintaining "normal" gamma globulin levels. This work indicates that the purine antimetabolites offer promising alternatives to the use of corticosteroids in the therapy of nephrosis, and suggest that other disease entities associated with autoimmune phenomena may also be amenable to such treatment; moreover, they suggest approaches to determine possible mechanisms by which the antimetabolites exert their effects.

Study of Delayed Allergy With *in Vitro* Techniques

The lack of an *in vitro* test system to substitute for skin testing and other similarly unsatisfactory procedures in the intact animal or human has prevented the evaluation of the role of the delayed type of hypersensitivity in the production of tissue lesions in the autoimmune diseases and in the rejection of tissue homografts. Reassessment of the "Rich and Lewis" test system for study of spleen and lymph node fragments in tissue culture is providing encouragement for approaching these issues. Migration of cells from explants of tissue from sensitized animals is measured in the presence of antigen, and specific inhibition of cell migration appears to reflect cellular or delayed type hypersensitivity. A heretofore unreported serum factor appears to confer on normal cells the property of exhibiting hypersensitivity to antigens. With this *in vitro* technique it may be possible to ascertain the relationship between circulating antibody and delayed-type hypersensitivity, and eventually to assess the role of delayed hypersensitivity in many host phenomena of immunological importance.

LABORATORY OF CLINICAL INVESTIGATIONS

This is the third year of operation of the Laboratory under the present Clinical Director. During this time a number of new programs have been initiated, notably that of clinical immunology, respiratory viral disease in volunteers, malaria, leprosy, and mechanisms of host response. In addition, strong programs continue in systemic fungus disease, biochemistry, immunology, and other activities previously in operation.

Purine Antimetabolites in Non-Neoplastic Diseases

In recent months a significant new trend in medical therapy has been extended by the observation that cases of nephrosis, of the type classified as of unknown etiology, have been greatly benefited by treatment with one of the purine antimetabolites, 6-thioguanine (6–TG). In addition to nephrosis, patients with chronic hepatitis and systemic lupus erythematosus have now been treated with some favorable results.

At present two patients who had received long courses of steroid therapy with varying degrees of response and many of the toxic consequences of steroid treatment have shown complete remission from nephrosis on 6–TG treatment alone or with 6–TG plus a previously inadequate dose of steroid. In one case, an adult male, whose condition was considered hopeless, restoration to apparent good health and return to full employment have occurred. He has been well for more than 1 year since treatment. The other patient, a 14-year-old girl, whose appearance was greatly marred by the plethora, striae, and rotundity characteristic of high-dose steroid treatment, has returned to a normal appearance in association with complete remission of nephrosis following a short course of 6–TG. She relapsed and was given longer treatment with a second remission.

Several patients with hepatitis were given 6–TG but presumably because of their disease, they are very sensitive to its toxic effects. Nevertheless, one adolescent male has apparently undergone remission after 6–TG treatment. One patient with systemic lupus improved with 6–TG, relapsed and then improved on retreatment. This sequence was repeated three times.

Toxicity of 6-Thioguanine

There is a considerable variation in threshold of tolerance of 6–TG, and toxic effects consist principally of agranulocytosis and thrombocytopenia, at which time severe infection can be a further complication. One severe episode of toxicity has occurred complicated by gram-negative bacteremia. The patient recovered.

A study in rabbits was undertaken of the effect of 6–TG on protein synthesis and antibody formation. It revealed a significant reduction in serum gamma globulin following 6–TG. The same findings as well as a significant reduction in isohemagglutinin titers were observed in nephrotic patients given 6–TG.

Action of 6-Thioguanine

Many observers believe that some cases of nephrosis, hepatitis, lupus, some anemias, and a number of other diseases are due to the effect of antibody produced in the host to its own body constituents such as liver, kidney, platelets, connective tissue, etc. The initiation of such a reaction could depend upon a viral infection as in hepatitis, but in other situations no adequate explanation is available. The rationale for the effect of 6–TG, at present, is that it suppresses protein biosynthesis in cells which form antibody globulins, thus preventing damage to host tissue from antigen-antibody combination. The findings in patients and in the rabbit studies are consistent with this concept, although possible anti-inflammatory effects of 6–TG are also being investigated.

From the limited studies now available it is difficult to predict the benefits of this new therapeutic approach to a group of diseases previously treated with only modest success. The tendency to relapse already evident in some cases and the pronounced hematologic and probably other toxic effects suggest some limits to the usefulness of the present agents. Nevertheless, two important points are already clear; first, a new principle of therapy has been demonstrated which is worthy of a careful follow-up; secondly, the fact that remissions can be achieved with antimetabolite administration in diseases of unknown eitiology opens a new avenue for investigation of the pathogenesis of these diseases. Both points are under further study.

Respiratory Viral Agents in Normal Volunteers

The use of prisoner volunteers in the study of viral respiratory agents was begun 2½ years ago. More than 500 men have come to the Clinical Center for these studies, 241 of them this year. One of the recent developments of the program has been the administration of a respiratory viral agent in a submicron size aerosol. This has permitted a rather precise estimate of the small dose of virus required to produce virus infection, illness, and antibody response. It has allowed comparison of the characteristics of disease produced by lower pulmonary tract inoculation with that of the upper tract, and is now leading to studies directed at answering the question of the mechanism of natural air-borne spread of respiratory viral disease.

In the studies undertaken with the staff of the Biological Laboratories at Fort Detrick, Coxsackie A–21 (Coe agent) has been found to produce disease (ID_{50}) with submicron size aerosol particles with 28 $TCID_{50}$ per volunteer in a 10 liter inhalation. The 95 percent confidence limits are 16 to 63 $TCID_{50}$. The smallness of this dose is emphasized when it is recalled that only about 50 percent of the dose is retained.

The equipment for this work was developed at Fort Detrick. Inoculation is quickly and simply performed in a truck semitrailer which contains all the necessary equipment and is brought to the Clinical Center for this purpose. It is apparent that this same equipment could be modified easily to provide rapid and accurate inhalation of viral vaccines, and in view of the stability of small particle aerosol clouds, it is apparent that larger scale inoculation would require only modification of the equipment along lines already well-understood. Part of the future effort in this program will be concerned with vaccine development.

Anti-Viral Therapy

In what is hoped may become a larger effort three antiviral substances have been tested in volunteers this year. The agent reported by Kaufman to be effective in herpetic conjunctivitis, 5-iododeoxyuridine, was found to be ineffective in conjunctivitis in volunteers caused by adenovirus type 27. A thiopyrimidine compound, active against influenza in mice, was found to be ineffective in treatment of induced human Asian (strain 305) influenza. More recently an irradiated preparation influenza virus (PR–8) which stimulated interferon production in tissue culture and in mice sufficient to protect against influenza and other viral infections, is being tested in volunteers. This program has moved slowly because less than 5 percent of prisoner volunteers are free of antibody to Asian influenza virus and getting sufficient numbers of such candidates for study is difficult.

Malaria

During the year the study of malaria antibody formation was continued. Studies in volunteers revealed that antibody to the simian strain, *Plasmodium cynomologi* (B), reacts to a greater extent with this parasite than with two human vivax strains (Chesson and Venezuelan). Antibody to the vivax strains reacts with them and to the simian parasite to the same degree. These findings were interpreted to indicate that the simian parasite possesses antigens in common with human vivax strains, but in addition, has an antigen distinct from the vivax plasmodia.

Study of sera obtained last year from Ghana has revealed a high titer of antibody when *P. falciparum* is used in a test, but low titers when vivax or the simian plasmodia were used. This evidence of specificity of the test for malaria antibody suggests a great area of usefulness of the procedure.

Biochemical Assay for Penicillin

Despite the many years of intensive investigation of pencillin derivatives no satisfactory biochemical assay for these compounds has been developed. In recent months, in the course of an investigation of penicillin allergy, an acid breakdown product of penicillin, penicillenic acid, was found to be stabilized by the addition of a mercuric salt, and could be quantitatively measured by UV spectrophotometry. This permitted an accurate assay of most penicillin derivatives on the market down to concentrations as low as 2 mcg per ml. It was further found that the reaction was less variable in the presence of albumen or serum. The test could be completed in about one hour, a much shorter time than with the bioassay using *Sarcina lutea*. In tests of unknown sera there was no significant difference in results by biochemical and bioassay. This test is therefore

applicable to the assay of biologic fluids and can be conveniently used as an adjunct in pencillin therapy.

Fungus Disease

This program continues to work toward improvement in the diagnosis and treatment of systemic fungus infections. Clinical reports are published regularly and serve to alert practicing physicians to attempt to find new cases. Such efforts benefit the program, since it stimulates referral of cases here for study. From such referrals, 67 patients were selected for study this year.

As experience accumulates new clinical syndromes are discovered. The report this year of five adults stricken with histoplasma meningitis is the first description of the disease diagnosed in adults prior to autopsy. Only one of the five patients died.

From the group of patients with cryptococcal meningitis, the largest group ever assembled in one institution, has come an encouraging report of the efficacy of amphotericin treatment. Of 30 patients with this usually fatal illness, only 5 did not respond to treatment. All five had some other serious disease, usually cancer or diabetes mellitus.

Plasma Cell Tumors and Antibody Production

Using ovalbumin as an antigen it was shown that mice with implanted plasma cell tumors did not respond to primary stimulation with ovalbumin. The antigen was given near the time of tumor implantation. Approximately 10 days later, however, when tumor growth was proceeding rapidly, response to a second dose of ovalbumin was followed by significant antibody response. It was the interpretation that tumor growth in some manner prevented development of primary response, but despite increasing tumor growth, the additional stimulation of a second dose of antigen was sufficient to cause antibody production in the few remaining antibody-producing cells of the mouse. It was felt that the tumor prevented primary response but that tumor cells did not participate in the production of antibody. These observations may have some bearing on mechanisms of immunity in humans with plasma cell and other diffuse tumors.

LABORATORY OF PARASITIC DISEASES

The work of this Laboratory has encompassed a broad range of subjects and approaches to parasitic disease problems.

Angiostrongylus Cantonensis

Quantitative experimental infections with *Angiostrongylus cantonensis* in rats and monkeys have revealed pathologic changes consistent with the clinical disease syndrome eosinophilic meningitis. Work with *A. cantonensis* has also shown that this worm undergoes larval development in a large variety of freshwater snails as well as slugs, and that larval stages can survive in both fresh and salt water. These findings suggest that the worm may be able to reside in various paratenic hosts (secondary carriers in which no development occurs). This could explain the epidemiological relation between the ingestion of raw fish and the occurrence of eosinophilic meningitis, suspected as due to *A. cantonensis*, in Tahiti.

Adults of *A. cantonensis* have been maintained *in vitro* for 80 days and metabolic products derived from these cultures have proven useful as antigens in a hemagglutination test on the sera of some rats. These antigens have come only from incubates of female worms during early cultivation, when they are still depositing eggs.

Experimental Schistosomiasis

Work on the pathophysiology of experimental schistosomiasis revealed shifts of worms to the lungs on treatment of mice that had developed portal-systemic collateral circulation. Repeated infection and repeated treatment of mice, allowing delivery of large numbers of eggs and worms to the liver, did not change the pathologic picture of the infection; and the mice rapidly returned to normal as judged by the physiologic and anatomic criteria. Treated mice showed no resistance to reinfection, except slightly slower development of worms. These results are of interest in relation to the management of human infections.

Snail Hosts of Schistosomes

Study of the biology and control of snail hosts of schistosomes has shown that two species of

Acanthamoeba-like protozoa parasitize tissues of snails. These organisms do extensive tissue damage but do not, apparently, decimate snail populations. However, they may be important in restricting populations, and adverse effects on the amoebae of some biocides could, conceivably, result in later increase in snail densities. Observations on seasonal changes in the apertural lamellae of *Australorbis glabratus*, and their relation to migration of snails out of water are important in understanding the ability of snails to survive dry seasons in some areas.

Antimony Compounds and Diet in Treatment

The efficacy of antimonial compounds against *Schistosoma mansoni* has been shown to be influenced by such dietary factors as acid-salt residues, Mg-Ca rations, and amount of crude fiber. However, these factors account for only a fraction of the enhanced (up to 30-fold) drug activity seen in mice on a purified semisynthetic diet. Antimony compounds given either by oral or parenteral routes have increased activity in such mice. These findings have considerable significance in relation to the chemotherapy of schistosomiasis in developing countries, where the diet of the people may be a major factor in failure to obtain good results on treatment. The findings that purified low residue diets induce self-cure of mice with various nematode infections, also have bearing on the management and control of intestinal helminthiases in many parts of the world. Aside from the practical aspects, these data provide a stimulus for basic work on the factors of toxicity, immunity, etc., that may be operative.

Axenic Cultivation of Amoebae

Work on the axenic cultivation of *Entamoeba histolytica* has been continued, with three strains now established in, and a fourth strain being acclimated to, axenic conditions. Noteworthy is the observation that the more hypotonic the overlay of the medium, the better its ability to support initial axenic growth. This suggests that the nutrition of amoebae is related to physical factors in the medium, stimulating pinocytosis. Chick embryo extracts of various types are still needed to support initial axenic growth, but once established, all strains can be adapted to growth without them. Substitution of a liver extract for yeast extract in the maintenance medium has increased amoeba yields. *Trichomonas tenax* has also been grown in axenic culture for the first time.

Biochemistry of Parasites

Continuing studies on the biochemistry of protozoa has added four enzymes to our catalog of those known from *Trichomonas vaginalis* and increased our knowledge of purification procedures in their study.

Further work on the absorption and excretion of sugars by *Taenia taeniaeformis* has shown that the uptake of glucose from the medium by adult worms is essentially independent of the glucose concentration, while on the other hand, more galactose is consumed at higher concentrations. Glucose is secreted by both larval and adult worms when kept in sugar-free Tyrode's solution; in the presence of low glucose concentrations, adults begin to absorb the sugar while larvae continue to excrete it until the concentration is eight times higher than the threshold for adults. The presence of other sugars appears to have little effect on larval glucose excretion. The lack of dependence on concentration of glucose absorption by adults suggests an active transport mechanism.

The activation of succinate oxidation by ATP, reported last year, appears to occur at the level of dehydrogenases, rather than in the respiratory chain. Uncouplers of oxidative phosphorylation, such as the halophenols, are assumed to be bound to mitochondrial lipids. However, work on lipid-depleted mitochrondria showed binding of pentachlorophenol (PCP) to the same extent as in lipid-rich controls. Furthermore, studies with plasma albumin, which reversed the uncoupling effect of PCP indicate that protein binding may be an important factor in the action of uncoupling agents. These findings are of general biochemical interest in regard to the theory of respiratory chain phosphorylation.

Toxoplasmosis

Research on toxoplasmosis has revealed cysts in the lung and intestine of mice. From these sites they can be shed to the outside environment. Studies on newborn mice from chronically infected mothers indicate that either immune paralysis or tolerance may develop when congenital transmission occurs during chronic infection. The finding of a toxin in the course of the serological studies is noteworthy. This toxin is entirely different from

that reported previously, about which there is considerable doubt, and is similar to sarcocystin or to that from coccidia. Results with the hemagglutination test have been so consistent that it is now adopted for routine diagnostic use.

LABORATORY OF BACTERIAL DISEASES

There has been a significant change in the research program of the Laboratory of Bacterial Diseases during the past year.

Pleuropneumonia-Like Organisms

Because of the growing importance of the pleuropneumonia-like organisms in human disease, the real need for basic studies on this group of organisms, and the long-standing interest of the Laboratory in the PPLO and the L-forms of bacteria, a new research program of basic studies on the PPLO and the L-forms of bacteria has been initiated.

To date, considerable quantitative growth data on representative strains has been accumulated, and some methods of preparation of antigens and antisera have been explored. Of particular interest are preliminary results of the use of certain cell fractions as erythrocyte sensitizing antigens. If such antigens can be developed in quantity, quantitation of immune responses may be made much more sensitive and accurate.

Intracellular Parasitism

The studies on the intracellular parasitism project have continued in the same general area as previously, that is, on antibody production *in vitro* of cells removed from immunized animals. Comparative studies have been done on cells from the spleen, lung, and peritoneal cavity of guinea pigs. No new antibody production was detected in macrophages perfused from the lung. Cells from the peritoneal cavity yield better results in our experiments than those from the spleen. The cell-type producing antibody is still uncertain. These studies are being continued.

LABORATORY OF PARASITE CHEMOTHERAPY

The main research effort continued to be centered on overall problems in malaria. However, investigations involving parasitic infections in general, with special emphasis on schistosomiasis, intestinal parasites and viruses were pursued.

Malaria—Human

The prophylactic effect of a new antimalarial drug appears promising. Twenty-two of twenty-four volunteers given a single intramuscular injection of CI–501, 5 mg/kg, and challenged by bites of infected mosquitoes on days 5 to 322 have been protected against vivax malaria for from 198 to 357 days. Two volunteers became infected after 169 days and 341 days following mosquito bites on days 167 and 322. Four volunteers given a single intramuscular injection of the same compound, CI–501, and challenged on days 9 to 12 with the Southern Rhodesian strain of Plasmodium falciparum, by blood inoculation, became infected. Four volunteers challenged on day 5 (after drug injected) by mosquito bites were protected. The Southern Rhodesian strain is not fully sensitive to chlorguanide and may be resistant to the triazine. Two volunteers challenged with the McLendon strain by blood inoculation on days 0 and 5 were protected.

A number of malaria strains exhibit resistance to chloroquine, the most important of modern antimalarials. The Colombia strain of *P. falciparum* was infective for mosquitoes for extended periods after chloroquine treatment which would cure a normally sensitive strain. The Thailand strain of *P. falciparum* was resistant to chloroquine, amodiaquine, pyrimethamine and chlorguanide but sensitive to the suppressive effect of quinine and to the sporontocidal effect of primaquine. Two cases of falciparum malaria acquired in Brazil were resistant to several normal courses of therapy with chloroquine. One patient was cured by higher doses of chloroquine and one was cured by a 10-day course of quinine totalling 10 gm.

Malaria—Simian

Continued studies of the susceptibility of man to monkey malaria have added important information. Serial passages of the M strain of *Plasmodium cynomolgi* by blood inoculation have been made in volunteers with a maximum of eight passages in one line. The peak parasitemia was not strongly correlated with passage number but the highest peak parasitemia (7,200 per cmm) was found in the eighth passage. In a smaller series

705-685—63——15

of subinoculations of the B strain the highest peak parasitemia was 1,150 per cmm. None of nine volunteers infected by mosquito bite relapsed after treatment of their primary attacks with quinine. One of three volunteers bitten by a single infected mosquito became infected and remained positive for 17 days.

Two of three volunteers were infected, by mosquito bite, with a strain of *P. cynomolgi* recently isolated from a Malayan pigtail monkey. The infections were benign and parasite counts did not exceed 50 per cmm.

One of two volunteers bitten by *Anopheles freeborni* infected with *Plasmodium brasilianum* became positive in 63 days. This is the first known infection of man by a New World simian malaria.

Two new species, *Plasmodium coatneyi* of *Macaca irus* and a tertian parasite of the white-handed gibbon were described. The former is a tertian parasite with asexual stages like *P. falciparum*. The latter resembles *Plasmodium vivax* but does not cause enlargement of erythrocytes. An additional new parasite of a non-simian host, the Malayan flying lemur, also has been described.

Infected salivary glands of *Anopheles freeborni* injected into a rhesus monkey produced an infection of *P. coatneyi* which was positive on the 15th day. *Anopheles quadrimaculatus*, *A. crucians* and *A. walkeri* were not susceptible to *P. coatneyi*. *Anopheles freeborni* was susceptible to *P. fieldi* but *Anopheles quadrimaculatus* was not. *Anopheles freeborni* and *Anopheles quadrimaculatus* were susceptible to both *P. gonderi* and *P. inui*.

Biochemical Studies

Although *Plasmodium berghei* is susceptible to folic acid antagonists (aminopterin, amethopterin and pyrimethamine) PABA is a complete substitute for folic acid while folic acid is only a partial substitute for PABA. Moreover deficiency of folic acid in animals supplied with adequate PABA favors rather than inhibits the parasites. A pyrimethamine resistant strain of *P. berghei* was found to have an increased need for PABA and an increased sensitivity to sulfonamide. These results are all consistent with the hypothesis that the parasite uses PABA to form a substance similar to but not identical with folic acid and that resistance to pyrimethamine involves a change in the nature of the folic acid analogue utilized in the metabolic system of the parasite.

Pantothenic acid deficiency as well as pantothenic acid analogues favor rather than inhibit *P. berghei*. The combination of deficiency plus antimetabolite has an antimalarial effect. These results are interpreted as meaning that the parasite needs pantothenic acid but is less sensitive to deficiency than the host. The more complete deprivation produced by the combined effect of dietary deficiency and antimetabolite is sufficient to inhibit the parasite.

The ability of malarial pigment to form a complex with chloroquine was predicted from theory and confirmed by both chemical methods and bioassay. The failure of chloroquine to inhibit the incorporation of labelled amino acids and the affinity of hematin for chloroquine suggest that the mode of action of chloroquine may involve degradation of hemoglobin rather than a synthetic process.

Immunological Studies

Fluorescent antibody techniques have revealed that antibodies to *Plasmodium malariae* appeared between the 15th and 28th day after inoculation and persisted until the 120th day. Antibodies to *P. falciparum* persisted up to 18 months but with a decline in titer by the 12th month. Cross reactions between *P. malariae* and *P. falciparum* occurred.

Far East Research Project Studies in Malaya

Four species of simian malaria including one new to science (*P. coatneyi*) have been isolated from wild-caught specimens of *Anopheles hackeri* by injection of sporozoites into uninfected monkeys.

A wild specimen of *Anopheles leucosphyrus* infected with *P. inui* was captured in the act of biting man. Also it was proved that *Anopheles balabacensis introlatus* is a vector of monkey malaria and other studies have shown that this species too attacks man about as readily as it attacks monkeys. An area in Cambodia was located where *A. balabacensis balabacensis* was common. The attack rate on monkeys in the canopy and man on the ground was nearly equivalent. Infected mosquitoes were found and identification studies of the sporozoites are underway.

Mansonia uniformis was consistently susceptible to experimental infection with two strains of *P. cynomolgi*, but sporozoites did not regularly ap-

pear in the salivary glands and attempts to infect monkeys by injection of sporozoites from oocysts have failed. Other susceptibility studies with over 20 species of Malayan *Anopheles* showed that *Anopheles barbirostris*, *A. umbrosus*, and *A. hyrcanus* were not very susceptible to *P. cynomolgi bastianellii* while *A. maculatus*, *A. kochi* and *A. sundaicus* were very susceptible. *A. balabacensis introlatus* was highly susceptible to *P. cynomolgi* but practically insusceptible to *P. c. bastianellii.*

About 40 inoculations of blood from aborigines with malaria into uninfected monkeys have not revealed strains that would grow in monkeys.

Schistosomiasis

Three of 104 compounds tested against *Schistosoma mansoni* were active. Two were less active derivatives of Miracil D and one was a confirmatory test of a compound produced by Abbott. Among the inactive compounds were 17 enzymes, mostly proteolytic.

Virus-Mosquito Larvae Associations

Larvae of *Aedes aegypti* and *Anopheles albimanus* mosquitoes acquired Semliki Forest virus from an experimentally contaminated aquatic environment. The resulting infections in adult mosquitoes were of low titer and prevalence.

Intestinal Parasites

Stilbazium iodide 10 mg/kg eliminated *Enterobius vermicularis* infection in children and *Strongyloides stercoralis* in adults. It was moderately effective against hookworm and *Trichuris.* Bephenium gave excellent results against hookworm and dithiazanine was highly effective against *Trichuris.*

LABORATORY OF INFECTIOUS DISEASES

This group of investigators, one of the largest in the Institute, pursued important studies on a variety of infectious processes. These ranged from the role of viruses in cancer, the viral etiology of respiratory disease, and antibacterial effects of sea water to the ecology, immunology and experimental treatment of fungus diseases.

Adenovirus Types 12 and 18 as Cancer Viruses

Trentin's discovery that type 12 adenovirus caused cancers in hamsters was promptly confirmed and his findings extended as follows: (a) Four separate freshly isolated strains of adenovirus 12 recovered from anal swab specimens of 4 Washington, D.C., infants readily produced similar cancers in animals; (b) The oncogenic activity was shown to be associated with replication of a particle inducing a unique specific complement-fixing antibody to adenovirus 12, but only in those hamsters developing cancer; (c) type 18 adenovirus, which share a number of unique properties with type 12, also produced similar cancers; (d) similar fatal but apparently nonmetastasizing fibrosarcomas were produced locally within 40 days following intraperitoneal, intracerabral, subcutaneous and intrathoracic injections.

Twenty-six other adenoviruses have not produced cancers in hamsters during 6 or 9 months' observation. Children infected with adenoviruses 12 and 18 are being followed and studies of the mechanism of adenovirus oncogenesis in animals more recent efforts.

Papilloma Viruses

Common complement-fixing antigens were found in bovine, canine and rabbit papillomas. These unsuspected relationships suggest a search for a similar antigen in human papilloma viruses. Interestingly enough, antibody reactions to animal papillomas can be demonstrated in human serums. Bovine papillomas were found to produce subcutaneous fibromas in hamsters and apparently a specific complement-fixing antibody response as well.

Natural History of Polyoma Virus

The natural cycle of polyoma virus was demonstrated previously in rural (agricultural) ecologies. Grain storage areas on farms and mills provide stable environments suitable for harboring large and dense populations of *Mus musculus* and numerous effective portable sources of polyoma infection. Similar results were obtained in surveys of grain mills in Indiana, Georgia and Florida. Polyoma was isolated from 49 of 109 grain specimens. A number of other virus infections were detected in mice in all these areas. Their importance to human and animal health is unknown, but two of them, reoviruses and lymphocytic meningitis (LCM), are known to infect man and domestic animals.

Respiratory Virus and Vaccine Studies

Much of the current activities of the respiratory program are now directed toward development of effective respiratory disease vaccines. Since the programs now underway involved setting up new commercial contracts during 1962, there is little that is new to report as yet. However, important new observation were made on certain respiratory viruses having direct importance to the vaccine effort. Volunteers were infected when fed adenoviruses 4 or 7 in enteric coated capsules. Selective infection of the intestinal tract, while bypassing the respiratory tract, provides a sound basis for pursuing live adenovirus immunization. Introduction of live virus by an atypical route may represent a solution to the thorny problem of adenovirus immunoprophylaxis. At present, effective safe adenovirus vaccines are not available because of difficulties in producing potent lots of adenovirus material free of simian virus (SV–40).

In other studies naturally occurring reinfection with RS virus was demonstrated in adults. This is an important finding in the natural history of RS infection. It also poses a problem regarding vaccination of adults, since upper respiratory illness can occur despite the presence of high levels of neutralizing antibody.

Complexity of the Rhinoviruses

Workers in LID, along with scientists in other laboratories, have identified at least 40 different serotypes and the end is not in sight. Before a rational approach to vaccination can be formulated, the rhinovirus group must be completely defined and the importance of the various virus serotypes established. A preliminary analysis of our volunteer data suggests that neutralizing antibody may be only moderately effective in preventing rhinovirus disease. The findings also suggest that reinfection may also be of importance in the natural history of the rhinoviruses.

Mycoplasma Agents in Respiratory Disease

The Eaton agent has now been clearly established as a mycoplasma (pleuropneumonia-like organisms) instead of a virus, and for the first time accurate, easily performed diagnostic tests for Eaton agent pneumonias have been developed. This represents the first association of a mycoplasma with human respiratory disease; in fact, with any type of illness in man. New techniques for recovery of Eaton agent on artificial media have provided simple methods for isolating the PPLO from infected individuals. This should facilitate future diagnostic and epidemiologic studies. Growth of the organism in broth suspension has made possible the preparation of a specific complement-fixing antigen, thus simplifying the problem of serodiagnosis. In addition, it provides the basis for growing the organism in bulk culture for vaccine production. It was found that a soluble hemolysin of *Mycoplasma pneumoniae* (Eaton agent) completely lyses guinea pig red cells, whereas this property is not possessed by other human PPLO. The implications of this finding to quantitation of the organism and to identification of field isolates are obvious.

In collaboration with the Laboratory of Clinical Investigations, infection of antibody-free volunteers with high passage artificially propagated *M. pneumoniae* was undertaken. Evidence of attenuation was obtained in this study. There were no illnesses among the 12 volunteers thus far infected indicating evidence of attenuation of the strain. These findings provide a basis for pursuing the development of an attenuated live mycoplasma vaccine.

Virus Infections and Human Cancer

A serological survey for viral infections in leukemia patients and closely matched nonleukemic controls, in St. Louis Hospital, Paris, France, revealed evidence of numerous virus infections. Although differences in virus experiences were observed in the two groups, the significance and possible explanation for these differences require further study.

Cooperating with the Laboratory of Clinical Investigations, NCI, and the Human Cancer Virus Task Force, comprehensive collections of serum specimens from patients with all types of cancer were begun. A serum center similar to that set up with NINDB for the study of birth defects is planned in conjunction with the new cancer-virus facilities proposed for our collaborative studies with NCI.

Cancer Viruses in Vaccines and Foodstuffs

In 1959, it was pointed out that studies on polyoma and extraneous viruses in cell systems and natural ecologies held serious implications for viral vaccines, particularly live vaccines. The

simian SV–40 virus was reported by others in poliovirus vaccines in 1961. Subsequently, its oncogenic behavior in hamsters and in cultures of human cells was demonstrated. In 1961, widespread contamination of cereal grains used for human as well as domestic animal nutrition with polyoma and other viruses of the house mouse was reported.

Recently, the frequent presence of chicken lymphomatosis virus in fertile eggs was reported. This has caused a "background noise" problem in chick embryo virus study systems. It also poses a problem to those concerned with the safety of virus vaccines made in chick embryo tissues and with the purity of widely used food staples.

Pacific Research Laboratory, Hawaii

In the summer of 1962, a new laboratory, the Pacific Research Section, was established in Honolulu, Hawaii, as a separate section of LID for the primary purpose of studying eosinophilic meningitis in the Pacific area. Further evidence that the widespread occurrence of eosinophilic meningitis in the Pacific area (especially in French Polynesia) may be due to *Angiostrongylus cantonensis*, a parasite of rats, was acquired by demonstrating the ability of larval forms to invade the brains of rhesus monkeys. Cases continued to occur in French Polynesia during 1962.

Rubella Virus Isolation in Tissue Cultures

Collaborative work with NINDB not only confirmed preliminary observations of the Walter Reed Army Institute of Research group on the isolation of rubella virus, but helped establish the viruses as the cause of rubella. The latter was achieved by sero-epidemiological studies and by the induction of rubella in volunteers shown by serological tests to be susceptible before inoculation. Immune volunteers did not develop the disease. Very recent preliminary results suggest that inactivitated rubella vaccines will present the experimental induction of rubella in susceptible volunteers.

Enterovirus Studies

The properties and natural behavior of enteroviruses received considerable investigation in 1962. ECHO virus type II was shown in Junior Village to be associated with a diarrheal illness in infants. A number of enteroviruses were shown to contain hemagglutinins which greatly simplified their typing and classification. Five newly recognized viruses (with entero-rhinovirus properties) were characterized and submitted for classification to the American Enterovirus Committee. Fifty more strains of reovirus were identified from feces of cattle; most were types as reovirus types 1 and 3. Inoculation of human volunteers with bovine reoviruses produced infection, but no recognizable disease.

Anti-Bacterial Effect of Sea Water

In the attempt to identify the macromolecule in sea water which is lethal for staphylococci, it was found that the activity is lipase-sensitive. This suggests that a lipid is the antibioticlike compound. This, except for a few steroids, is the first time this class of compounds has been implicated in such activity.

Studies on Hydrogenomonas

The electron-transport system of Hydrogenomonas has been broken down further into its component parts and it is now known that, following the reduction of DPN by H_2, the reduced nucleotide is oxidized via a flavin, vit K_3 (menadione) and a cytochrome. Some purification of menadione reductase has been achieved. It is emphasized that all of these enzymes in the complex are soluble, thus making investigation of the linkage between oxidation and phosphorylation more amenable to study.

Siderophilin

Carbon dioxide has been found to be essential for the formation of the siderophilin-iron chelators and indications are that only the 2 Fe :1 siderophilin complex is formed. Improved methods of purification of biologically active siderophilin have been devised and using such preparations, further observations have been made on the time sequence of the uptake of Fe by reticulocytes following erythropoietic stimulation. A new immunological method for the determination of small amounts of siderophilin has been worked out.

Bacteriophage Treatment for Urinary Infections

Studies on the utilization of specific bacteriophage for the treatment of urinary tract infections refractory to antibiotic therapy have been continued. Of clinical significance is the fact that,

following phage treatment, the urine was initially cleared of an antibiotic-resistant pathogen. After as much as 30 days later, baccilluria re-occurred, this time with a different pathogen, but now, in a high percentage of cases, the new offending cell was sensitive to those antibiotics initially found to be ineffective.

Detoxification

Detoxification studies have progressed further on potential tuberculostatic, fungistatic, parasiticidal, viricidal and antitumor agents. The antiviral activity extracted from various molluscs has been characterized as an acetylneuraminic acid-glycoprotein composed of sugars, sialic acid and 18 amino acids. Pyridyl- and guanyl-hydrozones have been tested for antitumor activity and have passed various preliminary tests carried out by the Cancer Chemotherapy Screening Center.

Cell Wall Formation

By the use of the immunofluorescence technique, it has been found that new cell wall is laid down in a diffuse, intercalated manner in the gram-negative *Salmonella typhosa* as opposed to the polarized, sharply demarcated partitioning in the gram-positive *Streptococcus pyogenes*. The same techniques have been used to follow formation of protoplasts induced by penicillin in *S. typhosa*. Fine structure of walls, membrane and protoplasm are being studied by electron microscopy using negative staining, shadowing and thin-sectioning.

The mechanism of the long-chain phenomenon induced by type-specific anti-M antibody has been investigated further and the location of the M protein, using immunofluorescence, has been verified as being more significant than the group antigen. After removal of the M substance by trypsin, new M protein was laid down only in areas of new cell-wall formation, thus indicating that M substance is not resynthesized by nonproliferating cells.

Environmental Sources of Infection in Mycoses

The presence of *Cryptococcus neoformans* in pigeon manure, has significance as evidence accumulates that man may frequently be exposed to this fungus and that most cases of cryptococcosis may be mild respiratory infections instead of fatal central nervous system disease. In one sample of pigeon manure were found 50,000,000 viable *C. neoformans* cells. When *C. neoformans* cells are plated directly from pigeon manure instead of obtained by mouse passage, it is apparent that strains vary widely in mouse virulence just as strains from human disease vary. This point is being examined more critically by testing several strains from a given sample and from samples of diverse origin. Absence of Histoplasma from pigeon manure, in which *Cryptococcus* is so abundant, seems to lend circumstantial support to the belief that there is a common, self-limited, respiratory form of *cryptococcosis*. This concept, although not generally acknowledged, is probable, and would align cryptococcosis with other mycoses of respiratory origin.

Chemotherapy of Mycoses

Poor or erratic response of patients with systemic mycoses to therapy indicates an urgent need for better antimycotic agents. A compound, X–5079C, which was tested first in this laboratory and has had encouraging clinical trials, is not yet available in sufficient quantity for final evaluation, especially with respect to relapses. Fifty drugs and antibiotics have been tested *in vivo* and 17 drugs by conventional *in vitro* tests. None of these appears to merit clinical trial. An improved method of measuring *in vitro* sensitivity of *Cryptococcus neoformans* to Amphotericin B minimizes error due to decay of Amphotericin B and indicates an inhibitory level of 0.05μg/ml for most strains of *C. neoformans* from human cases. This is many times the sensitivity formerly recognized and is more consistent with clinical findings and therapeutic effect.

Physiology of *Coccidioides Immitis*

Three strains of *Coccidioides immitis* have now been cultivated serially in the spherule form in a defined medium. The metabolism of mannitol by strain M–11 has been studied since the two modifications of this strain differ in their ability to use mannitol as a carbon source. Cell-free extracts of both the mycelium and spherules have been found capable of dehydrogenation of mannitol-6-phosphate but not of mannitol. The dehydrogenase is active in extracts of glucose grown cells suggesting that mannitol-6-phosphate is a normal metabolite in these cells. Presumptive evidence has been obtained for the existence of mannitol within the cells of this fungus. Since the spherule form does not grow on mannitol, the inference is that there is a difference in permeability or trans-

port with respect to mannitol in the two forms of this fungus.

Immunity in the Mycoses

Using mice experimentally infected with *Candida albicans* nonspecific factors influencing immunity were investigated. A sublethal infection with *C. albicans* protected mice against a later challenge infecting dose. Killed cells of *C. albicans*, other species of Candida and nonpathogenic fungi did not afford this protection, although killed cells of Coccidioides, Histoplasma and Blastomyces did give some protection. Plated tissues from mice killed at intervals during the experiments showed a decreased Candida population in the protected mice. X-radiation of mice and, to less degree, thorotrast injections blocked the protection. Serum of mice which received sublethal infections with *C. albicans* or killed Coccidioides and Freund's adjuvant decreased growth of *C. albicans in vitro*. The type of protection studied is nonspecific in nature and, although serum factors may contribute, the cells of the reticuloendothelial system appeared to play the major role.

Cryptococcus Antigen

Limited use of an antigen prepared from ruptured cells of *Crytococcus neoformans* has induced delayed dermal reactions in 4 of 9 patients, with active cryptococcosis, 14 of 16 patients with inactive or cured cryptococcosis, 8 or 17 patients with various systemic mycoses, and 15 of 22 "normal" volunteers. An analysis of other fungal skin tests and serologic studies suggests that cross reactions with the other major systemic mycoses is not an important factor in causing this high incidence of hypersensitivity. The test appears to be specific in animals and evaluation of its significance in man is being attempted. If the high percentage of reactors in a normal human population is a specific test of hypersensitivity to Cryptococcus and is evidence of exposure to Cryptococcus with resultant subclinical and immunizing infection, it will greatly clarify and expand knowledge of the epideminology of cryptococcosis.

NATIONAL INSTITUTE OF MENTAL HEALTH

INTRAMURAL RESEARCH

Introduction

The year 1962 has been a busy, successful, and relatively stable one for the research programs of the laboratories and branches in the NIMH intramural program. The achievements of the year are summarized in the statements to follow, prepared by the Director of Clinical Investigations and by the Chiefs of the laboratories and branches. I shall not attempt any further summary here.

Though stability is not the highest goal of a research organization—new scientific knowledge is—there are times when a measure of stability can contribute in important ways to the atmosphere from which new knowledge springs. During the past year, despite the continuing attractive offers from universities and other institutions, not only did none of our senior scientists leave the NIH, but two distinguished alumni of the program returned from universities where each had spent a year. It was a pleasure for all hands to welcome back Dr. Seymour S. Kety, who returned from The Johns Hopkins University Medical School on July 1 to take over his former post as Chief of the Laboratory of Clinical Science, and Dr. Edward V. Evarts, who returned on October 1 from the Duke University Medical School to become again Chief of the Section on Physiology of the Laboratory of Clinical Science. That Laboratory and the program as a whole are greatly strengthened by the presence of these two outstanding scientists.

We were fortunate during the year in the appointment to permanent positions of leadership of two men who last year were acting chiefs of their units. Dr. Melvin L. Kohn, who has been a member of the Laboratory of Socioenvironmental Studies almost since its inception, accepted an invitation to become Chief of the Laboratory. Dr. Lyman C. Wynne, also a longtime member of the NIMH staff, accepted the equally arduous responsibility as Chief of the Adult Psychiatry Branch. The appointment to positions of such responsibility of scientists whose professional and scientific growth to this stature has occurred within the relatively brief life span of this intramural program marks a kind of coming of age. It is a source of pride to the entire staff that the program has been of such a quality as to attract and hold such outstanding young men, and to provide them with the opportunity for maximum personal and professional growth.

As the year ended, a third similar appointment was recommended. Dr. D. Wells Goodrich, now Acting Chief of the Child Research Branch, was proposed for Chief of the Branch. Like Drs. Kohn and Wynne, Dr. Goodrich has grown up in the NIMH, and also like them, he has provided most effective leadership for his research group. I am personally grateful to all three for their interest, devotion, enthusiasm, and willingness to complicate the life of a scientist with the often frustrating problems of a laboratory chief.

Though no senior scientist has left the NIH, the year's end did mark the departure from this program of Dr. Robert B. Livingston, Chief of the Laboratory of Neurobiology, who transferred to the Division of Research Facilities and Resources to assume direction of the large institutional research grants program. After his notable service as Director of Basic Research for NIMH and NINDB and as a laboratory chief, he will be missed. The work of the Laboratory of Neurobiology, pending a decision as to its future, is being carried on under Dr. Ichiji Tasaki as Acting Chief.

In addition to the research carried out during the year, several laboratories and branches devoted considerable time to the planning of new facilities which are scheduled to become available in from 18 months to four years. The basic biological laboratories, together with the Section on Technical Development, completed detailed layouts of space in the new basic research building for the NIMH and the NINDB, now expected to be com-

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pleted in the fall of 1966. Scientists from the Laboratories of Psychology and Neurophysiology continued their planning for the animal behavior research facilities to be constructed on the NIH farm near Poolesville. Completion is scheduled for the late spring of 1964. Dr. D. Wells Goodrich and his staff of the Child Research Branch continued, with many frustrations and hopefully some progress, to work out the complicated problems of contracting for leased space designed to suit the unique needs of this group. Additional animal research facilities were planned during the year for both the Addiction Research Center at Lexington and the Clinical Neuropharmacology Research Center at St. Elizabeths Hospital. All of the proposed new facilities are badly needed to take care of the present program adequately and to permit very modest expansion of the work in certain important directions.

The program continued this year to receive the valued counsel of the NIMH Board of Scientific Counselors. Members this year were Dr. Jordi Folch-Pi, Chairman, Dr. Leonard S. Cottress, Jr., Dr. Howard F. Hunt, Dr. Lawrence C. Kolb, Dr. Stephen W. Kuffler, and Dr. F. C. Redlich. They met March 16–17 in Bethesda, when members of the intramural staff reviewed for them the 5-year study on "Human Aging: Biological and Behavioral Aspects." On October 18–19, the Board met at the PHS Hospital, Lexington, Kentucky, for a comprehensive review of the research program of the Addiction Research Center by Dr. Harris Isbell and members of his staff. On both occasions the Board showed its usual high interest in the program; members contributed many helpful suggestions and raised many stimulating questions.

As one looks back over the year he realizes, as it is not always possible to do day by day, the important contributions which have been made by what might be called the supporting staff of the intramural program—the nonscientists. Chief among these has been Mr. Gordon J. Klovdahl, Administrative Officer, whose energy, devotion, resilient good temper, administrative skill, and ingenuity in finding solutions to unsolvable problems have had their impact on the work of every laboratory. I would like to express my gratitude to him, to those who work with him, and to the supporting staff throughout the program for their contributions to this important effort.

CLINICAL INVESTIGATIONS

It is now 10 years since the Director of Clinical Investigations assumed his duties and began recruitment of the staff whose current studies are reported in the following pages. It is an appropriate time to pause briefly, to consider our present situation in the light of early experiences, and to venture a guess as to the possible course of events in the near future. As I review the reports of the branch and laboratory chiefs, it seems to me that each of them has good reason to feel a sense of achievement and satisfaction in the development of his program. This is not to say that continuing problems do not exist. In every instance, however, there has been a substantial commitment to one or more major projects; in some cases important findings have already been made; in others, the progress of the work has been sufficiently promising to justify fully the long-term investment of time and effort.

But 10 years are a short time in the life of an institution; ours is an infant compared to the universities and institutes which have been the major bearers of tradition in scientific education and research. Now, by virtue of the great and continuing expansion of American science, we no longer enjoy the temporary security afforded by the unique position we held in early days. At that time, our resources for research were unparalleled; now support is available in many settings; questions are raised not only as to the relative advantages of these various facilities, but also as to the proper function of a large government research group in the structure of a new scientific community which was not even anticipated when the Government laboratories were planned.

At the inception of the Clinical Investigations program, two alternative modes of organization were considered. One would have called for the establishment of a highly flexible clinical facility in close association with a research institute. In such an arrangement, each of the research groups in the institute would have had as one focus of its interests, problems which derived from the actual clinical operation. Some of these problems would have been susceptible of generalization and might have been explored in depth in ways and places appropriate for their exploitation, but their relevance to the clinical situation would have remained ever in the foreground. There is one con-

sideration which even now would strongly favor such a plan. A national Institute which has responsibility for the stimulation and support of mental health research, for the training of personnel both for service and research, and for the organization of community services to meet the mental health needs of the nation should have available to it a mental health center both as a source of, and a testing ground for, some of its recommendations. It was felt that the Clinical Center did not lend itself to the development and support of such a program, however, and the idea was therefore rejected.

The second plan, which was the one actually followed, promised to fit more closely the patterns of research for which the Clinical Center was organized. The focus of this program would be that of making contributions to a general theory of behavior. In this case, the clinical situation as an example of maladaptive behavior might cast light on some of the forces which influenced behavior, but it would be only one of a number of approaches to the problem. There would be a broader representation of other social and behavioral science disciplines. Although it was expected that some contributions to psychiatric treatment might spring from our clinical studies, this program would emphasize basic investigations and would be only incidentally concerned with some of the applied studies which might have been a major segment of the first plan. It was hoped that this type of behavioral research institute in which many of the relevant disciplines were strongly represented, where investigators were freed from service and teaching burdens, would provide a favorable setting both for further advances in each discipline per se and also for fruitful collaborative studies when suitable research problems could be identified. The ultimate significance of such a program for the prevention of mental illness and for positive contributions to the improvement of mental health is obvious.

As one views the scope and variety of the investigations underway the question might well be raised as to why we have cast so wide a net. Certainly no one person in the program possesses the breadth of knowledge which would enable him to provide sophisticated and sensitive direction to all of the research groups. Would we have made greater progress had we channelled our resources into a smaller number of studies, each of which might have been pursued more intensively? Even with the advantage of 10 years' hindsight, I cannot answer that question to my own satisfaction. The total program was not constructed by selecting areas which seemed particularly promising for study. Rather, since it was felt that the most unusual feature of the National Institutes of Health was the opportunity afforded for interdisciplinary research, primary emphasis was placed on the recruitment of senior investigators who had a lively curiosity and a specific interest in pursuing such studies whenever suitable occasions should arise. These program chiefs were given the opportunity—or perhaps it would be more accurate to say that in order to recruit them it was necessary to afford them the privilege of choosing their associates and selecting the areas in which their research efforts would be spent. This overstates the case slightly, but it is important to emphasize the fact that we made our investment primarily in men and only secondarily in problem areas, feeling fully confident that this will ultimately prove the wiser course. A lengthy volume could be written about the events, decisions, and institutional structures which supported and those which impeded the development of the program. These matters assume added importance in these days when many new research centers are being founded, but this is not the time or place to discuss them. In any event, our decisions were not always reasoned and logical. As Einstein pointed out, "The great mistake of the 19th Century theorists was to believe that theory could be derived inductively from facts." We proceeded on the principle that even in science some allowance has to be made for art, and that senior investigators should be free to use their intuition in deciding how to approach problems and how to organize their laboratories. One cannot institutionalize or prescribe a posture for creativity.

Recruitment was a difficult task; it was 5 years before chiefs were appointed in each of the branches and laboratories. By that time, too, a new research facility was added to the program, the Clinical Neuropharmacology Research Center developed in collaboration with Saint Elizabeths Hospital. One major concern of this laboratory is the study of some of the new pharmacological agents both for their effects upon patients with different psychiatric disorders and as blocking or

facilitating agents in studies of nervous system function at a variety of levels. The more important goal, however, has been the establishment of a sophisticated, clinical group in close relationship with a group of basic scientists—a conception which closely resembles the one first considered as a possible pattern of organization of the entire Clinical Investigations program. The achievements to date and problems encountered in the development of the Center are thoughtfully reviewed in Dr. Elkes' report on the following pages.

I believe it is fair to say that the program today is stronger than it has ever been. The fact that a number of former staff members have been appointed to important teaching and research posts is testimony to the high opinion in which their work is held. Those who never left and those who have returned have received numerous evidences of the esteem of their scientific colleagues; these have included lectureships, awards, editorships, and office in professional societies. It is a rare conference on psychiatric research to which several members of the staff are not invited. Most important of all is the impact some of our studies have had on other investigations in their particular fields.

It appears also that the pattern of organization has been successful in that more multidisciplinary and interdisciplinary studies are being undertaken. I hasten to say that this in and of itself is not regarded as a good and necessary goal to be followed by everyone on the staff. After all, the most important idea or discovery that may ever come from our program might very well come from a solitary worker in a lonely corner in our smallest laboratory. But it is the other types of investigations which we are almost uniquely equipped to support, and it is gratifying to see that many such have been undertaken. Contributions to a developmental psychology and to a definition of the biological correlates of behavior are gradually emerging as two strong unifying currents into which many of our projects fall. Another area which will probably assume increasing importance is that of cross-cultural studies; here we may examine the experiments of nature which are the only legitimate experiments available to us for the evaluation of certain psychosocial processes on personality and behavior. I believe that projects which fall into these categories could not be as efficiently mounted in a much smaller organization; that, for example, we could not have brought together in a small institution one anthropologist, one psychiatrist, and one psychologist with equal success. It has been the eternal plaint concerning interdisciplinary studies that they tend to degenerate, to take on the coloration of the dominant member's discipline and to operate at the level of the lowest common denominator. This does not occur nearly so readily when each member of the interdisciplinary team continues his close ties with his own laboratory and maintains his professional identity thereby. Much of the merit possessed by some of our studies on aging; on the biological aspects of schizophrenia; on the influence of family interaction patterns on the modes of perception and thinking of the individual members; and on the effects of certain biosocial factors on the emerging behavior patterns of the infant and preschool child springs from the fact that each member of the multidisciplinary team has held true to his own identity and, in so doing, has contributed what only he could bring to the synthesis achieved by the group. I believe that the results achieved to date and the progress of our current work warrant a degree of optimism about the future; we are steadily adding bits of information to our knowledge of how the nature and ordering of experience and which structures and biological processes determine the level of adaptation.

I have stressed the predominantly basic nature of our investigation and indicated my opinion that the decision to engage in and continue this program is an appropriate and fully justifiable one. It is another question, however, as to whether from the standpoint of the National Institute of Mental Health it is desirable to maintain an intramural program which is limited to such basic studies. I have long felt that the answer to this question is "No." As it grows larger and its influence spreads more widely than before, it becomes more important for the Institute to have a program of applied and operational research as well. There is a fundamental importance to the setting in which research is carried out. As the national center for mental health research, training and services, NIMH has an overview of problems in the field which can not be shared by university departments or local psychiatric hospitals whose functions are more parochial. We can expect a rise in pressures to study specific and urgent problems—pressures which will

come at least as much from inside as from without as the need for reliable information becomes more and more critical. Under such circumstances, it may seem natural to turn to the already large intramural research group and to press them for answers. Were this to occur, it would have an adverse effect upon our present program. Large though it is, it takes a critical mass of investigators in any one field to create and support an atmosphere favorable to the development of productive basic studies. We have brought together such a group. Its members were specifically recruited because they are largely devoted to the study of basic problems. An attempt to divert their energies by direction would destroy the foundations upon which the program is built. I feel that the time has come in the development of the National Institute of Mental Health for us to give serious thought to the establishment of another research program within the Institute, one which would be primarily concerned with clinical research in the broadest terms, in which all branches—extramural as well as intramural—might participate. In my opinion such an operation would be a unifying force within the Institute and would augment its leadership potential for improving the care and treatment of the mentally ill.

LABORATORY OF CLINICAL SCIENCE

The Laboratory of Clinical Science is concerned with the broad area of biological psychiatry and attempts to bring the basic biological sciences in closer juxtaposition to the problems and phenomena of psychiatry. Its senior staff consists of scientists who have won distinction in biochemistry, pharmacology or physiology and who, through medical training or experience, are cognizant of the unanswered problems of diagnosis, etiology, and therapy in psychiatry. The organization and direction of the Laboratory is based upon the thesis that the most effective means for achieving the acquisition of practical, useful knowledge in this area is through the creativity, dedication and resourcefulness of the individual scientist in an environment which permits those qualities to flourish.

The research activities of the Laboratory may arbitrarily be divided into three areas: basic biology, clinical biology, and psychiatry, although this is at best a loose and fluid division since the work of many of the investigators spans all three areas or moves freely from one to the other in the course of the development of a particular problem.

Basic Biological Research

The Metabolism, Storage, Release and Inactivation of the Catecholamines

These substances, primarily epinephrine and norepinephrine, occupy an important place in medicine and psychiatry as essential mediators in the sympathetic nervous system and important contributors to affective state. Over the past 4 years, the Laboratory has led a wave of widespread research activity in this area largely through the brilliant work of Dr. Julius Axelrod, Chief of the Section on Pharmacology, who discovered the important enzymatic mechanism for the inactivation of catecholamines, the enzyme (catechol-O-methyltransferase) responsible, and defined the major and minor metabolic pathways by which these important substances are degraded in the body. There is reason to believe that Axelrod's choice of that problem was determined not only by his longstanding interest in sympathomimetic amines and his knowledge of the state of the field, but also by the interest of the Laboratory in the possible relationship between catecholamine metabolism and schizophrenia. In the past year a number of significant contributions to knowledge in this area have emerged from the work of Axelrod, Kopin, and their collaborators. Biochemical, pharmacological and radio-autographic studies (Potter, Wolfe, and Richardson) have demonstrated more definitively than was hitherto possible the storage of norepinephrine in vesicles within sympathetic nerves. These vesicles have been partially isolated and their chemical and physical properties are under study. A number of drugs which affect sympathetic nervous activity or emotional state, including bretylium, guanethidine, reserpine, and the monoamine oxidase inhibitors, have been studied with respect to their actions on the release or inactivation of noradrenaline. The work of Kopin has quite clearly demonstrated a difference in the nature of the products of noradrenaline released by sympathetic nervous activity or by a drug such as reserpine. These observations have led to the concept that monoamine oxidase serves to destroy norepinephrine prior to its activation and thereby limits the tissue levels

of this catecholamine. Active free norepinephrine is inactivated by being rebound, O-methylated in the tissues, or washed into the circulation with subsequent O-methylation or rebinding. It would appear that normetanephrine excretion is an index of activation of norepinephrine while VMA excretion provides information about total synthesis or not necessarily activation of the catecholamine. release or norepinephrine (as by reserpine) but

Biological Transmethylation

This fundamental process, the requirements of which were defined for the first time by Cantoni, has a number of interesting implications for biological psychiatry. Axelrod has shown that it was involved as a principal mechanism for the enzymatic inactivation of the catecholamines, and studies by Pollin, Cardon and Kety have suggested the possibility that this process may have some role in schizophrenia. In the past year Axelrod has found an enzyme in rabbit lung which is capable of methylating a variety of amines. This enzyme can convert normally occurring compounds, such as serotonin and tryptamine, to psychotomimetic metabolites, form adrenaline from noradrenaline and methyldopamine from dopamine. It can also synthesize morphine and nicotine from the corresponding precursors. In collaboration with Märki and Witkop of NIAMD, the normal occurrence in mammalian tissues of the catecholamine, N-methyldopamine, was demonstrated for the first time.

S-adenosylmethionine is one of the major methyl donors in the body and crucial to most biological methylations. Baldessarini and Kopin have developed a method for assay of this key intermediate in body tissues. This is being applied to the study of the effects of methionine loading, toxins, drugs, dietary deficiencies, and other processes which may affect the tissue concentration of this methylating agent and may serve to modulate the processes of biological methylation. Studies such as these represent basic contributions in their own right, but also contribute to the evaluation of the possible role of biological transmethylation in schizophrenia.

Mechanism of Action of Thyroxine

The interest of Dr. Louis Sokoloff in this important problem stemmed from his earlier studies in which it was demonstrated that in patients with hyperthyroidism the brain did not participate in the general increase in metabolic rate. This led him to the hypothesis which, in collaboration with Seymour Kaufman of the Laboratory of Cellular Pharmacology, he has amply confirmed, that an important action of thyroxine lies in its ability to stimulate protein biosynthesis and turnover. During the past year his group has clearly established that the thyroxine effect is localized to one step in the sequence of reactions in protein biosynthesis, namely the transfer of soluble-ribonucleic-acid-bound amino acid to microsomal protein. They have furthermore demonstrated that the effect is not secondary to an effect on the generation of ATP, GTP, or reduced glutathione, cofactors which have been found to influence the transfer step. This step in itself probably includes a number of component reactions and preliminary results have suggested the possibility that the thyroxine effect on amino acid incorporation into protein is the result of an increased rate of stripping of synthesized protein from the microsomal template RNA. Such a mechanism would be compatible with a thyroxine stimulation of both protein synthesis and breakdown, and experiments are currently in progress designed to evaluate this possible mechanism more definitively.

The thyroxine effect is characterized by an absolute dependency on the presence of both mitochondria and an oxidizable substrate in the incubation system and involves a lag of some five minutes preceding the period of stimulation. The processes occurring during this lag period have been subjected to intensive study with the isolation of what appears to be a crucial intermediate substance. Isolation and identification of this factor is one of the immediate major goals of the program.

Studies of the thyroxine effect in brain preparations have also been expanded. It was previously shown that the rate of amino acid incorporation in preparations from adult rat brains was slow and inhibited by thyroxine while that of infant rat brains was high and stimulated by this agent. Recent studies have demonstrated that these differences between infant and adult brains are associated with their mitochondrial fractions. Surprisingly high amounts of amino acid incorporation into the mitochondrial fraction and, particularly, in preparation from infant brains, were observed. Since this fraction also contained myelin, studies are now being carried out to deter-

mine if the amino acid incorporation is actually in the myelin component and reflects myelin synthesis *in vitro*. The effect of thyroxine in stimulating protein synthesis and its differential action on infant as opposed to adult brain could account for the defective cerebral and intellectual development found in cretinism.

During the past year the scope of the thyroxine project has been widened to include studies related to the physiological actions of the hormone *in vivo*. It has been found that puromycin, an agent which inhibits protein biosynthesis by blocking the step which is stimulated by thyroxine, also blocks the calorogenic effect of thyroxine and reduces the metabolic rate of thyroxine-treated rats to the euthyroid level. These results are consistent with the hypothesis that the increased oxygen consumption in clinical hyperthyroidism may be secondary to the effect on protein synthesis.

Autosensitization Phenomena in the Central Nervous System

For a number of years Dr. Marian Kies and her associates in the Section on Biochemistry have been carrying out studies on the immunochemistry of brain, using as a model the phenomenon of experimental allergic encephalomyelitis, a demyelinative disease, like multiple sclerosis, which is precipitated in animals by the parenteral injection of appropriately treated brain tissue. The work in recent years has centered on the purification and characterization of the antigen present in brain tissue. In the past year modification in the myelin preparation, which was previously described by Laatsch and Kies, has resulted in a fraction completely soluble in chloroform methanol which still retains its lamellar structure on electron-microscopic examination. The high antigenic activity and protein content of this preparation suggest that the protein-lipid bonds which existed in whole tissue are still intact. Thus, the opportunity is available for identifying the specific lipid attachment of the antigen in myelin and studying the effect of this lipid on the antigenicity of the protein. A technique developed in this section has made possible considerable advance in the purification of the antigen and offers general usefulness in the fractionation and purification of tissue extracts which contain protein and lipids in stable combination.

In conjunction with Dr. Peter Mueller of the Psychosomatics Unit, further studies have been carried out defining the interesting protective action which is exerted by a vitamin C deficiency against the appearance of experimental allergic encephalomyelitis in guinea pigs. Studies are also under way on the components of Freund's adjuvant in the production of allergic encephalomyelitis. This material (a water in oil emulsion containing killed microbacteria) has been used empirically for years in immunological reactions. During the past year the section has examined the contribution of the individual adjuvant components to the disease induction and has obtained information suggesting that the physical state of the emulsion may be the most important factor in creating an effective vaccine. Gram negative bacteria may be substituted for mycobacteria and in some cases are as effective as the tubercle bacillus itself in possessing the adjuvant effect.

Biochemical Aspects of Membrane Function

These studies, conducted by Dr. Jack Durell of the Section on Psychiatry, are directed at the fundamental molecular processes involved in the production of nervous activity. Further information has been gained in the past year on the mechanism of chlorpromazine action on yeast hexokinase. In addition, the effect of acetylcholine on the incorporation of radioactive phosphate into the phospholipid of brain microsomes has been investigated. This effect has been related to ion transport and is therefore of marked interest. It has been confirmed that acetylcholine has a definite effect upon this process but the mechanism appears to be different from that suggested by previous investigators; this mechanism is now under investigation.

The Activity of Individual Cortical Neurons in Sleep and Waking

This section has devoted considerable effort to analysis of the activity of single neuron in the visual cortex during sleep and wakefulness. Various modifying conditions have been imposed upon the experiments, including photic stimulation and visual inspection or attention. Two forms of sleep have been studied, that which is associated with slow waves in the electroencephalogram and that

associated with low voltage fast activity. The latter type of sleep is of particular interest in view of the fact that it appears to be an analog of that type of sleep which in man is associated with dreams. These studies have revealed that under certain conditions there may occur alterations in total amount of neuronal activity within a particular area of cortex, but most interesting are the findings that under most conditions alterations in the state of wakefulness or sleep are associated with differences in the organization of activity within the neuronal population rather than with respect to its total amount. These findings support the earlier findings of other members of the Laboratory of a relatively uniform energy utilization by the human brain in sleep, wakefulness, and attention.

Clinical Biology

A substantial segment of the program of the Laboratory is concerned with the application of basic biological studies to man under normal conditions and in disease. In the past year the major effort has been biochemical and has represented the interests of a number of the members of the Laboratory in the catecholamines and in free fatty acids.

The Production, Metabolism, and Excretion of Catecholamines in Man

The important contributions by Axelrod, already alluded to in defining the metabolic pathways of adrenaline and noradrenaline, have made possible for the first time an approach to the measurement of the secretion of these important hormones by man under a variety of physiological and psychiatric conditions. Three major products which appear in the urine account for 80 to 90 percent of adrenaline and noradrenaline which are produced in the body and therefore some effort has been devoted to the development of suitable methods for the estimation of these metabolites and the applicaation of those methods to clinical problems. The development of an accurate, specific method for one of them (VMA), the major urinary metabolite of both catecholamines in man, by McDonals and Weise, has permitted evaluation of the effects of drugs on the excretion rate of this compound and a comparison of normal subjects with schizophrenic patients. The increase in this excretion produced by reserpine and the decrease with chloromazine are equally present in normal and schizophrenic subjects. Methods for the estimation of the major and minor derivatives of epinephrine and norepinephrine are currently being developed and improved by Kopin, LaBrosse, and Henkin, respectively. Such methods will be used to estimate the rates of production of norepinephrine and epinephrine in a variety of clinical states. Studies on the relative magnitudes of the pathways of metabolism of the catecholamines, using radioactive tracers in normal subjects, in patients with mental disorder, and in autonomic nervous system disease, are being continued and extended.

During the past year Dr. Elwood LaBrosse has carried out extensive studies of catecholamine metabolism in patients with neuroblastoma. In addition to metabolites which had been previously identified, his studies have revealed a significant excretion of 3-methoxylated amines. Examination of the tumor tissue itself revealed the presence of significant amounts of catechol-O-methyltransferase. This finding of an important catecholamine inactivating enzyme in the tumor itself suggests the mechanism to explain the presence of elevated urinary excretion of the O-methylated metabolites in this condition and the absence of comparable elevations of the free catecholamines or the hypertensive and other symptoms associated with their release. On the basis of this work it appears possible to differentiate this type of tumor from pheochromocytoma, in which the active catecholamines are discharged into the blood stream. Studies on the tumor tissue have also revealed the presence of other enzymes necessary for the synthesis and degradation of the natural sympathetic nervous system hormone, norepinephrine.

Changes in the Blood Levels of Free Fatty Acids (FFA) in Association With a Number of Clinical States

Drs. Mueller and Cardon of the Unit on Psychosomatics have conducted an extensive series of studies on these readily mobilized components of ingested and stored fat under a variety of physiological and pathological states. Among the affective states, anger appeared to be associated with significant changes in FFA levels, whereas depression and fear did not. Patients with clinical depression do not show a consistent pattern in blood FFA levels. The relationship of these sub-

stances to satiety and obesity are being actively investigated as well as the important question of the relationships between degree of unsaturation of dietary fat, hypertension, and atherosclerosis. The hypothesis that the atherogenic effect of high fat diet is the result of a greater tendency for fat mobilization by norepinephrine is not supported by their findings. During the process of breakdown of adipose tissue to provide the fatty acids, equivalent amounts of glycerol are also liberated. Both FFA and glycerol changes in blood have been measured during the past year in conditions of fasting and during glucose, epinephrine, or insulin administration in normal subjects. Data of this nature have not been previously obtained.

Clinical Physiology

In a joint study with Dr. Birren of the Laboratory of Psychology, Dr. Cardon has obtained evidence for a highly statistically significant difference in reaction time in man during different phases of the cardiac cycle. The chemical and neurogenic possibilities for explaining this phenomenon which may, in turn, throw some light upon factors which affect so fundamental a neural function as reaction time are being investigated.

Dr. Sokoloff, in conjunction with Dr. Feinberg of the Clinical Neuropharmacology Research Center, has initiated a study on cerebral blood flow in senile dementia with special emphasis upon the question of whether restriction in blood flow to the left hemisphere is more apt to result in mental deterioration in right-handed individuals. This represents an attempt to answer a question previously raised by the Section on Cerebral Metabolism in earlier studies in which such a possibility was suggested by data on cerebral blood flow in this condition. The present study requires the perfection of a technique for estimation of cerebral blood flow on each side of the brain by means of external counting. If such a development is successful, it will have usefulness to the broad field of cerebrovascular disease and diagnosis, in addition to contributing to an answer to the interesting relationship between cerebral dominance.

Psychiatry

The Laboratory has been aware of the unique nature of the phenomena which constitute psychiatry and the requirement for sophistication and sensitivity in the approach to these, not only from the point of view of eliciting the most meaningful correlations with biological observations but also as the basis for studies in their own right. The psychiatrists within the Laboratory have conducted and participated in various programs of biological research and, in addition, have conducted research at a behavioral, sociological, or purely psychiatric level.

Research in Schizophrenia

In the past year the Laboratory has continued its program of studies in the biological aspects of schizophrenia in an effort to discover and define significant and relevant variables at that level. Studies on the relationships between the catecholamines and schizophrenia, which initially stimulated the major effort of the Laboratory in the field of catecholamines, have continued. In the past year Dr. Cardon carried to completion an examination of the responsivity of blood pressure and blood glucose among schizophrenic patients to the infusion of norepinephrine. Differences from the normal in this regard were not as great as those which were previously found with norepinephrine. Studies on the excretion of VMA, a major urinary metabolite of the catecholamines, in schizophrenia and its response to reserpine and chlorpromazine did not reveal any significant differences from results obtained in normal controls.

The possibility of the presence of a characteristic serum factor in some schizophrenic patients, which has been suggested by a number of groups, is being evaluated by Drs. Libow and Durell. The question of physical fitness which has arisen as a possible explanation for differences which have been reported between schizophrenic and normal populations has been examined by Dr. Cardon with no evidence to date to suggest a significant reduction in physical fitness in the patients under examination.

Analysis was completed during the year of data obtained in a study of the effects of oxypertine—one of a family of tryptamine derivatives—on a population of chronic schizophrenic patients. The results indicate a moderate to significant improvement in two-thirds of the patients and, more important, a correlation between the response to oxypertine and a previous response by the same patients to the administration of tryptophan and a monoamine oxidase inhibitor which, in fact, had prompted the oxypertine study. These observa-

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tions, in addition to introducing another class of compounds into the group with ataractic effects, are of some relevance to the hypotheses which relate amino acid metabolism to certain forms of psychosis.

Toward the end of the year with his return to the Laboratory, Dr. Kety, in collaboration with Drs. Kopin and Durell and their associates, has reestablished the program in amino acids and schizophrenia with special emphasis on methionine and biological transmethylation. Findings initially reported from this Laboratory by Pollin, Cardon, and Kety in 1961 have been confirmed by three other groups. They are now being reexamined and extended with the purpose of elucidating the biochemical and psychological changes which accompany the administration of this amino acid.

The study of the siblings and family of schizophrenic individuals was continued by Dr. Pollin and his collaborators. A total of 35 families have now been studied. The goal is the achievement of three groups of 15 families each, schizophrenic, juvenile delinquent, and control, well matched by independent, external judges. During the year a beginning was also made upon the next and more definitive stage of this study in which the same approach in a more intensive manner will be employed in families in which there are identical twins discordant for schizophrenia. As the result of a nationwide screening for such pairs, 73 referrals have been made of which approximately one-third appear to offer considerable likelihood of meeting the rather rigorous criteria of the study. It is anticipated that the data obtained in the sibling and twin studies will be relevant not only to questions of etiology of schizophrenia and juvenile delinquency, but also pertinent to more basic issues of personality formation. Dr. Pollin will be continuing and extending these studies in the Laboratory of Adult Psychiatry, to which he moved during the current year.

Studies in the Acute Psychotic Episode

Under Drs. Durell and Kellam, a ward has been organized and operated for the treatment and study of patients with schizophrenia and related psychoses. The goal of this unit was the creation of a context suitable for the effective social, psychological, biochemical, physiological and psychiatric investigations of acute psychotic episodes. During the course of the first year of the operation of this unit, some new information was gained relating the regulation of thyroid function to fluctuations in the psychotic state. In addition, a large amount of data involving various physiological and psychophysiological measurements have been accumulated, and there has been some indication of change in phase with changes in psychosis.

Considerable data have been collected relating to social contact, ward events, and symptom fluctuations, which represent another major interest of that unit. Observations have included clinical course ratings for each patient daily, social contact measurements, and an evaluation of staff attitudes. The analysis of the data has partially begun and several tentative hypotheses have been generated.

Research in Aging

Dr. Robert Butler has completed this year a highly productive collaborative program which was largely focused on geriatric psychiatry. In the past year he finished the collaborative 5-year followup of an initial group of 47 community resident geriatric subjects who had been intensively studied in the Clinical Center 5 years before. Statistically significant correlations were found within this group between mortality and a number of physical, sociological, and psychiatric observations: clinical evidence of arteriosclerosis on initial examination; heavy cigarette smoking; low weight; psychiatric rating of relative poor level of adaptation; psychological ratings indicating relative absence of future goals and feelings of discontent on initial rating; and widowhood. It is hoped that continuing intensive case analysis of the followup studies will make it possible to evaluate the relative significance of the several factors which the above correlations suggest are related to mortality in this group.

Mental Retardation

In addition to studies of Sokoloff on the mechanism of action of thyroxine in the young and adult brain which have obvious implications for cretinism but have not as yet been extended to that condition, the Section on Biochemistry has made an interesting contribution to this field. A case of histidinemia has been described in which precocious puberty, chronically dislocating patel-

lae, hemivertebrae, mental retardation and speech defect occurred. Column chromatography of the patient's urine, following oral administration of C^{14}-labeled L-histidine, revealed a pattern of metabolite excretion which could be explained only by a deficit of the enzyme histadase. Resultant alterations of the other metabolic pathways of histidine were delineated in a study carried out in collaboration with Dr. Stanley Berlow of Marquette University. These studies were made possible by previous work in this section by Dr. Donald Brown in which the pathways for histidine metabolism in normal man were elucidated.

LABORATORY OF PSYCHOLOGY

This year the annual report of the Laboratory of Psychology has been organized by Sections. In some cases it deals with the specifics of individual research projects and in other cases with overall orientation. In general it can be seen that there is an increasing tendency for projects to extend beyond Sections and develop collaborative efforts with other units.

Office of the Chief

The Office of the Chief retains its twofold major interest: (1) the psychology of schizophrenia, (2) the nature of the therapeutic process.

A special effort has now begun to bring together a large body of experimental data on schizophrenia collected over many years into a series of monographs developing a theory of the pschology of schizophrenia centered around the concept of "segmental set." A detailed analysis of the body of experimental data already available, and of new data to be gathered on our wards here and at St. Elizabeths, will be carried out to test certain hypotheses as to the importance of difficulties arising particularly during the period of preparation for response in schizophrenics with a background indicating good or poor prognosis. Senescent and brain damaged subjects will be used for control purposes.

During the past year the principal effort on the study of heredity and environment in schizophrenia has been in the writing and editing of a book which is based on multidisciplinary studies of a family with monozygotic quadruplets concordant as to schizophrenia. A conceptual framework involving three theories for thinking about the etiology of schizophrenia is being proposed. The theories are tested point by point (using data from this study and others) with respect to various characteristics of the disorder, such as subtype, time and type of onset, severity, outcome, sepcial features of the illness, and premorbid personality characteristics.

The twins project became operational this past year. The first families were investigated to determine whether they fitted the essential criteria, and a few were brought to the NIH. Only one family has so far been included in the study but other potentially acceptable cases are already available. The project is based on monozygotic twin pairs discordant as to schizophrenia. It involves not only an investigation of the environmental factors which contribute to the discordance, but in a broader sense, the partialling out of variance contributed to various cognitive, affective, psychomotor, psychophysiological, motivational, and social responses by heredity, specific environments, and interactions between the two.

In addition, studies of cognitive processes in schizophrenia are being continued. A small number of chronically ill schizophrenics have been tested intensively with the Heuristic Evaluative Programmer. Some of their parents and some control subjects have also been tested. Preliminary reviews of the findings are highly encouraging with respect to illuminating characteristics of schizophrenic patients' logical thinking, problem-solving, and their educing and processing information.

The studies of preparatory set, psychophysiological responsivity and arousal in schizophrenic patients and normal controls are also being continued.

In earlier studies on the development and maintenance of preparatory sets in schizophrenic patients by the reaction time method, it was found that when trials with long and short preparatory intervals were given in an irregular manner to schizophrenic patients, a trial with a long (10 to 15 second) preparatory interval detrimentally affected subsequent trials with shorter preparatory intervals, but the impairment in normal subjects was much less or absent. This detriment may have resulted from the patient's different expectancies or their inability to shift from a set for a long interval to a set for a short interval, even when the short interval was expected. Experiments designed to test these alternative hypotheses

indicated that schizophrenics are both adversely affected by unexpected stimuli, and also have difficulty in shifting from a long to an expected short trial. Normal subjects seem to show less effect of shifting in this way.

Another hypothesis was that a trial or series of trials with a long preparatory interval may slow down reaction time on subsequent trials because it produces a change in the level of subjects' arousal. This hypothesis is being tested by recording several psychophysiological variables continuously while subjects are performing a reaction time task. An earlier study of the orienting response is being continued to investigate the effects of variations in the signal value of a stimulus on the psychophysiological responsivity of schizophrenic and normal subjects. This involves comparing responses to an auditory stimulus when: (1) nothing is required of the subject; (2) he is told to press a lever; (3) he responds as quickly as possible to a stimulus. The psychophysiological variables recorded are skin resistance, heart rate, respiration, and finger pulse volume. In addition, EEG, forehead pulse volume, skin temperature, and the mechanical force with which the reaction time key is depressed are sometimes measured.

A preliminary study has revealed a striking differences in the increase of psychophysiological responsivity with increases in the signal value of the stimulus, controls increasing markedly and schizophrenics showing no change from one condition to the next. Patients did not show the marked relaxtion over the course of the experiment shown by the controls. Patients depressed the reaction time key with greater force than controls, a finding which helps to explain the general slowness in reaction time in schizophrenia. These procedures are currently being applied to acute patients.

Also tested were several patients whose psychoses showed marked episodic features. Large changes in baseline levels and in measures of psychophysiological response correlated with changes in clinical condition. The nature of these changes depended on the individual case. The plan is to correlate these data with psychiatric, bichemical, and other physiological data to elucidate the mechanisms responsible for the behavioral fluctuations, as well as to study the effects of degree of pathology on the behavioral and autonomic tests.

A collaborative study of schizophrenic twins has been initiated. In addition to the aforementioned psychophysiological methods, autonomic conditioning and one or two reaction time tests are utilized.

The following areas of concern to research in psychotherapy have been examined: 1. The upper limits of health to which therapy may be pursued; a small number of subjects have been seen in therapy with an eye to making observations that are potentially relevant to this problem. 2. The physiological correlates of therapeutic change; it is expected in the near future to investigate various possible monitoring and recording techniques to find ones which may be feasible, relevant, and promising. 3. The effect of psychotropic drugs upon the basic processes of therapy, in particular upon the patient-therapist relationship and upon the patient's capacity of "experiencing." A film project called "The analysis of the psychotherapeutic process, particularly the psychoanalytic process" has been completed.

The investigation of patterns of body movement which differentiate moods or feeling states, and of the wide individual differences in patterns of body movements has been continued. By the use of filmed interviews with a series of young, normal subjects it has been possible to count movements for successive 30-second intervals, and to compare interindividual and intraindividual differences. Because of the limited usefulness of such counting from film records, there is now under development instrumentation whereby accelerometers are attached to subjects, and the outputs of these are used to control paper tape punches so that data can be fed directly to a computer.

In the study of judgment of effect from visual expression in brief motion picture excerpts, data have been obtained from a number of judges, including trained psychotherapists and professional dancers. Through the selection of 6-second excerpts in which the face and the rest of the body both had pleasant expression, others in which both had unpleasant expression, and still others in which the face was pleasant and the body unpleasant, it was found that judges tended to respond as if they were looking at the face only. Despite brief training in looking at body expression only, psychotherapist judges continued to view the whole excerpt in terms of the facial ex-

pression. When dancers were used as judges, however, they proved much more sensitive to body expression, and capable of distinguishing between inconsistent face and body expressions.

During the year members of the Laboratory staff made a comprehensive review of the needs for local electronic data processing facilities. The study showed that a computer available in the Laboratory would (1) be of great value in assisting the investigative staff to develop its competence in the use of computers; (2) provide the close contact between investigator and computer which is necessary in preliminary and exploratory experiments, where methods of analysis are in the process of development; and (3) make possible the on-line use of the computer in a number of experiments on problems of learning, psychophysiology, and social interaction. The study showed that all investigators were willing to learn computer programming. A review of available small computers was made and a request was submitted for the procurement of an IBM 1620 system.

Section on Early Development

During this year, though there have been several changes in the composition of the Section's staff, the general nature of the research programs continues very much as it has been, but with increased emphasis on studies of maternal and infant behavior in the subhuman mammal.

The investigation of exploratory behavior and the resulting conditioned behaviors in the infant and young child has been continued. The crib apparatus devised for this purpose has been utilized in two studies of the reinforcing properties of novel visual stimulation. In one study, infants between the ages of 3 and 6 months and older children, aged 2 to 5 years, were placed in an environment which they would manipulate to increase visual stimulation. The first part of the problem was to discover whether the rate of the exploratory response could be increased or decreased; the second and related part was to discover whether the stimulus was reinforcing.

In the older children conditioning of the response occurred very rapidly. Within one session fixed ratio control was established, and frequency curves characteristic of extinction were obtained when the stimulus did not appear. These results demonstrated that complex visual stimulation can function as a reinforcer for young children and that exploratory behavior can be increased.

In a second session, a week later ratio control was established more rapidly but the behavior was not maintained. The children left the task earlier. Their performance suggested that what was learned had been retained, but that a more powerful (more novel) reinforcer would be required to maintain their response at the level of the first session.

The results of a similar study of the young infant were not so clear cut. There is considerable evidence that the rate of response was altered by the stimulus, but performance was not maintained. With infants so young some other response or some other reinforcer may give better results. But it is also possible that the course of learning in the immature organism may differ from that in the mature organism. We have discovered, for example, that novel visual stimulation increases visual exploration at the expense of hand manipulation. The infant pauses to look and this in itself may disrupt other motor behavior.

In another study, the effect of novelty upon the child's choice of toys was investigated. The subjects were 3-, 4- and 5-year-olds. Three pairs of similar but not identical toys were used. The child was given opportunity to play with both toys of a pair, then with whichever one he preferred. Both were then offered again and the test was whether he chose to play with the one he had just played with or the other, presumably more novel, one. As a group the subjects chose the more novel one. Some of the youngest children, however, chose the more familiar one. Not only were they the youngest but they were also more often girls than boys, and most of them required the presence of their mothers during the testing. Therefore, age, sex, and dependency on mother are variables to be investigated in the future. May it be that awareness of the strangeness of a situation increases the likelihood that a familiar, rather than a novel toy will be chosen? The first and simplest step, however, will be to increase the amount of time the young child is given to play with the toys in the beginning, for the younger child may not satiate as quickly as the older.

Anticipatory mouth opening in the infant, and the effect of noncontingent reinforcement upon the

rate of the infant's social vocalizing, and on the effectiveness of the individual components comprising reinforcement are also being studied.

The wealth of data obtained in Israel, during the past 2 years, on the adaptive and social behaviors (smiles and vocalizations) of infants reared in four different types of environments is now being analyzed. The subjects were some 600 babies under 18 months of age, living in institutions, kibbutzim, 1-child middle-class families and multiple-child middle-class families. In one study smiles and vocalizations were recorded during a 12-minute period in which a woman observer (essentially a stranger to the child) looked at the infant with unchanging, unsmiling behavior, followed a half minute later by a 2-minute period of smiling and repeatedly speaking the child's name. Preliminary analyses indicate that smiling is most frequent at 20 weeks of age in the institution infants and at 16 weeks in the kibbutz and private home infants. Some infants smiled at every age, 4 weeks through 18 months. The private home infants smiled most, those living in institutions least. Vocalizations involving consonants were absent until about 6 months and the incidence then increased with age, most markedly in Kibbutz and private home infants. These findings indicate that there are some obvious differences in stimulus to these social behaviors afforded by different environments. A second study in this area is designed to investigate the specific stimulus properties of these environments. In the same four types of living conditions infants at ages 8, 16, 24 and 32 weeks were observed, each child by a trained observer for two half days under everyday conditions with continuous coded notations made on the infant's ongoing behaviors and the variety of stimuli provided by the environment. The data lend themselves to elaborate statistical analyses, which are now in process.

The work on assessing developmental status (mental, motor, and behavioral) in a 1½-hour standard testing situation, during the first 2 years of life has been continued. The current emphasis is on data collection for the ages 18, 21, 24, 27 and 30 months, and on cross-cultural comparisons of the three types of developmental scores. For the first 15 months, the test forms in use are those based on the normative standards derived from the analysis of 1,409 cases, 994 of which were supplied by the NINDB Collaborative project tested in 1958–60. The 18–30 month tentative norms are those derived at the University of California in 1961 on tests of 175 Berkeley children. Tests are now being accumulated on local children in the 18–30 month range, and for either or both first and second year tests on infants in Ohio, California, Hawaii, England, India and Israel. Most of these data are not yet available for analysis. In 1961 normative profiles by age were developed for the Infant Behavior Profile 1–30 months (emotions, activity, and attitudes, as observed in the standard testing situation). At present a detailed analysis of these behaviors is under way on 1,200 cases ages 1–15 months. The analyses include studies of possible behavioral differences, not only for age, but for race, sex, socioeconomic status, and motor and mental scores. Also intercorrelations and factor analyses of the items in the 12 behavior categories are being computed. Interpretations will be made when the information is available after data processing. There are preliminary indications that these ratings are reliable and meaningful and that they will add significantly to the evaluation of performance on the mental and motor scales in the early diagnosis of mental deficiency. They may also reveal at an early age stable reaction tendencies that are the precursors of later modes of response and adjustment.

The analysis and writing on several aspects of accumulated longitudinal (30-year) records of the Berkeley Growth Study has been continued. One of the most significant findings in this study is the accumulated evidence that the boys' school-age intelligence scores are most clearly related to maternal behaviors observed in the boys' first 3 years of life. The girls' school-age intelligence, on the other hand, is mostly independent of maternal behaviors and is more clearly correlated with indicators of parental ability. These findings, which appear to be supported by results from other published studies, have led to an hypothesis of genetic sex differences in the determiners of mental functioning, indicating the greater impact on boys of early significant experiences and the greater role for girls of genic determiners.

Another aspect of these longitudinal studies has dealt with physical growth and the prediction of adult height. One analysis on age means and annual increments in head circumference from birth through 25 years, provides age norms for a healthy sample, as well as evidence of a small puberal spurt

of growth in head size. Another, on prediction of adult height in the first 3 years of life suggests that, in general, the error in prediction at 2 years is about 2 percent. This error can be reduced somewhat by a formula that includes the heights of the child's parents. Two other studies done with several San Francisco endocrinologists indicate, by statistical treatment of pre- and post-treatment prediction of adult stature, that efforts to reduce the stature of tall girls by estogens or to increase the stature of short children by two anabolic agents, 17-Ethyl-19-Nortestosterone (Nilevar) or Methandrostenolone (Dianabol), are of little avail.

In another area of study an attempt has been made to construct theoretical models of parent behavior and child behavior, and to utilize these models to develop rating scales that will describe relevant behaviors and reliably characterize the persons rated by them. A previously devised inventory on the child's perception of his parents' behavior, given to normal and psychotic adults proved to be reliable. A factor analysis revealed a major factor of love vs. hostility, a factor of intrusiveness and control through guilt, and one of lax discipline and autonomy. Self-reports of adjustment on these same subjects showed maladjustment to be correlated with the love-hostility factor. Studies to test whether the child's perception of his parents' behavior is more important for the child's adjustment than the actual behavior of the parent are now being carried out.

A checklist for use by teachers, to evaluate school children's social and emotional behavior has been devised. This behavior checklist is made up of specific observable behaviors that were selected to represent the more general concepts of the theoretical model. These concepts, in turn, represent general factors that have been repeatedly isolated from personality data. The checklist as used by the teachers yielded reliable measures which were factored into two major dimensions: I. Verbal expressiveness and gregariousness as opposed to self-consciousness, submissiveness and withdrawal, and II. Cruelty, destructiveness and resentfulness as opposed to kindness, consideration and cooperativeness. This checklist should prove useful in mental health screening in the classroom.

In another approach to maternal behavior, a study is being made of maternal emotions during pregnancy, as they relate to aspects of labor and delivery and to postnatal status of the infant. For this, use is being made of an inventory which had been developed previously, of psychosomatic anxiety symptoms and of psychological reactions to pregnancy. This inventory is now being applied to a Dutch population whose scores on the inventory will be related to obstetrical records, findings of a neurological examination of the infant and behaviour observations of the infant in the first 10 days of life and possibly later. In an earlier study on U.S. women, pregnancy adjustment was found to be related to length of labor, and to lacerations during delivery of primiparae. It will be important to test these findings on a population that is culturally and geographically different. If the earlier findings were verified, and their relations to the offsprings significant, we will have further information on the importance of prenatal conditions for the normal development of the child.

Section on Perception and Learning

The collection of data for the studies on rat behavior at the Rockville Barn has been completed. These studies, begun in 1956, are concerned with the exploration of interactions among behavioral, physiological, social, and environmental factors in groups of rats reared under semi-natural conditions. Preliminary analyses have indicated two major trends: increasing group size leads to the social withdrawal of individuals, and a continuing elevated level of Vitamin A in the diet leads to deleterious consequences for the performance of complex behaviors such as sexual and maternal activities. During the past year the data have been tabulated and prepared for subsequent automatic machine-processing and much more detailed analysis. In addition, the rats have been sacrificed and made available to a number of collaborators for various pathological studies. This type of study is now being extended to a comparative survey of mammalian social groups with reference to the typical group-size of each species.

The studies of perceptual adaptation in normal human subjects have been continued. When one is exposed to an abnormally organized array of stimulation, the perceptual system gradually adapts to the new situation; when the normal organization of stimulation is restored, there is an aftereffect which decreases with time and presumably represents readaptation to the normal state

of affairs. The process of perceptual adaptation is at present understood to consist of a shift in the zero point and scale values of the basic dimensions of perceptual experience such as temperature, color, curvature of contours, etc. A special test of perceived velocity has been developed for the purpose of studying adaptation and aftereffect in the perception of motion. This test utilizes the response-time of the subject as a measure of perceived speed, and the subject himself is not aware of the occurrence of any adaptation or aftereffect.

Initial work with this procedure indicated that adaptation to a continuously moving pattern changes the scale values for perceived motions which are in the same direction as the adapting velocity but not for motions in the opposite direction. This finding is at variance with the fundamental notion of perceptual adaptation as a normalization process, at least with respect to the perception of motion, and it is of the utmost importance to determine the validity and generality of the method used. One experiment was undertaken to ascertain the precision and form of the function relating response-time to variations in stimulus-speed. The reciprocal of response-time was found to be a linear function of stimulus-speed, extrapolating through the zero-point, and the correlation between the two variables was extremely high. In another experiment the kind of adaptation pattern, duration of adaptation, and the time-intervals between adaptation and test velocities are being varied. Collection of these data has not yet been completed.

The same perceptual velocity test is being used with schizophrenic patients in a somewhat different application at the Clinical Neuropharmacology Research Center. It had been found previously that some schizophrenics develop extraordinarily long absolute response-times when tested repeatedly over a number of weeks, without manifesting a concomitant deterioration in relative accuracy. The increase in response-time appears to reflect a progressive change in the perception of time. Since some schizophrenics develop lengthened response-times, while others do not, it is pertinent to try to determine what other factors might differentially characterize those who do exhibit the effect. Through the auspices of the Mental Health Research Institute of the University of Michigan, it was possible to administer the perceptual velocity test to a group of 50 schizophrenic patients for whom a large number of psychological, physiological, and other measures are available. The results of this study very satisfactorily replicated the original findings with respect to perceived velocity, and a correlational analysis will be undertaken to see whether individual differences in this performance are associated with differences in the other measures.

An experiment assessing the effect of poor visual acuity on size-constancy judgments was completed. Size-constancy refers to the fact that perceived object-size remains approximately constant even though the angular size projected onto the retina varies inversely with object-distance. Accepted views of perceptual constancy would generally predict a positive correlation between acuity and constancy. One view, however, derives higher constancy in aging subjects from a failure of accommodation of the lens, implying a negative correlation between acuity and size-constancy. Some previous work on the effects of drugs questioned the assumption that visual acuity is related to size-constancy at all, except perhaps indirectly as a psychological factor affecting the subject's attitude or approach to the task.

Two groups of young adults were tested for visual acuity and their size-constancy judgments obtained under two different instructional conditions. Two groups of aged subjects were tested similarly, except that they were required to perform the tests once without their normal eyeglass corrections and again wearing their glasses. With the younger subjects there was complex interaction between acuity, size-constancy, and order of receiving instructions, indicating a psychological rather than strictly visual relationship between acuity and size-constancy performance. For the older subjects visual acuity was very much poorer without glasses, but size-constancy was not significantly affected. The chief difference between the age-groups was that the older subjects were much less differentially responsive to variation in the instructions. The conclusion from this study is that the perceptual capacity underlying size-constancy does not depend directly upon visual efficiency and does not deteriorate with age.

Section on Personality

The research pursued by this section during the year 1962 represents the continuation and extension of two major research programs: (1) crea-

tivity; and (2) formal (noncontent) aspects of speech.

1. Creativity

During the past few years our research in this area has focused on testing the Maltzman and Brainstorming techniques for increasing original and effective responses of subjects on open and closed-ended problem-solving tasks. This past year the program has been broadened to include a survey of a variety of variables, individual and environmental, which may affect creative performance. These include: cognitive and personality characteristics of leaders and members of three-man groups; effects of psychotherapy on productivity; and motivational factors influencing specific and generic learning.

A. Group Creativity Variables

Since many scientific investigations involve collaboration among members of different disciplines or subspecialties, it is important to determine the individual and group influences which act to enhance or detract from group creativity. In association with Dr. Fred Fiedler of the University of Illinois, a study was undertaken to relate creative performance of 60, 3-man groups to (1) the cognitive abilities and personality factors of group leaders and group members; (2) the nature of the group interaction; and (3) group performance under three stress conditions. Inasmuch as the analyses of some of the data have not been completed, those findings now available must be viewed as very tentative. There is evidence that characteristics of the leader, as measured by the "Least Preferred Colleague" technique developed by Fiedler, appear to influence the quality of the group product. It appears that the behavior of the group leader may be a better predictor of the quality of the group product than are measures of cognitive capacities of individuals comprising the group. The particular stress conditions utilized in the study do not appear to have exercised either a primary or secondary influence on the creative performance of the groups.

A variety of techniques for the analysis of the interaction of members of the group have been explored; however, none has been found to be fully satisfactory.

B. Effects of Psychotherapy on Productivity

Increasingly, the stress on "positive mental health" includes the assumption that effective psychotherapy may facilitate creative output and thereby enhance a person's potential contribution to his society. It is generally assumed that to the degree that the individual is freed from intrapsychic conflicts, he will have greater access to internal and external experiences and will be able therefore to utilize his energies in a creative rather than a stereotyped fashion. In order to assess the effects of various kinds and durations of psychotherapy on the productivity of behavioral scientists, a survey of a selected sample of phychologists has been initiated. To date a sample of approximately 1,400 individuals have been surveyed with an 81% return. The experimental group consists of individuals who have entered psychotherapy no sooner than 3 years after receiving their Ph. D. and have terminated psychotherapy no later than 1959. The control and experimental groups are matched for the socioeconomic status of parents, age, field of professional training, etc. The assessment of quantity and quality of "scientific productivity" is currently under way. No results are available.

C. Motivational Factors

1. Curiosity and Achievement. High curiosity and intense intrinsic interest in a topic appear to motivate the creative individual. It has frequently been suggested that the current educational system stresses the achievement of high grades as a motivation for learning rather than intrinsic curiosity. Therefore an attempt to assess the comparative effects of curiosity and achievement motivation on learning is being made. Within the framework of Atkinson's Motive-Expectancy-Value (MEV) theory of motivational arousal, we have also attempted to determine the combination of personality and situational factors affecting the strength of arousal of these two motivational dispositions.

The study had a number of specific aims. First, it sought to test the adequacy of the MEV model within each of two orientations toward the same learning material, a *test-achievement orientation* (assumed to activate motivation both to achieve

and to avoid failure) and a *curiosity orientation* (assumed to activate motivation to seek information). Second, it attempted to determine the effects of differential strength of motivational arousal in each condition on two aspects of learning: (1) acquisition of facts (rote learning) and (2) acquisition of general principles (comprehension). Third, it sought to compare the effects on rote learning and comprehension of strongly aroused achievement and information-seeking motivations.

It was found that the MEV model adequately predicted the performance of subjects in both the achievement and curiosity conditions. In the achievement condition, it was generally found that amongst Ss with a moderate expectancy of doing well (indexed by high past grade attainment) comprehension was, as predicted, a significant positive function of achievement motive strength and a significant negative function of fear of failure motive strength. For these same Ss rote learning, as expected, was not adversely affected by fear of failure but, contrary to expectation, was not facilitated by achievement motive strength (this latter finding perhaps being a function of the simplicity of the rote questions). Amongst Ss with a *low* expectancy of doing well (low past grade attainment), as predicted, neither comprehension nor rote performance were related either to fear of failure or achievement motive strength. An unanticipated but reasonable finding involved the overall superiority on both comprehension and rote learning of high over low "grade-attainment" Ss. If grades are regarded as an index of the incentive value of academic achievement per se as well as of expectancy, then this result would not be inconsistent with the MEV model.

In the curiosity condition (where no attempt was made to measure curiosity motive strength), it was found, as predicted, that both comprehension and rote learning were a positive function of (1) expectancy of understanding the learning material and (2) degree of involvement or interest in the area covered by the material. Only those individuals who had a strong interest in the area *together with* a high expectancy attained an adequate level of comprehension and rote learning. Those individuals with high interest alone or high expectancy alone apparently exposed themselves as minimally to the material (with resulting failure to acquire facts and to comprehend) as those who were neither involved nor expectant of understanding.

In comparing the influence of curiosity and achievement motivation, it had been predicted that those subjects with strongly aroused information-seeking motivation would learn more comprehensively and with less emphasis on rote learning than those with strongly aroused achievement motivation (provided that fear of failure was controlled). The results did not support this prediction for both groups performed equally well on both comprehension and rote learning. This finding is open to such alternative explanations as (1) the information-seeking motivation may not have been generated at as high a pitch as the achievement motivation in this study, or (2) rote memorization has ceased to be rewarded in the public school tested.

The above findings are based exclusively on a male sample of high school students since the validity of the personality measures used for females appears to be very doubtful. An overall comparison of males and females, however, indicated that while the females were significantly superior on rote learning, the males were superior on comprehension in both experimental conditions. This finding raises the question whether the frequently reported observation that girls are academically superior to boys at almost all grade levels may be due to the fact that such school performance reflects higher facility with rote memorization.

2. Arousal and Habituation. The question of arousal and curiosity motivation is also being investigated. Its point of departure was the theories proposed by Berlyne and Sokolov to explain the arousing effects of novel or unexpected stimulation, and the habituation of arousal (or orienting reactivity) in the absence of novelty. This study was aimed both at hypothesis-testing and broadening the range of considerations usually brought to bear on the occurrence and maintenance of habituation, which has been experimentally approached in one fashion only; namely, the repetitious, physically exact duplication from trial to trial of a nonreinforced stimulus or stimulus pattern. In the present experimental paradigm, each trial involved the presentation of physically novel stimulation; namely, numbers presented seriatim (1, 2, 3. 4, etc.). It was assumed that with the adult

human subject, each successive presentation would shortly become highly predictable or expected.

Constriction in finger blood vessels, detected with a photoelectric plethysmograph, was used as the indicator-measure of arousal or orienting. Twelve of twenty subjects run in the experiment manifested reaction patterns indicating that selective habituation had occurred to physically novel but expected stimulus presentations, i.e., nonreactivity (habituation) was abruptly terminated upon presentation of an out-of-sequence (unexpected) number. These results were interpreted on the one hand, as suggesting a complexity in brain events required to mediate habituation beyond that which Sokolov has hypothesized, on the other, as providing support for such theories as Berlyne's on the arousal value of surprising or unexpected stimuli and Osgood's on the nature of thought-process "predictive integration."

II. Formal Characteristics of Speech

Heretofore the underlying purpose of this research has been to study the shifts in noncontent speech variables as a function of changes in affective states of the speaker. This focus has now been broadened to include the study of interrelationships between speech and thinking, as the latter may be influenced by a number of factors of which affect is but one. The previous identification of three categories of speech disturbances—filled pause, editorial correction, and articulation error—was fruitfully applied in a study of the relationship between speech disturbance and body movement. It was found that with a sample of 39 speech excerpts, the amount of body movement correlated highest with the filled pause category ($.41$ $p < .01$); somewhat less with editorial correction ($.32$ $p < .05$) and insignificantly with articulation error ($.24$).

A second variety of nonfluency, the hesitation pause, was investigated. A psychophysical study of threshold for pause discrimination revealed that pauses following junctions—natural division points corresponding roughly to "phrase" boundaries—have a significantly higher threshold than pauses which occur within such phrases. On the basis of these findings, differential linguistic functions have been proposed for these two kinds of pauses.

A preliminary study which attempted to assess the differentiation and stereotypy of speech indicated that the so-called "function words" (and, if, or, etc.) represent a fairly constant proportion of words spoken. Variation from sample to sample, if any, is confined to shifts in the relative proportion of subclasses of lexical items—nouns, verbs, adverbs, etc. During the past year a larger sample of data has been subjected to computer analysis. In collaboration with Dr. Samuel Greenhouse, these new data will provide the empirical base for an attempt to develop a precise mathematical model of the distributional characteristics of speech samples.

Section on Neuropsychology

I. Perceptual Functions of Posterior Association Cortex

Vision

An experiment initiated in collaboration with Dr. George Ettlinger of Queens Square Hospital, London, attempts to determine which cortical area is responsible for recovery of visual discrimination learning after the focal area for this function (the inferotemporal area) has been damaged. Preliminary results suggest that the prestriate area is mainly responsible for the recovery, a finding which seems consistent with an earlier conclusion that the prestriate area serves as a relay between the visual system and the inferotemporal cortex, and thus may assume inferotemporal functions, though less efficiently, when the inferotemporal area has been removed.

Experiments involving visual preference and visual incentive are continuing in an attempt to study visual perception uncontaminated by associative learning factors. Should a reliable technique be found, it will then be used to investigate the effects of posterior cortical lesions, and thereby help to answer the question as to whether postoperative visual discrimination deficits are due to disturbances of perception or of associative learning.

It is possible that this question may be more easily answered using human subjects. Accordingly a project has been undertaken to analyze the defects produced by right and left temporal lobe removals in man. Approximately 80 patients are to be examined on a variety of tests which demand little or no associative learning or memory. Should these subjects show impairments it would

strengthen the impression derived from studies in monkeys that deficits following temporal-lobe damage are related to perceptual functions.

Still another approach to these problems is being developed through the use of prisms which distort visual input. It is known that the process of learning to coordinate motor movements with the experimentally transformed visual environment is analogous to that used by infants in acquiring mastery of visually guided movement. The use of prisms thus provides a method for studying the processes of visual learning in relation to brain functions. Accordingly a study is being undertaken to compare the rate of mastery of adaptation to distorted images in monkeys with lesions in different parts of the visual system.

Olfaction

After several unsuccessful experiments, a clear-cut impairment in olfactory discrimination has been obtained in a monkey with a bilateral anterior temporal lobectomy. Replication of this finding will be attempted; if successful, this will lead to a study of the effects on olfaction of selective anterior temporal lesions, i.e., a search for an "associative" area serving olfaction comparable to the one serving vision.

Audition

An "associative" area serving audition seems to be located in the superior temporal convolution, adjacent to the primary acoustic area. However, auditory impairments produced by lesions here have been found to be similar to auditory impairments produced by dorsolateral frontal lesions. In an attempt to dissociate these deficits, a test of auditory discrimination thresholds is now being designed. Results from other studies suggest that once animals with frontal lesions have been retrained on the easy form of this task it will be found that their thresholds are unimpaired. This deficit should therefore be dissociable from a true auditory deficit, expected after superior temporal lesions.

Somesthesis

The principal aim of these studies is to find the locus of the lesion which will disrupt the memory trace for previously learned tactual discriminative habits. The method is to train one hand on these habits, remove a portion of the contralateral ("trained") hemisphere, and test for retention with the untrained hand. Since the untrained hand presumably has no sensory or motor impairment, failure to transfer may be ascribed to loss of the memory trace. Two types of lesion are studied: sensorimotore (premotor, motor, postcentral, and posterior parietal) and nonsensorimotor (all cortex exclusive of the sensorimotor region, as defined above). Results so far indicate that normal monkeys and those with sensorimotor lesions show good transfer of the habits to the untrained hand. Nonsensorimotor lesions appear to produce deficit, but the nature and extent of such deficit require further study.

A second aim is to find out whether sensorimotor or nonsensorimotor lesions affect initial learning of tactual habits with the *ipsilateral hand.* The results show that sensorimotor lesions produce deficit in learning difficult form discriminations, whereas nonsensorimotor lesions do not impair tactual learning. Whether this deficit results from sensory impairment is being studied by determining sensory thresholds.

A third aim is to study the effects of sensorimotor and nonsensorimotor lesions on the *contralateral hand.* Surprisingly, a monkey with a nonsensorimotor lesion who had learned all discriminations at a normal rate with his ipsilateral hand not only showed no transfer to the contralateral hand, but a severe deficit in reacquiring these habits. Other monkeys with this lesion will be studied.

The effects of lesions on tactual functions is also being studied in man. Seven patients admitted to the Clinical Center for surgical therapy of Parkinson's disease (lesions of n. ventralis lateralis) have been tested pre- and post-operatively. This nucleus is the origin of the major afferent system to the motor cortex, a region which may subserve somatosensory functions as well. Quantitative tests of punctate tactual 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 impairment. Preliminary results indicate elevation of thresholds in some patients but not in all. Effort will be made to discover characteristics of the lesion, or of the patient, which correlate with impairment.

II. Problem-Solving Functions of Frontal Association Cortex

Delayed-response type deficits occur after lesions not only in prefrontal cortex but also after lesions in such subcortical structures as caudate nucleus and hippocampus. It thus appears that dorsolateral frontal cortex, caudate nucleus and hippocampus may be part of one system. Recent anatomical findings lend support to this conclusion and suggest further that orbital frontal cortex, medial sector of dorsomedial nucleus, and amygdala may be part of another system. A study has therefore been designed to test this possibility by comparing the effects of combined lesions in the caudate nucleus and hippocampus with combined lesions in dorsomedial nucleus and amygdala.

Additional evidence has been gathered in the last year suggesting that perseverative behavior is more a consequence of orbital frontal lesions than of dorsolateral frontal lesions. The new experiments involved spatial discrimination reversal, object discrimination reversal and one-trial learning tests. These data thus appear to contradict an earlier view that the delayed-response deficit after dorsolateral lesions is due to abnormal perseverative tendencies.

The notion that an increase in perseverative behavior follows frontal lesions arose from the observation in many studies that frontal animals failed to suppress responding when responding was no longer appropriate. However, if the perseverative notion is correct, frontal animals should also perseverate not-responding if this has been previously dominant in the animals' repertoire. Accordingly a study has been undertaken in which before operation the great majority of reinforcements are given for not-responding in the test situation. The prediction is that after orbital frontal lesions this tendency will predominate, and be perseverated even though it is no onger appropriate. If the prediction is confirmed not only will support be given to the explanation of the effects of orbital frontal lesions in terms of perseveration, but it will rule out an alternative interpretation that frontal lesions result in a loss of response inhibition.

III. Emotional-Motivational Functions of the Limbic System. Anatomical and Functional Organization of the Forebrain Alimentary System

In this study the concept that mammalian hunger-satiety mechanisms are critically dependent on discrete, mutually antagonistic centers in the hypothalamus was investigated. Although analysis of the data is still proceeding, some tentative conclusions are possible. Food and water intake in sated animals have been elicited not only from hypothalamic placements but from extrahypothalamic placements as well. The effective loci included in addition to lateral hypothalamus, posterior orbitofrontal cortex, olfactory tubercle, substantia innominata, diagonal band of Broca, amygdala septum, preoptic area, and anterior cingulate gyrus. Food ejection without gagging in hungry animals also has been evoked from both hypothalamic and from extrahypothalamic placements. The effective loci include posterior orbitofrontal cortex, olfactory tubercle, substantia innominata, diagonal band of Broca, amygdala, preoptic area, and lateral hypothalamus. Gagging and vomiting also have been evoked. The effective loci for these include n. ventralis anterior, lateral septal nucleus, and amygdala. These findings suggest that the neural substrate for ingestion, ejection, and vomiting is distributed over extensive areas of the primate forebrain. Motivational and autonomic correlates of the evoked alimentary behaviors were also studied. It was found that evoked intake patterns were associated with increases in heart rate and with reward as measured by self-stimulation. Ejection and vomiting, on the other hand, were associated about equally with increases and decreases of heart rate but only about half of the ejection points were rewarding. The evidence suggests that self-stimulation, rather than being associated with parasympathetic activity, as has been proposed in the literature, is associated with activation of the sympathetic nervous system.

Interaction Study

Electrodes were implanted in four animals and the behavior which could be evoked from each

point was observed. It was then determined whether or not the monkey would press a bar to selfstimulate or to escape from stimulation through each electrode. The behavioral effect of stimulating through two electrodes simultaneously was also studied. These measures were compared with autonomic effects of the same simultaneous stimulations. It is expected that the method of studying the interaction between effects will lead to an understanding of the relationship between somatic, autonomic and motivational aspects of certain given behavior patterns. Early results suggest that there is no simple pattern in the effects of interaction; rather a variety of complex effects are obtained which depend not only on the motivational systems involved but also on intensity and timing of the electrical stimuli.

Another aspect of these interaction studies was undertaken in Switzerland in collaboration with investigators there. Using the Hess method of implanted electrodes, a study was made of the distribution of points within the hypothalamus from which defensive and escape behavior could be evoked in the cat. In general the earlier findings with respect to the localization of these functions were confirmed but it became apparent that there is much more overlap of the areas subserving these two functions than had been suspected. With respect to the effects of simultaneously stimulating both the defensive and the flight areas it was found that as a rule there is facilitation. However, which particular behavioral components will be facilitated depends on relative intensities as well as the time at which one or the other component is initiated.

Intensity Study

This study was undertaken to clarify the distinction, if any, between the motivation systems as defined by self-stimulation and escape from brain stimulation and the behavior patterns evoked by the same stimulation. Data so far deals only with self-stimulation. The effect of varying intensities of stimulating current on rates of self-stimulation has been obtained from 38 electrodes in 4 animals. The shapes of the curves derived from this data where then compared to the shape of the curve denoting changes in the intensity of the evoked behaviors as the current varies. Preliminary analysis suggests that for certain behaviors the behavior and self-stimulation curves intersect in such a manner that the slopes are of different sign. Thus it is probable that the neural systems involved in behavior and reward are not identical but are independent to some degree.

IV. Alerting Functions of the Reticular Activating System

This area of investigation was terminated when Dr. A. F. Mirsky left NIMH. Although the data analysis of this material continues, some of it is far enough along to permit some conclusions. In one study of this series the behavioral and electrophysiological effects of high and low dosages of secobarbital on normal subjects during tasks requiring sustained visual attention was observed. The pattern of changes found in the EEG, finger pulse and respiration records indicate that the physiological condition of the subject during the period when the majority of errors occurred is not the same as that found in previous studies with chlorpromazine administration and sleep deprivation. A pattern of low voltage fast EEG activity was found to accompany errors under the barbiturate condition. This EEG pattern has, paradoxically, been associated with both relatively deep sleep, lowered vigilance preceding sleep, and arousal. Correct responses, on the other hand, are accompanied by high voltage alphalike waves, a pattern more usually associated with the absence of visual attention. These findings suggest that the maintenance of visual attention as measured by CPT performance is not necessarily accompanied by the physiological changes usually associated with wakefulness and that, therefore, separate neural systems may be involved.

Section on Aging

The Section on Aging has been organized to carry out research on age changes in behavioral capacities. As a naturally occurring phenomenon, aging is a product of biological and environmental circumstances. The research is directed at obtaining and developing experimental conditions under which the more important implicated processes can be manipulated. The Section has two units of special emphasis: the Unit on Higher Cognitive Processes, and the Unit on Psychophysiology.

Unit on Psychophysiology

This unit has continued research on the nature and basis of the slowing of psychomotor responses with increased age. A collaborative study with the Federal Aviation Agency was reported which correlated psychological and vascular functions in a relatively healthy group of 161 men between the ages of 23 and 60 years. The obtained correlations were higher between the psychological measurements and age than between age and the physiological measurements. The results make it difficult to accept an explanation that over this age range the vascular changes lead to a group of behavior changes typified as a slowing in psychomotor speed, despite the fact that other evidence has been gathered that both cardiac disease and hypertension are associated with significantly greater slowing than expected for age alone. One of the possible interpretations is that the behavioral changes are early manifestations of a common factor of altered nervous control. Further efforts are being made to study possible elementary psychophysiological bases of the slowing of behavior as well as to its consequences for complex behavior.

One hypothesis suggested by the previous work was that the level of activity of the sympathetic nervous system was inversely related to speed of discrimination. To test this hypothesis it was thought necessary to relate measures of sympathetic activity and reaction time in the same individual. One method of study adopted was to compare variations in reaction time as a function of the phase of the cardiac cycle in which the stimuli are presented. Auditory stimuli were presented to subjects who had to respond by raising a finger as quickly as possible. Simultaneous electrocardiograph records enabled the investigators to analyze response speed in terms of the phase of the EKG record in which the individual stimuli were presented. Results obtained on over 50 normal subjects, using 100 stimulus presentations for each, have shown a significant relation between response speed and the phase of the cardiac cycle in which the stimulus is presented. These results provide evidence that to some extent excitability of the brain may vary from moment to moment in phase with the cardiac cycle. The relationship of increased sympathetic activity to psychomotor retardation appears to be an important topic to pursue.

Also within this Unit, three studies of human aging were completed this year; two of set in perceptual behavior and one of set in relation to reaction time (RT). The RT set study tested 4 hypotheses: with advanced age more time is required to (1) develop a state of optimum set, or to program a response; (2) overcome the effects of inaccurate, overestimation of the time available to organize response; (3) respond in general, and aspects of set are not independent of the overall slowing processes; and (4) disengage attention from prior involvement with the signal that prepared the responder for the oncoming stimulus. In this experiment 32 men and 32 women aged 18–27 years, and 24 men and 12 women aged 61–80 years were individually tested with 300 RTs which included 6 separate series of 5 preparatory intervals in each series. For men, the data did not support hypotheses 1, 3, or 4. Hypothesis 2 was found tenable. Women followed a different pattern in that old and young were more nearly alike. It was suggested that the interaction between age and sex in relation to RT is an area needing elucidation.

The data of the two perception studies in relation to age demonstrated the role that instructional sets may play. In one study it was indicated that given appropriate, structured sets, rather than opportunity to develop sets older subjects may perform with minimum difficulty. As uncertainty is introduced, older subjects may find the task relatively more difficult than will younger subjects.

The study in press reported limens for old and for young subjects under different instructional sets for perceiving critical flicker fusion and apparent motion. Differences between age groups in the correlations between limens suggested an interaction between age and instructional set.

Unit on Higher Cognitive Processes

This unit has continued its effort to identify, analyze and evaluate deficits of the aging indivdual in an area of behavior that requires the exercise of "complex mental processes." For this purpose it is developing a Heuristic Evaluation and Problem Programming device (HEPP) which presents on extensive series of logical problems to a subject and records on punched tape all of the actions that he takes relevant to his effort to solve them. The

subject's reaction panel consists of a set of 12 display lights that he can turn on or leave off and an evaluation button that indicates how the machine classified a given on-off selection. He is free to choose any display and to have it evaluated; every selection is classified as either Green or Red by the machine through its logical controls. It is always the subject's task to discover, by interrogating the machine, through his selection and evaluation operations, just what condition HEPP is placing on a display in order for it to be classified Green. Preliminary work with this device has been completed. In order to estimate the range of abilities that is likely to be encountered in the type of population usually sampled in a Mental Health project, the following groups of subjects were tested during the current year: nine aged people, nine schizophrenics, three sets of normal parents of schizophrenics, and six young controls. The schizophrenics were tested for about 10 hours each, the other subjects for about 5. An IBM–1620 program has been designed to evaluate some of these performances.

The results obtained and the analyses performed during the current year support last year's tentative conclusion that HEPP is more flexible than LAD and much more adapted to the capacities of our subjects. Most schizophrenics and all oldsters were able to work with rewarding success at the two lowest HEPP-levels of difficulty and several of the aged subjects were able to achieve success at the moderately high Level. Though well below the ceiling of HEPP this class of problem presents considerable difficulty for the average young normal. Two distinctive heuristic deficits were apparent in our aged subjects: (a) an extreme difficulty in holding a well defined and clearly understood goal-direction when required to shift between two different sets in an irregular order from problem to problem, (b) a surprising inability to invent rather obvious techniques for reducing cognitive strain when increasingly complex problems created a need for such techniques. Both of these findings, though considerably more specific, are in agreement with our earlier conclusions from performances on LAD. The prominent peculiarities of the schizophrenic's performance are (1) an apparent inability to perceive regularities in the relations between external events, (2) a preference for direct but inefficient approaches over indirect but effective ones, (3) a marked tendency to employ risky techniques that depend on "luck" rather than slightly more elaborate procedures that guarantee success.

Animal Studies

The general proposition that advancing age tends to be associated with increased difficulty in modifying ongoing behavior, has been investigated in many studies of human subjects. To test the phylogenetic generality of this proposition, three rat studies were undertaken which were completed this year.

In one study, Sprague-Dawley rats were trained to either a right or a left position response in a single unit Y-maze. Modifiability was tested by performance on the successive reversals of the position response in relation to the performance on the original position learning. Rats of three age groups at the start of the study were old (approximately 2 years), middle-aged (approximately 10 months), and pubescent or post pubescent (approximately 3 months). Motivation for food reward was 23 hours of food deprivation. Old rats were statistically poorer than younger rats in both the first and second reversal tasks, but not in original position learning or in the third and fourth reversal tasks. These data suggest that advanced age is associated with difficulty in modifying behavior, but this difficulty is overcome with continued experience in reversing. This conclusion, however, was made equivocal by further analysis of the data and by similar data collected in a different way.

When age groups were matched by individual performances on original learning, the age differences in reversal performance were no longer found statistically significant, although mean and median performances were still poorer for the old rats. When the study was repeated by making the food reward larger and by reducing the time spent in the goal box, age differences were not found.

A second study was carried out with the hypothesis that age decrements in reversal behavior would be increased if the task were made more difficult. A serial four-choice maze was used with Sprague-Dawley rats of ages similar to those

above. Old rats were found to be poorer in performing the maze than younger ones, but age decrements in reversal performance were not found.

A third study testing the same proposition with the rat, involved extinction of food-reinforced operant. Rapid extinction was taken as ability to modify behavior, whereas resistance to extinction was taken as an index behavioral "rigidity." Rats were of five age groups: 4, 6, 14, 22, and 27.5 months. Statistically significant age differences were not found. However, the age trend was in the hypothesized direction and it may be that the natural age decline in general speed of response counteracted the age trend of increased rate of response during extinction.

The project on Learning and Transfer of Training has been continued as an operant conditioning study using light aversion as the drive. It is the purpose of the current series of experiments to evaluate light aversive activity as a behavioral indicator of physiologic damage from detrimental treatments such as drug injection of X-irradiation. Terminal examination of the specimens in such studies usually should precede death but yet be delayed until the experimental agent has produced a detectable effect.

In a preliminary study of this type, 12 rats (9 mos.) were tested for half an hour a day for 4 weeks during which they were X-irradiated once a week, 6 animals with 100r and 6 with 300r. Lever pressing activity stopped almost completely on the day before death but continued at a pre-irradiation rate up to that time. Thus light aversive activity was found to be a reliable and precise indicator of impeding mortality. It is now planned to extend the test population and to employ contrasted age groups for a comparison of the effects of 100, 300, 600, and 900r on light aversive activity.

In another set of observations undertaken with a similar purpose, the same drive was studied in 20 rats during the development of tolerance to the following dose-increment schedule of morphine: 15, 30, 45, 60, 80 and 100 mg/kg. At each dose-increment time, activity was depressed for two or more days and then increased, as a given dose was continued, so that recovery of near-normal activity could be taken as an indicator of the development of tolerance. The cogency of this exploratory study was somewhat attentuated by the need for forming a number of subgroups to provide information on several possibly significant variables. Thus by testing separate groups at 0.5, 1.5 and 2.5 hours after injection, it was found that the most effective time to test is about 1 hour after treatment. In another group, by administering saline placebos periodically, it was shown that mere injection of the standard mass did not disturb performance. Still other animals were tested throughout the 4-month observation period without any treatment in order to establish norms of performance over this exceptionally long test interval. These control animals showed no decrement in the effectiveness of the light aversion drive. It is proposed to continue this work during the present year in order to (1) increase the population size and (2) investigate the development of dependence as well as the development of tolerance.

In a collaborative study with the Clinical Pharmacology and Experimental Therapeutics Service, NCI, it was demonstrated that the cerebrospinal fluid/plasma distribution ratio for thiocyanate was a function of the plasma thiocyanate level. The ratio was further elevated by the simultaneous administration of bromide, and it was shown that the effect was independent of the turnover rate of the cerebrospinal fluid. The intracisternal administration of hypertonic solutions reversibly inactivated the blood-cerebrospinal fluid barrier, temporal restoration of function depending upon the molarity of the solutions employed.

Almost completed is a collaborative study with the Clinical Neuropathology Section, NINDB, in which it was determined that the cerebral edema produced by cold injury to the cortex is due to an expansion of the extracellular compartment of the brain. It had previously been shown in the Section on Aging that cerebral edema produced by interference with cerebral metabolism (tin poisoning) did not result from enlargement of the extracellular space, but probably originated from cellular swelling.

The variety of the studies in progress within the Section on Aging creates an impression of diversity that should be interpreted in relation to the particular structure of this field of investigation. As an area of scientific inquiry, aging, in addition to its rapidly growing core of theory, contains numerous beliefs and/or facts concerning

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the phenomena of aging that cannot be systematized in the present state of the discipline. The Section's animal studies are usually designed to test the generality of particular practical judgments in the area and to attempt to systematize them, at least, to the extent of identifying the physiological mechanisms through which they operate. Thus, the experiments with morphine derive the well accepted belief that aged patients are hypersensitive to morphine and consequently should be denied its analgesic action except in cases of extreme urgency. The study on thermoregulation also possesses obvious roots in common observation. The work with human subjects, on the other hand, tends to be limited to the more theoretically oriented study of age-related changes in centrally controlled behavior, psychomotor and cognitive, which may well be explained by a slowing and/or dissynchrony of nerve impulses.

LABORATORY OF SOCIO-ENVIRONMENTAL STUDIES

The Laboratory, this year as before, has been pursuing a broad range of studies on the effects of social structure upon personality and behavior. There have, however, been some shifts in emphasis. We continue, to concentrate much of our efforts on studies of the family, with a further increase in cross-national comparative research. But there has been a shift of attention away from studies of the mental hospital. And we have begun new research on the social psychological consequences of job, occupation, and career.

This Laboratory is characterized by considerable diversity—in the disciplinary identifications of its members, in the theoretical and methodological approaches they employ, in the concrete problems on which they choose to work. This has been one of the principal strengths of the Laboratory; and so long as investigators are free to choose the objects and methods of their own research, this will undoubtedly continue to be the case. There is always a delicate balance, however, between the advantages of diversity and the advantages of cumulativeness. For that reason, it is encouraging that several new studies represent conscious attempts to concentrate more of our efforts on a few of the most exciting problems that have emerged from our previous research.

I. Studies of the Family

Parent-Child Relationships and the Personality Development of the Child

Much of the Laboratory's research on parent-child relationships is being conducted at an experimental nursery school in Washington, D.C., where, in cooperation with the staff of the school, Drs. Marian Yarrow, John Campbell, Roger Burton, and Mrs. Doris Hawkins are developing both substantive and methodological studies. In general, their research deals with the ways in which parents, teachers, and peers influence the young child's behavior. Their strategy is to use a continuously available sample of subjects to investigate concurrently a set of research questions, which, though distinctly different in focus, have considerable overlap in revelant variables. Common to most questions of socialization and child development are certain core variables measuring (a) adult actions and values assumed to determine various aspects of the child's behavior; (b) characteristic behaviors of the child (usually viewed as the consequent variables); (c) parental responses to characteristics of the child; and (d) characteristics of the relevant environment. Data will be gathered on these variables as standard information on each child and on his family, such data having common use and relevance for a series of specific projects. Both direct observations and verbal reports are being obtained, in both natural and experimental situations.

Several studies are now sufficiently far along to merit more detailed discussion:

(1) The first study to utilize the resources of the school is an investigation of the reliability and validity of retrospective data on parent-child relationships. The aims of the research are to assess the nature of differences between earlier events and parents' recollections of such events, and to determine how retrospection is influenced by such factors as the length of the time interval between events and recall, intervening events, and the current psychological situation. Two hundred and twenty mothers of children on whom data were collected by the school 2 to 25 years ago have been interviewed. The child's sex, current age, and birth order positions are factors on which a systematic sampling of mothers has been based. The

interviews obtain mothers' retrospective reports concerning specific aspects of child development and parent-child relationships. Data from these interviews are compared with a baseline consisting of direct observations, interviews, and ratings gathered at the earlier times.

The first results show a statistically significant correspondence between baseline measures and mothers' current reports on child characteristics and child rearing; however, considering how often retrospective data have been used in past studies, the correlations are disappointingly low in magnitude. A median correlation coefficient of approximately +.35 obtains in comparisons of initial and recall data on the personality characteristics of the infant and of the growing child, child rearing practices, parent-child relationships, child personality characteristics, the child's relationships with his peers, and early traumatic experiences.

Examination of the direction of differences between baseline and recall data reveals a large number of items on which there are highly significant shifts in one direction. These shifts show a tendency for the mothers to downgrade the father's role and to upgrade their own role in recalled earlier child rearing events. For example, fathers are remembered as more distant and cold than they were earlier though to be, while mothers remember themselves as having been closer to the child than they felt themselves to be at the time. There was a significant shift toward recalling the children's earlier characteristics in a more favorable light than before; similarly, the children's reactions to mother's rearing practices are now seen more favorably. On the other hand, mothers restrospectively report using many more kinds of discipline than they acknowledged at the time.

(2) A second methodological inquiry, recently inaugurated by these investigators, assesses a method long advocated but little used in family research: direct observation. The purpose of the project is to compare two methods of obtaining observational data in a real-life situation. One method, which has been labeled the "stream of behavior" technique, presents a sequential narrative account of the ongoing activities in a given setting. In the second method, which utilizes precoded categories of behavior, the observer records the appropriate category whenever it occurs. The study is designed to ascertain: How closely do these two methods correspond in their assessment of behavior? Do they correspond more and less well for different areas of behavior? Is there differential reliability of report for different settings? What are the reliabilities of the operations for each stage of the two methods?

To provide the data for these analyses, simultaneous observations of the same child have been recorded by three observers. This permits the recording of observations of the same behavior by the two techniques and the appraisal of the reliability of each of the observational schemes. Sampling procedures have been systematically balanced over settings, children, time of day, individual recorder, and observational method. Eighteen children have been observed over a period of 16 days, with an average of eight 12-minute observations on each child. Analyses of these data are now being done.

(3) Dr. John Campbell has this year begun planning an investigation of patterns of variation in family members' attitudes, values, beliefs, perceptions, and behavior concerning illness and health. His goals are to examine ways in which selected personal and situational factors relate to health orientations. The potential value of the research extends beyond the confines of sickness and health. These phenomena offer a strategic opportunity to pursue such general social psychological issues as role learning, developmental changes in family relationships, and role behavior in stressful conditions.

At this date, Campbell is engaged in designing his research instruments—which will include both structures interviews and a semiprojective test intended specifically for eliciting children's orientations to illness.

(4) In a series of experimental studies, Dr. Roger Burton has investigated the development of conscience in children, with special reference to the effects of familial relationships on this development. In previous reports, we noted that these investigations show that the same child rearing techniques have different effects for boys' and for girls' ability to resist temptation: the more controlling the mother, the greater the girl's ability to resist temptation; a more permissive approach to child rearing is more effective for boys. There is also evidence that direct, physical forms of discipline are most efficient during the child's early years with the more symbolic forms gaining in effectiveness as the child grows older.

Further analyses of the mother-child interactions *in the test situation* indicate that mothers of children who deviated from the rules of the game (who yielded to the temptation to cheat) were more directing and restricting of their child's activity than were the mothers of the nonyielders. The mothers of yielders were more task oriented and specifically encouraged the child to do well in the game. Furthermore, mothers of yielders more actively encouraged behavior they considered good and discouraged undesired behavior. Mothers of yielders were also more likely to make their affection contingent on the child's behavior. Mothers of nonyielders tended to talk more and to initiate more general conversation, unrelated to the task at hand. They were also more likely to accept and to meet their children's requests. Analyses of the children's behavior reveal some expected dovetailing with the differences between mothers' behavior, in that the yielders were more task-oriented than the nonyielders, and also were likely to lie to their mothers about the rules of the game.

In connection with this research, Burton has reanalyzed the data of Hartshoren's and May's classic study of honesty, using newer statistical techniques and taking into account his own and others' more recent studies of moral development. (This was discussed in last year's report. In brief, Burton's reanalysis shows an underlying generality in moral behavior, even though much of the variance in the honesty tests is clearly due to specific test determinants.) Burton has now proposed a model to account for the several sets of relevant data. The model involves two generalization gradients which may be thought of as theoretically independent: a gradient involving the specific stimulus elements of particular situations, and a gradient pertaining to verbal mediation in which certain cognitive elements are abstracted from one situation and generalized to new and different situations. His paper discusses the organizing and heuristic value of this model with respect to the major variables found to be relevant to the development of moral behavior.

Research on the Family in Japan

Simultaneously with these studies of the family in the United States, Dr. William Caudill has been continuing his research on the family in Japan. Caudill has been aided this year by the presence in the Laboratory, as a Visiting Scientist, of his long-term collaborator, Dr. L. Takeo Doi of St. Luke's International Hospital, Tokyo.

The work with Japanese families is an outgrowth of Caudill's study of psychiatric patients and their treatment in Japan. The general goal of this research is to gain a sharper understanding of human social and psychological development over the life cycle through analysis of data obtained from several cultures. The most recent analyses make use of data secured from staff and patients of mental hospitals in Japan in response to a "picture interview" designed to ascertain which impulses are thought to be allowable and which to need restraint, in various life situations. The three projective pictures utilized in this analysis depict minor illness, adult heterosexual interaction, and bathing. Two main patterns emerged in response to these pictures. The most frequent pattern is a positive emotional response to the situations of sickness and bathing, treating the third situation in an emotionally warm but nonsexual manner. The second is an absence of positive emotional response to the situations of sickness and bathing, with a reaction either of distaste or of lack of feeling to the heterosexual situation. The first pattern might be thought of as "an emphasis upon nonsexual satisfactions," the second as "a denial of pleasure and emotion." These two patterns are probably very common among Japanese, who value very highly simple physical pleasures in situations of close contact with other persons. One way of assuring the continued existence of these pleasures is to ignore, to exclude, or to isolate any sexual feelings that might arise. But a significant number of persons have not been able to meet the emotional impact of events by an emphasis upon nonsexual satisfactions. For these persons, sexual feelings do tend to intrude, insistently and uncomfortably, into such situations. Their "answer" is a general restriction of impulse gratification, in the extreme, a denial of emotional feeling, whether positive or negative.

Caudill has also applied the precise observational techniques, developed by Dr. Harriet Rheingold of the Laboratory of Psychology, NIMH, to the measurement of mother-child interaction in Japanese families. Preliminary analysis of results with an initial sample of 30 mother-infant pairs (half from middle-class families, half from working-class; half the infants boys and half girls) show

some fairly large differences from data obtained with American mothers and infants. The Japanese mother is apt to spend a great deal of time with the baby and to respond exceedingly promptly to a "protest, fuss, or cry" on the part of the child. These findings, together with interview data from 300 Japanese mothers describing very close physical contact of parents and infant (bathing together, sleeping together), indicate a very high degree of interdependency.

Social Class and Parent-Child Relationships

During this past year Miss Eleanor Carroll and Dr. Melvin Kohn have been reanalyzing the data from their study of the effects of social class upon parent-child relationships to assess the degree and types of sex-role differentiation in middle- and working-class families. (Overall, there is far more sexual differentiation in working- than in middle-class families, both in the parents' roles and in the ways they treat their children.) Dr. John Clausen, at the University of California, has also been utilizing the data of this study for his analysis of authority relationships in the family.

On the basis of the data of this and other studies, Kohn has attempted a general interpretation of the processes by which class position affects parents' values and behavior. Much of the analysis is an attempt to specify the conditions of life of middle- and working-class parents that are apt to have the greatest effect on their values, and hence on their child-rearing practices. The focus is on basic differences in occupational roles—in particular, that middle-class jobs deal more with the manipulation of interpersonal relations, ideas, and symbols while working-class jobs deal more with the manipulation of things; that there is a greater degree of self-direction in middle-class than in working-class jobs; and that advancement is more subject to self-direction in middle-class jobs and to collective action in working-class jobs.

These differences parallel the differences previously shown to obtain between middle- and working-class parents in their values for their children—the middle-class emphasizing self-direction and the working-class conformity to external authority. At minimum, one can conclude that there is a congruence between occupational requirements and parental values. It is, moreover, a reasonable supposition, although not a necessary conclusion, that middle- and working-class parents value different characteristics in children *because* of these differences in their occupational circumstances. In particular, working-class parents want their children to conform to external authority because the parents themselves are willing to accord respect to authority, in return for security and respectability.

This argument, of course, assumes that other differences in the conditions of middle- and working-class life are congruent with, and reinforcing of, these occupational differences. The analysis concludes that, under present circumstances in the United States, this is indeed the case. One cannot, of course, assume that this would be the case in other places or other times.

Social Class and Parent-Child Relationships in Italy

Drs. Leonard Pearlin, Pier Brunetti, and Melvin Kohn are in process of replicating and extending, in northern Italy, the study of social class and parent-child relationships previously conducted in Washington, D.C. The intent of the new study is to determine whether or not social class has similar effects on parent-child relationships in Italy. The expectation is that there will be certain basic similarities, but that the different historical traditions and present conditions of life, particularly in the working-class, ought to make for some substantial differences. Furthermore, several aspects of family life not especially prominent in the Washington research—e.g., relations with a larger network of kinfolk—will probably be important to an understanding of family relationships in Italy.

The study faces such serious methodological problems as to be something of a methodological experiment: the aims of conducting an inquiry truly comparable to the one done here, yet realistic and meaningful in a different culture are, if not contradictory, at least difficult to reconcile. To do no more than translate into Italian the questions asked of Washington, D.C., parents would clearly be inadequate; to design the inquiry entirely anew so that it was no longer comparable would be equally so. What is needed is to redesign the inquiry to develop new and valid indices for the principal concepts, together with such further data as to make the discrepancies interpretable. Perhaps the collaboration of American sociologists (Pearlin and Kohn) with an unusual-

ly sensitive and knowledgeable Italian psychiatrist (Brunetti) will make this possible.

II. Studies of the Mental Hospital and of Psychiatric Patients

The Social Structure of the Mental Hospital

Dr. Leonard Pearlin has recently completed his research at St. Elizabeths Hospital on the effects of the social structure of the hospital on nurses' attitudes and behavior toward patients. His data were derived from a survey of all nursing personnel, together with an inventory of the characteristics of the 156 wards of the hospital. This approach made possible the investigation of the ways in which the social structure of the hospital and of its constituent wards, in interaction with the individual characteristics of the personnel, affect the therapeutically relevant orientations of personnel. Past reports of this research have shown that the social context of the hospital has a direct bearing on the thinking and feeling of hospital workers on a variety of issues relevant to the functioning of mental hospitals and to the care of mental patients. Specifically, the social organization of wards was found to contribute to these attitudes of personnel: (1) their liking or disliking of patients as persons; (2) their psychological withdrawal from patients; (3) their enthusiasm or disdain for the use of drugs in the treatment of patients; and (4) their feelings concerning personal fulfillment in their hospital work.

In further analyses conducted this year, Pearlin showed that the social context of the hospital also has a direct bearing on (5) the extent to which personnel resist or accept a set of presumably-therapeutic proposed changes in hospital policy. Attitudes toward change were found to be related to both the values of personnel and to their positions in the nursing force. The highest status nurses, the RN's, are least resistant—especially those who hold to a body of treatment values sympathetic to the types of change studied here. What matters for the resistance of nursing assistants, however, is not an abstract ideology of treatment but much more concrete issues of ward management. And even where nursing assistants do espouse a treatment ideology favorable to the changes studied here, they remain resistant to the changes *unless* their supervisors are favorable. What appears to determine the relevance of various precepts of patient care is their centrality to the things that personnel actually do in their jobs and to the issues they deal with in meeting the demands of their work.

The Behavior and Characteristics of Patients

A continuing aspect of the Laboratory's mental hospital research concerns the social relations of patients—with special emphasis on the *lack* of sociability manifested by chronic schizophrenics. Dr. Carmi Schooler's research with chronic schizophrenics has been directed to exploring the motivation behind their apparent aversion to social interaction. His earlier findings indicate that chronic schizophrenics have a capacity for social behavior that is not generally recognized or exploited in treatment; nevertheless, there is a very real fear among chronic schizophrenics of being placed in a position were they might be called upon either to give or to receive expressions of either positive or negative feeling.

While these predilections for and aversions to social behavior describe most chronic schizophrenics, there are important differences between male and female patients. Newly inaugurated work is designed to compare male to female patients both with respect to their behavior on the ward and with respect to the relationship of ward behavior to internal psychological functioning.

In further experimental work conducted this year, Schooler attempted to assess the effect, on schizophrenics' level of functioning, of being able to affect the fate of another either positively or negatively. The results suggest that the performance of patients in such a situation is a function both of the type of illness and of culturally defined sex role. But over the course of time, the hospital environment seems to deemphasize initial differences between diagnostic subcategories so that the behavior of all patients eventually tends to conform to a common norm. Thus, among male patients, the catatonics are initially more hostile and the paranoids more benevolent; in time, the catatonics become more benevolent and the paranoids more hostile. Reverse trends occur among the female patients, catatonics being initially more benevolent and paranoids initially more hostile. This leveling effect of hospitalization supports the contention that the environment of large mental hospitals deemphasizes individual differences and produces conformity around a common norm.

An analysis, by Dr. William Caudill, of the

ordinal position of patients admitted in 1958 to four psychiatric hospitals in the Tokyo area shows an overrepresentation of eldest males and youngest females. The pattern is especially clear when the analysis is limited to living siblings of the same sex as the patient. It holds, however, only for psychotic patients—not neurotics. Caudill is securing further data in Japan, and Schooler in the United States, for further comparisons of the relationship of sibling rank to hospitalization in the two countries.

III. Studies of Job, Occupation, and Career

Both the research on social class and parent-child relationships, and that on the social structure of the mental hospital, highlight the importance of occupation as a determinant of values and behavior. We have now begun to make this one of the foci of the Laboratory's research.

The problem to which this research is addressed was aptly posed 30 years ago:

"What does any occupation do to the human being who follows it? * * * We know that some occupations markedly distort the personalities of those who practice them, that there are occupational patterns to which one conforms his personality as to a Procrustean bed by lopping off superfluous members. Teaching is by no means the only occupation which whittles its followers to convenient size and seasons them to suit its taste. The lawyer and the chorus girl soon come to be recognizable social types. One can tell a politician when one meets him on the street. Henry Adams has expanded upon the unfitness of senators for being anything but senators; occupational molding, then, affects the statesman as much as lesser men. The doctor is always the doctor, and never quite can quit his role. The salesman lives in a world of selling configurations. And what preaching most accomplishes is upon the preacher himself. Perhaps no occupation that is followed long fails to leave its stamp upon the person" (Willard Waller, *The Sociology of Teaching*, 1932, pp. 375–6).

Many hypotheses have since been advanced as to the effects of some particular dimension of occupation on such diverse aspects of personality and behavior as cognitive style, emotional expressions, values, and patterns of familiar relationships. There have not, however, been any systematic studies that have attempted to unravel the many dimensions of occupation to ascertain, if not the effects, at least the social psychological correlates of each. This is what Drs. Carmi Schooler, Morris Rosenberg and Melvin Kohn are hoping to do. To date, their efforts have been concentrated on an exhaustive *a priori* analysis of the dimensions of job, occupation, career and of a number of hypothesized dependent phenomena—various aspects of personality, values and off-the-job behavior. The most difficult problems of research design—including being able to differentiate the consequences of occupational experience from the processes of occupational self-selection—have been deferred, on the optimistic theory that these can best be solved after a thorough-going *a priori* analysis of the relevant dimensions.

A few of the principal dimensions that will certainly be included in whatever study is finally designed have been included in the Italian study of social class and parent-child relationships discussed above. So there will probably be some basis for cross-national comparisons here.

Another approach to the study of the social psychological consequences of occupation and career has been taken by Dr. Roger Burton. Several years ago, while a graduate student, Burton studied a group of highly successful professional musicians—their success attested to by their having contracts with motion picture studios. (The object of that study was to assess the personalities of the musicians by comparisons to norms for adult males generally. It turned out that, despite all stereotypes, the musicians were not so strange a lot at all.) Recently, the motion picture studios dropped all their contracts, and these formerly elite musicians became part of the "free lance" or "catch-as-catch-can" group of musicians in the Los Angeles area. Simultaneously, other conditions further narrowed the demand for performing musicians. Burton's study will focus on the changes, if any, in personality which have accompanied this environmental stress. It will also investigate the influence of selected personality and social structural variables in affecting their behavior at this time. It is hoped that the study will shed light on the stability of personality during stress. Further, it should indicate the importance of personality in affecting reactions to a stressful situation, and may also indicate some of the coping mechanisms by which a highly trained person adjusts to the necessity to change his occupation.

IV. Searching for Relevant Social Variables

The last two studies to be discussed do not fit into the arbitrary categories used thus far in organizing this report. Instead of being keyed to families, hospitals, or occupations, they are attempts to ascertain *which* social variables affect some important aspect of personality.

Self-Esteem Among Adolescents

The first is a broad-scale investigation by Dr. Morris Rosenberg of the determinants (and consequences) of level of self-esteem among adolescents. This study is based on a survey of over 5,000 high school students in selected schools throughout New York State. Level of self-esteem has been measured by a series of questions addressed to the student's conscious self-evaluation; these questions prove scalable, and there is evidence of the validity of the measure from a small series of intensive interviews with and observations of high- and low-scorers.

Last's year's report summarized Rosenberg's findings with respect to (a) the relationship of self-esteem to depression and to psychosomatic symptoms, (b) the effect of parental interest and disinterest in the child on his level of self-esteem, (c) the effects on the child's self-esteem of growing up in a neighborhood where most of his peers are of a different religious background from his own, (d) the effect of the adolescent's level of self-esteem on his participation in high school activities, and (e) the effect of his level of self-esteem on his interest in national and international affairs.

Further analyses extend our knowledge of the determinants of level of self-esteem:

The self-esteem of adolescents varies with their social class, nationality, and religion, but not as a simple reflection of the social status of these groups. For example, boys whose families are of middle or higher social class levels have somewhat higher levels of self-esteem than do students from lower social classes, but this difference does not obtain for girls. The evidence suggests that the class difference in boys' level of self-esteem is in good measure a reflection of different patterns of father-son relationships in various social classes. Lower class fathers are less likely than higher class fathers to have close relationships with their *sons;* and the father's relationship to the son matters greatly for the son's level of self-esteem. But social class makes little or no difference for how close fathers are to their *daughters.* Hence, social class seems to matter for boys' level of self-esteem not because of the prestige attached to higher social class status, but because of the subcultural norms and practices of various social classes. Similarly, the often substantial differences between various religious and nationality groups in levels of self-esteem appear not to be associated with the general social status of the group but with its norms and practices.

Second, divorce and remarriage have important effects for the adolescent's self-esteem level, but not in an obvious way. In groups in which divorce is very rare, the child of divorce has considerably lower self-esteem than others, whereas this is not true in groups with higher divorce rates. If the mother was very young at the time of the marital rupture, then the child's self-esteem is lower than if she were older. Finally, children whose mothers remarried appear to be more disturbed than children whose mothers did not remarry; the negative effect of remarriage is particularly strong among older children.

Family composition was also found to be associated with self-esteem. Being an eldest, youngest, or in-between child is not associated with self-esteem. But "only" children—especially "only" boys—appear to have higher self-esteem than do other children. It appears, too, that boys who are mostly surrounded by sisters—particularly older sisters—have unusually high levels of self-esteem.

Finally, it appears that one of the socially significant consequences of self-esteem is for occupational orientation. In reflecting on their occupational ideals, students with low self-esteem are likely to be concerned with the avoidance of power or conflict in the world of work. In addition, the youngster whose self-esteem is low anticipates occupational frustration—he is at least as eager as others to be occupationally successful but he is far more likely to expect to fail.

The Social Psychology of Aging

The healthy, aged man studied 5 years ago by Dr. Marian Yarrow and Mrs. Harriet Murphy, of this Laboratory, with Mr. Paul Blank of the Social Service Department, are now being restudied, in order to examine stabilities and changes that

have occurred with time, and to determine the predictive value for current status and functioning of physical and psychological indicators obtained 5 years ago.

Of the 47 men studied in 1955–57, 39 had survived to 1961–62. Correlations between assessments of their attitudes and behavior as of 5 years ago and now are highly significant (md+0.58) indicative of considerable individual consistency. There is no evidence of a general movement of the group in one direction. For example, men were as likely to show more complexly organized daily behavior at the later period as they were to show a lower order of complexity; they were similarly no more likely to have moved toward less than toward more social participation beyond their immediate family groups. The data of this study do not support an image of withdrawal of the individual from his social environment, at least not among the physically healthy aged.

ADDICTION RESEARCH CENTER

The Addiction Research Center had another successful year in 1962. The older sections of the unit continued to be highly productive. The major event of the year was the activation of a social science program under the aggressive leadership of Dr. John A. O'Donnell.

As reported last year, there is now reason to believe that potent analgesic drugs with no, or minimal, physical addictive properties can be developed. Methotrimeprazine, a phenothiazine tranquilizer which is analgesic in man, has been shown to be nonaddictive. The compound however is irritating to tissue and induces marked postural hypotension. Two indanes, which are as potent as codeine as analgesics but somewhat more toxic, were also shown to possess only minimal or no addictiveness. Interest in narcotic antagonists as analgesics has increased greatly. This development traces to observations made at the Addiction Research Center many years ago that nalorphine, the original narcotic antagonist, was not addictive even though it was an analgesic in man. Nalorphine, unfortunately, produces aberrant mental reactions and so is not a clinically useful pain-relieving compound. Pharmaceutical companies have therefore been developing other antagonists in the hope of eliminating the mental side effects while retaining the analgesic activity and non-addictiveness of nalorphine. Two outstanding compounds have been studied this year: (1) 2′-Hydroxy-5,9-dimethyl-2-(3,3-dimethylallyl)-6,7-benzomorphan (WIN 20, 228, NIH–7958, ARC II–C–2) is one half as potent as an analgesic as morphine and is essentially a non-addictive compound; and (2) 2-Cyclopropylmethyl-2′-hydroxy-5,9-dimethyl-6,7-benzomorphan (NIH–7981, WIN 20, 740, ARC II–C–3), which is twenty times as potent as morphine as an analgesic, but only about three times as potent as nalorphine as an antagonist. It probably produces fewer untoward mental reactions than does nalorphine in analgesic doses and is better tolerated by former addicts on chronic administration. It will not suppress or create physical dependence. Further developments with this compound will be watched with interest. Emphasis in the analgesic program in the immediate future will continue to be focused on the antagonists, as recommended by the Committee on Drug Addiction and Narcotics of the National Research Council.

The psychotomimetic program was continued through the year with emphasis on tolerance and cross tolerance to and between various psychotomimetic agents. This work is being carried on in an effort to classify the various psychotomimetic compounds into related groups, in the hope that such a classification would yield clues to the neurophysiological or biochemical mechanisms by which such drugs create mental aberrations. The patterns of effect of the cholinergic blockers, scopolamine and N-Ethyl-3-piperidyl-benzylate (JB–318) were shown to be different from those of LSD. Partial tolerance to the cholinergic blockers developed on chronic administration of these drugs. Patients tolerant to the cholinergic blockers were supersensitive to LSD rather than being cross tolerant; hence the mechanisms underlying the effects of cholinergic blockers are quite different from those of LSD. 6-Hydroxy-dimethyltryptamine does not account for the psychotomimetic activity of dimethyltryptamine. The emphasis in the psychotomimetic program in the immediate future will continue to be centered on problems of tolerance and cross tolerance. Studies on chronic intoxication with marihuanalike drugs will be added if time permits.

The biochemical unit continued to improve methods for the detection of drugs in body fluids. Thin film chromatography is now the basic method used for this purpose in the ARC. This program

continues to be important because of the need of the states for chemical methods useful in monitoring the effects of parole and probation programs for addicts. In addition, the biochemical unit has been concerned with biochemical changes in the central nervous system during cycles of addiction. Addiction to opiates results in increased concentration of catecholamines in the brain of rats; when drugs are discontinued catecholamine concentrations fall to normal. Tolerance and dependence on opiates may therefore be related to increased release, increased metabolism, or decreased production catecholamines. No significant changes in serotonin levels were observed. Emphasis in the biochemical program will continue to be placed on brain neurochemistry in the immediate future.

The Addiction Research Center Inventory (ARCI) for measurement of the subjective effects of drugs has been further developed by the psychology section. By multiple group factor analyses, 10 separate factors have been isolated from the inventory. Additional data after the administration of morphine, pentobarbital, amphetamine and alcohol have been collected in patients who have also taken the MMPI and other personality tests. Correlations of these personality tests with the various ARCI factors will be done in the hope of developing scales in the ARCI which are approximate measurements of personality variables, and which can be used as criteria for evaluating some characteristics of the meaning and content of the items in the ARCI. Specific drug scales of the ARCI have been abstracted and used in clinical work by the opiate and psychotomimetic sections. Further data comparing alcoholics and addicts were collected and partly analyzed. A group of studies on the effects of a variety of drugs on physiological responses have been initiated and are in progress. In the future, work on the methods for measuring and predicting subjective drug effects and the work on psychophysiological changes will be continued. A new group of studies designed to obtain measurable changes in individuals with a character disorder will be undertaken.

It was a very active year in the experimental psychology unit. The potent analgesic drugs do not block the conditioned emotional response (CER) by causing marked sensory motor impairment, by intensification of feeding behavior, by enhancement of the unconditional stimulus, or by the animal learning that shock is not aversive after analgesics. It was further shown that the animals perceive and discriminate between the conditional stimuli used. Morphine acts by altering effector or response mechanisms; contrariwise, pentobarbital and amphetamine act by general impairment of receptor or perceptual mechanisms. Methods for addicting rats to morphine were developed, and the manifestations and time course of abstinence in the rats charted. Voluntary shaking of the entire body and skin ("wet dog" phenomenon) is the most useful manifestation of abstinence for this purpose. Etonitazene (I–G–2) suppresses abstinence from morphine in rats addicted to morphine. Etonitazene also creates physical dependence of moderate degree. Rats withdrawn after being addicted to morphine will drink more etonitazene than control rats in relapse tests in which the animals have a "choice" between etonitazene and water. Experiments are now underway to determine if rats given choices will learn to drink chiefly water when intoxicated with morphine, and chiefly etonitazene when abstinent from morphine. In the future, the experimental psychological program will continue to emphasize these studies on conditioning of drug-seeking behavior.

The neurophysiological section showed that convulsions occurred following withdrawal of barbiturates from chronically intoxicated decorticate and decerebellate dogs. Thus the processes underlying barbiturate withdrawal convulsions are probably diffuse and not localized. Emphasis in this program is to change to the determination of levels of neuronal excitability during withdrawal as compared with control without regard to localization.

It was shown that dilantin and scopolamine did not prevent barbiturate withdrawal seizures. Aminooxyacetic acid may afford partial protection against barbiturate withdrawal seizures, probably because of a direct depressant effect rather than by increasing the concentrations of gamma-amino-butyric acid in brain. Screening of additional pharmacological blockers will continue. If leads are uncovered which suggest any specific type of chemical change in brain, direct biochemical determinations will be done on tissues.

In conjunction with the psychology unit, the neurophysiology unit outlined the course of abstinence from morphine in rats that had been given morphine in doses increasing to 160 mg/kg intra-

peritoneally twice daily for 38 days. Signs of abstinence included weight loss, irritability, increased motor activity, fall in basal metabolic rate, fall in body temperature, polyuria, diarrhea, polydispsia, and generalized shaking of body and skin ("wet dogs"). The symptoms of abstinence were maximal after 24 to 48 hours. After the fourth day many of the signs disappeared, but polydipsia and an increased number of "wet dogs" continued for as long as 79 days. The method for producing acute physical dependence in dogs by infusing morphine or similar drugs for an 8-hour period was used as a screen for the addictiveness of new drugs. It was found that ARC I–K–1 had actions similar to those of morphine and codein in the spinal dog. Using this same technique the section is now studying the morphine antagonists ARC II–C–2 and ARC II–C–3. Morphine, imipramine, chlorpromazine and pentobarbital induced specific patterns of action in chronically decerebrate cats.

The Social Science Section was activated in June of the calendar year with HSO Dir. John A. O'Donnell as chief. Necessarily most of the activities have been concerned with recruitment and planning for future activities. The section now has a social science analyst GS–14 (Dr. John C. Ball), a Fellow in sociology (Basil J. Sherlock) and six full-time supporting personnel. Another social science analyst has been recruited and is initiating a followup project on patients discharged from the Lexington hospital to Puerto Rico. Members of the unit have made many contacts with individuals concerned with the narcotic problem in the Federal, State, and local governments. During the coming year the projects that will be initiated include: development of methodology for following up addicts in different environments and in different cultures, collecting and analysis of information on addicts from the records of the Fort Worth and Lexington hospitals, and the development of a system for uniform data collection on future hospital admissions.

Studies on Addictive Properties of New Analgesics

These studies are designed primarily to provide information on the human addiction liabilities of new drugs (chiefly potent analgesics with morphinelike properties) for use by authorities responsible for recommending measures for control of such agents at national and international levels. They also assist the medical profession in evaluating the therapeutic and toxic properties of new drugs in clinical use and provide opportunities for basic research on the mechanisms of tolerance, addiction, and habituation.

During the current year the effects of addiction to intravenous heroin as compared to those of placebo on patterns of physical activity were evaluated in a double-blind study. The daily measures of activity selected were: (a) hours lying horizontal on bed, (b) hours of sleep, (c) hours off the ward, and (d) miles recorded on a pedometer. As compared with placebo, heroin increased activity when given in single intravenous doses or in repeated doses for 3 or 4 days, but significantly depressed activity when administered chronically over a longer interval. When these veteran addicts received heroin four times daily they showed a tendency to retreat from all forms of activity and social contacts and to go to their individual rooms where they would lie alone on their beds, with eyes closed, and frequently with radios turned on, "nodding" (sedated, but usually not sleeping).

The effects of graded doses of pentobarbital (150, 200 and 250 mg) and of morphine (8, 16 and 32 mg) were compared in 12 former addicts. In the case of morphine, only miosis and itching were strongly and positively correlated with dose. In the case of pentobarbital, only postrotatory nystagmus, sleep, and drunkenness were dose-dependent. The subjects' "liking" for morphine was positively correlated with increasing dose levels, but "liking" for pentobarbital was negatively correlated with increasing dose levels.

Addictiveness of Seven New Synthetic Morphine-like Compounds

Addiction Research Center numbers (ARC) will be used in the test to designate compounds. An annual report of drugs investigated is made to the Committee on Drug Addiction and Narcotics, NRC.

1. *2,2-Diphenyl-4-(1-[4-(N-piperidine)-4-carboxamide]-piperidine)-butyronitrile (ARC I–D–21)*. This is a new derivative of meperidine and was developed as an analgesic. Although found to be slightly less addicting than morphine, the compound was quite irritating on intramuscular injection and therefore is not clinically useful.

2. *1-Dimethylamino-3-phenylindane hydrochloride* (*ARC I–N–1*). ARC I–N–1 and the succeeding compound represent a new chemical series with an analgesic potency in the range of codeine. ARC I–N–1 has been evaluated for addictiveness by the oral route using the single dose, substitution, and direct addiction procedures. Since it did not cause morphinelike subjective effects, suppress abstinence or create physical dependence, it was concluded that it does not possess morphinelike addictiveness.

3. *2-Amino-indane hydrochloride* (*ARC I–N–2*). When administered orally I–N–2 did not induce morphinelike subjective effects, suppress abstinence from morphine or create physical dependence. However, I–N–2 is a stimulant drug; it increases blood pressure, induces insomnia, and in high doses can cause grand mal convulsions. Although nonaddictive, the adverse side effects limit its usefulness as a therapeutic agent.

4. *Mixture of phenazocine plus N-allylnorphenazocine* (*ARC I–H–1 plus ARC II–D–1*). The mixture employed consisted of nine parts of phenazocine to one part of antagonist. The mixture was studied because it was a satisfactory analgesic in man. In former addicts, the mixture causes morphinelike euphoria, suppresses abstinence from morphine and creates physical dependence. It was concluded that the addictiveness of this mixture is comparable to that of morphine or to that of phenazocine alone, hence the mixture would have no greater advantage therapeutically over morphine or phenazocine *per se*.

5. *1-2′ - Hydroxy-2,5,9-trimethyl-6,7-benzomorphan HBr* (*ARC I–H–2*). ARC I–H–2 was studied because it did not suppress abstinence in the monkey, yet it was equipotent to morphine as an analgesic in man. I–H–2 was equipotent to morphine in inducing euphoria in nontolerant patients but was only one-seventh as potent as morphine in suppressing abstinence in addicted patients. When chronically given, I–H–2 was less well liked than morphine, but induced a moderate degree of physical dependence. Although I–H–2 is somewhat less addictive than morphine, interest in the compound centers on the discrepancy between potency in inducing euphoria, potency in suppressing abstinence, and potency in creating physical dependence.

6. *2 - Hydroxy - 5,9 - dimethyl-2-(3,3-dimethylallyl)-6,7-benzomorphan* (*ARC II–C–2*). ARC II–C–2, a weak opiate antagonist, is about one-half as potent as morphine for clinical pain relief. In nontolerant addicts II–C–2 does not cause significant morphinelike euphoria regardless of the route of administration; it is relatively ineffective in suppressing abstinence; and former addicts will not continue taking it chronically. All of these experiments indicate that II–C–2 has a low degree of addictiveness, probably no greater than that of *d*-propoxyphene.

7. *2 - Cyclopropylmethyl - 2′ - hydroxy - 5,9-dimethyl-6,7-benzomorphan* (*ARC II–C–3*). This phenazocine derivative is a potent morphine antagonist in animals. In man it is approximately 20 times as potent as morphine as an analgesic. In nontolerant former addicts, 0.4–0.8 mg subcutaneously of ARC II–C–3 induce subjective effects which resemble those of nalorphine and which are disliked by the patients. 0.75 mg of II–C–3 precipitated abstinence in addicted patients. It therefore will not substitute for morphine in addicted individuals. Direct addiction tests are being carried out.

Acute and Chronic Intoxication with Drugs other than Analgesics and Barbiturates

1. Single Doses of Drugs Related to Marihuana

In 2 separate tests 6 to 19 nontolerant human subjects received single oral doses of tincture of cannabis and a synthetic marihuana derivative, 1′-2′ - dimethyl - heptyl cannabinol (VII–B–4). This latter drug consists of a mixture of isomers but has a structural formula similar to CA–101. CA–101 has been previously studied at the ARC and induces subjective and objective marihuana-like effects at total doses of 0.5–1.5 mg. Similar effects lasting as long as 8 hours were observed with VII–B–4 with a total dose of 1–2 mg and consisted of relaxation, hunger, laughter, mild alterations in body image, postural tachycardia and hypotension. A dose of 100–150 mg/kg of our sample of tincture of cannabis was required to produce similar effects, except that postural hypotension was minimal or absent.

2. Single Doses of Tetrahydroaminoacrine (*ARC XV–B–1*)

In doses of 30–60 mg this substance has been reported to abolish the hallucinogenic effects of cholinergic blocking drugs, presumably by in-

hibiting of brain cholinesterase. In preliminary experiments 50 mg i.m. of this drug produced nausea and vomiting, but no definite mental effects.

3. Single Doses of 3-Quinuclidinol Benzylate (ARC IV–2B–5)

This substance is reported to have cholinergic blocking properties and to produce long lasting psychotomimetic effects in man, with small doses. We have found that 3 mcg/kg, orally and intramuscularly, produce definite subjective and objective effects in man resembling those of scopolamine except for the duration of action. Effects do not appear for 3–6 hours and have lasted for 30 hours or more after a single dose.

4. Comparison of Single Doses of 6-Hydroxy-N-Dimethyltryptamine (ARC V–C–8) With N,N-Dimethyltryptamine (ARC V–C–3)

Five subjects received each drug intramuscularly. V–C–3 (0.75–1.0 mg/kg) induces marked subjective and objective effects resembling those of LSD, except for a shorter duration of action. Prominent features include anxiety, perceptual distortions, visual hallucinations, pupillary dilatation and a rise in blood pressure. However, V–C–8, the 6-hydroxy derivative, failed to produce any significant effects in doses up to 2.0 mg/kg. These results suggest that the 6-hydroxylated metabolite is not responsible for the effects of dimethyltryptamine in man.

5. Cross Tolerance Between N,N-Dimethyltryptamine (ARC V–C–3) and LSD

Six subjects tolerant to LSD (1.5–3.0 mcg/kg) will be tested for cross tolerance with V–C–3 (0.5–1.0 mg/kg). These studies are now in progress. Due to the lack of information on chronic toxicity of V–C–3, studies of direct tolerance to this compound will not be attempted at this time.

6. Direct and Cross Tolerance Between LSD and Cholinergic Blocking Drugs

Six subjects have now completed this experiment. Definite but slowly developing and incomplete direct tolerance to the mental and cardiovascular effects of the cholinergic blockers, scopolamine (10 mcg/kg) and N-ethyl-piperidyl benzylate (JB–318) (150 mcg/kg) occurred when these drugs were administered three times daily for 19–21 days. No tolerance was observed to the pupillary dilatation or depressed salivation with scopolamine.

Subjects who were tolerant to either scopolamine or JB–318 were not cross tolerant to LSD; instead, the LSD reaction appeared to be enhanced in subjects that were tolerant to either of the cholinergic blockers. This was manifest chiefly by an increased duration of the LSD reaction rather than an increase in peak effect.

Subjects developed complete direct tolerance to LSD (1.5 mcg/kg) but were not cross tolerant when challenged with either of the cholinergic blockers. Also the reaction to the cholinergic blockers was not enhanced in subjects that were tolerant to LSD.

Clinical Studies of Intoxication with Alcohol, Barbiturates and Related Drugs

During the year a paper was published on the similarities and differences in the personalities of addicts, alcoholics and criminals, using the MMPI. Similarities of these groups were stressed although differences of slight significance were found. A more theoretical paper will be published in the near future. A further study to differentiate alcoholics and addicts, using items on the MMPI, was completed. In a preliminary analysis using 50 subjects in both a test and a validity group, only 5 items "held up" under cross validation, and some of these, such as 1 concerned with excessive drinking, were also correlated with age. The finding of such few differences was consistent with previous findings on MMPI scales. However, an original scale was developed on a test group which did maintain discrimination under cross validation. It thus appeared that the paucity of significantly differentiating items mentioned above must have been associated with the number of subjects employed. For this reason subjects from the two studies were combined. In this manner 89 items of the MMPI were found to differentiate narcotic and alcoholic addicts. Narcotic addicts endorsed the more obviously psychopathic items and items on social adequacy. Alcoholics, in contrast, showed more religious preoccupation, hostility, impairment of work performance, and symptoms which might conceivably be related to prolonged excessive use of alcohol. The scale, scored in the "alcohol" direction, is correlated with maladjustment, femininity, and performance on

some drug scales of the Addiction Research Center Inventory (ARCI), including the alcohol scale. The new MMPI scale is not correlated with drug preference or drug use as shown by the MMPI and a rating scale. Although it might be readily assumed that the alcohol-addict differentiating scale would correlate with drug preference and history of excessive use in narcotic addicts, such is not the case. This lack of significance possibly may be due to the lack of an appropriate "alcoholic-addict" criterion group for measuring excessive drinking, since both groups acknowledge excessive use of alcohol to a significantly greater degree than normal persons. This possibility can be "checked" by using scales developed from "alcohol-normal" criterion groups; or the low correlation may be attributed to the paucity of questions in the MMPI which can be related to a dimension of excessive use. The factor analytic study (Monroe and Astin) of the items contained in Psychopathic deviate scale of the MMPI support this reasoning since it was found that the item on excessive drinking was nonspecific. In a review of about 200 scales listed by Welsh and Dahlstrom the item about excessive drinking was found only on a few scales, e.g., alcohol scales or those that measure psychopathic deviation in a broad sense. It is hoped that the Inventory of Habits and Attitudes developed by Hill and Monroe will be more sensitive to a dimension of excessive use. Other than for the lack of relation between various criteria of excessive drinking certain predictions were confirmed. For example, subjects who are high on the new MMPI scale are usually low on the first MMPI typological factor. "High" scores on this factor are obtained by individuals who wish to portray themselves in a favorable light or wish to indicate adequacy.

Using the Guilford-Zimmerman Temperament Survey (GZTS) scales of extroversion, differences between addict subjects "on" and "off" alcohol were nonsignificant. Also the correlations between the principal extroversion scale (restraint vs. nonrestraint) and empirical ARCI drug scales for alcohol and amphetamine were nonsignificant. In addition, the partial polarity between some of the effects of alcohol and amphetamine that are observed on the ARCI (found after correcting for general drug effects) is not accounted for by the extroversion dimension (GZTS). This finding suggests that some results that support Eysenck's theory might be more highly correlated with an efficiency dimension (ARCI), previously referred to as motivation or as the amphetamine specific scale, than with extroversion.

Subjects under alcohol, pentobarbital, chlorpromazine, amphetamine, morphine, pyrahexyl and LSD were compared on an ARCI index of carelessness. It was thought that the index, in addition to measuring carelessness and literacy, would be related to drug induced confusion, i.e., the subject who is confused may be more likely to answer logically opposite questions or exactly restated questions in an inconsistent way. This hypothesis was upheld in a general sense, for subjects under alcohol and pentobarbital obtained the highest scores on "carelessness." However, it is noteworthy that amphetamine produced lower scores than the "no-drug" condition, but it was also concluded from this study that a person must be quite confused and disoriented before he is unable to answer in a consistent manner questions of simple structure. Subjects who are overtly careless or illiterate are more likely to obtain high "carelessness" scores than those who are regarded as confused. The "confusion" score was most highly correlated with the alcohol drug scales, as might be expected.

Biochemistry of Addiction

The Effect of Morphine on the Catecholamine Level of Rat Organs

Morphine has long been known to release epinephrine from the adrenal gland, probably due to stimulation of central autonomic centers which in turn stimulate the adrenal medulla via the splanchnic nerves. It has been reported that moderate to large doses of morphine decrease brain catecholamines in certain species. Our investigations were designed to provide information in the following areas: (1) to determine how morphine affects brain catecholamine content, (2) to relate changes in brain catecholamine levels to physiological effects of morphine, and (3) to determine if alteration of catecholamine metabolism is related to abstinence.

Responses to single doses of morphine were reported in 1961. Data during abstinence in one group of rats was also presented. Similar methods have been employed this year in further study of

addiction and abstinence. Four groups of Wistar albino rats, equally divided as to sex, were injected subcutaneously twice daily with morphine sulfate of mg/kg/day doses beginning at 5 and attaining maxima of 160, 260 or 400 over a 40-day period. Concurrently 12 saline treated controls were studied.

Addiction

One group (maximum of 400 mg/kg/day) was sacrificed about 2 hours after the final injection of morphine. Addicted animals failed to gain weight as rapidly as saline treated controls, and had very little body fat. Morphine addiction did not alter brain weight, but did significantly decrease heart weight. Spleens tended to weigh less than those of controls, while adrenal glands were hypertrophied. Brain catecholamine levels showed significant increases over saline controls. Serotonin levels were unchanged. Heart and splenic catecholamines, both on the basis of concentration and total content per organ, tended to decrease. Splenic serotonin, calculated as content or concentration, was significantly elevated.

Abstinence

Three other groups of animals (maximal doses of 160, 260, or 400 mg/kg/day) were sacrificed at 24 or 48 hours after the final injection in order to study the effects of abstinence. These rats lost weight during withdrawal and had very little body fat. Brain weights were unchanged, weights of heart and spleen were significantly decreased, and adrenal glands were hypertrophied. Brain catecholamine levels returned to normal. Serotonin was unchanged. The concentration and content of heart catecholamines were significantly depressed. Concentrations of catecholamines and serotonin in spleen were significantly increased but the total amounts per organ were the same as those of controls.

Future Experiments

In collaborative studies there are indications that addiction to morphine may produce a prolonged alteration in oxidative and water metabolism in the rat. Further studies will attempt to determine if these changes are due to a slowly reversible disorder in hypothalamic-pituitary function, and whether this disorder is related to an alteration in brain catecholamine metabolism. Experiments measuring the effects of morphine addiction and withdrawal on food and water intake, urinary excretion of catecholamines and adrenocortical hormones, and thyroid function are planned. Emphasis will be placed on the changes in those variables apparent during the protracted secondary abstinence syndrome.

Excretion of Catecholamines and Their Metabolites During a Cycle of Addiction to Morphine (Pilot Study on Man)

This is a collaborative study with Dr. Weil-Malherbe of St. Elizabeths Hospital, Washington, D.C., in whose unit the chemical analyses are done; ward observations are made here. Epinephrine, norepinephrine, 6 metabolites, dopa and dopac were followed. Throughout three successive 20-day periods, excretion of all the compounds was erratic. All compounds were for the most part excreted at higher levels than during the control period. In general, levels of all compounds fell on withdrawal to values lower than those of the preaddiction period. Thirty days after withdrawal, the metabolites were being excreted at about 50 percent of the preaddiction rate. The subject was recalled after 4 months (5½ months after withdrawal). He was then excreting metabolites at levels comparable to preaddiction controls. This experiment is being repeated on another subject, who will be followed to find at what time after addiction normal excretion returns.

The Pathways and Neuronal Substrates for Morphine-Induced Hyperglycemia in Dogs

Studies of glucose levels in response to single doses of morphine, with and without inhibitors, have been made. Future experiments will involve concurrent determinations of oxygen and carbon dioxide equilibria on response to morphine.

Neurophysiology and Neuropharmacology of Chronic Intoxication With Barbiturates and Related Drugs

1. Elevation of Electrical Seizure Thresholds

After bilateral adrenalectomy the usual electroconvulsive threshold elevation developed in cats and dogs while the daily maintenance doses of desoxycorticosterone and cortisone were held con-

stant. Control data on two intact dogs and one normal cat have been obtained to complete the project.

It will be important to determine whether the electroconvulsive threshold elevation results from a local or a more generalized cerebral adaptation. It is intended to stimulate across two anteriorally placed electrodes twice daily until the usual threshold elevation effect occurs and then to measure the electroconvulsive threshold across two posteriorally placed electrodes. If a similar threshold elevation occurs in the latter position, evidence that the cerebral adaptation is a generalized phenomenon will have been obtained. Such a finding would indicate the feasibility of making whole brain extracts in smaller animals in order to determine whether an inhibitory substance can be obtained as a result of daily electroconvulsions and the "tolerance" that develops thereto.

2. Effect of Decerebellation on Barbiturate Withdrawal Convulsions

Thirteen beagle dogs were subjected to surgical ablation of the entire cerebellum prior to chronic and progressive barbital sodium intoxication. Eight of these preparations survived to be withdrawn (along with eight intact control dogs) from final dose levels of sodium barbital ranging from 135 to 191 mg/kg. Three control dogs and one decerebellate dog developed convulsions under these conditions. The project is not completed, but, unless autopsy proves the cerebellum to have been incompletely removed in the preparation that had abstinence seizures, it is apparent that the cerebellum is neither the sole site of origin nor an obligatory neuronal substrate of barbiturate abstinence convulsions. However, decerebellate dogs may be less susceptible to these withdrawal seizures than are intact animals.

It is planned to reintoxicate the decerebellate dogs using higher final dose levels in order to obtain barbiturate withdrawal convulsions in more than one such preparation, and to establish the dose effect relationship in comparison to normal dogs.

3. Effect of Nondepressant Chemicals on Barbiturate Abstinence Convulsions in Dogs

This screening program has been continued with the purpose of finding leads to possible biochemical mechanism underlying the abstinence seizures.

Thus far the only chemical that has reduced the number of seizures has been aminooxyacetic acid (AOA). This substance induces an accumulation of gamma-amino butyric acid (GABA) in brain, and presumably has inhibitory effects therein. Two of six dogs (33%) that received 5 mg/kg of AOA every 8 hours during barbiturate withdrawal did not have convulsions. Four of seven dogs (57%) that received 5 mg/kg of AOA and two of five dogs (40%) that received 11 mg/kg of AOA every 8 hours during barbiturate withdrawal were protected from abstinence seizures. These results indicate that GABA may be directly or indirectly related to barbiturate abstinence seizures (BAS). In the former instance a GABA deficiency would be postulated as the direct cause of BAS. In the latter case GABA would act as a nonspecific inhibitor of BAS when excess levels of GABA are obtained in the brain during barbiturate withdrawal. Direct measurements of this substance in brain during barbiturate intoxication and withdrawal will be required to prove which possibility is correct.

During the coming year attempts will be made to prevent BAS by administering hydrazine during withdrawal. This chemical is also supposed to cause an increase of GABA in brain. If it proves effective against BAS, direct measurements of GABA in brain may be carried out as suggested above.

Psychological Studies of Addiction

With some minor changes, the studies subsumed under this title remain much the same in purpose as those described in 1961. The changes are chiefly concerned with methods and less frequently with formulation of problems. The investigations are designed to: (1) provide further information on the psychopathology of the narcotic addict, (2) develop methods for measuring specific and nonspecific subjective effects of analgesics, hypnotics, tranquilizing, analeptic and psychotomimetic drugs and relate these effects to personality characteristics, (3) develop techniques to isolate variables which control behavior in the social deviant and relate these to the misuse of narcotics and alcohol, and (4) devise methods, using animals, for investigating anxiety and re-

lated phenomena and for screening of analgesics and tranquilizing drugs.

Previous work on the personality structure of the addict was confined chiefly to use of the Minnesota Multiphasic Personality Inventory (MMPI). A paper was published during the year comparing narcotic addicts, alcoholics and criminals on factor analytically derived scales. Since this test is based on classical Kraepelinian nosology, it appeared necessary to provide a broader base within which the personality characteristics of addicts, including psychopathology, might be more meaningfully defined. All the original subjects tested under no-drug, placebo, and drug conditions on the Addiction Research Center Inventry (ARCI) (approximately 250) were also tested concurrently on four personality tests under no-drug conditions. Factor analytic techniques were then applied to selected scales of the latter four inventories to establish more extensive personality typology and at the same time provide intra- and inter-correlations for these tests on narcotic addicts. The next step in this particular investigation will be to correlate these typologies with drug effects as indicated by the ARCI.

During the year the application of factor analytic and other statistical techniques to data gathered on the ARCI under drug conditions have been mainly directed toward further analysis of the LSD–25 condition, and retesting of the efficacy and power of all previously developed scales. Ten factor analytic scales were developed from analysis of responses under the LSD condition on the original test and cross validity groups (N=100). These scales were then standardized on the no-drug and placebo conditions and tested for reliability on another sample of subjects. All scales showed some degree of independence, with the greater proportion being very satisfactory in this regard. Suggestions were obtained from the analyses that most drug effects may be fairly well defined by changes on two factors; one tentatively labelled "Reactivity," and the other, bipolar factor, "Efficiency." While this set of scales seems especially appropriate in analyzing psychotomimetics, the structure of the scales suggests that they may also have more general applicability. From reevaluation of all previously developed scales it is evident, as might be expected, that the original empirically developed scales are more effective in showing nonspecific drug effects than are scales based on patterning of action. These two forms of scales, however, are equally effective in demonstrating "dose-effect" and "placebo-drug" differences. Relatively specific patterning is most obviously produced by the highest doses of drugs used in the present series of studies.

In a "probability learning" study social deviants (psychopaths) were compared with normals on procedures involving choice and decision making and the effects of drug thereon. Analysis of the collected data has only begun and is inadequate for drawing conclusions. However superficial inspection suggests that addicts overestimate (overplay) the most frequent alternative and underestimate the least frequent alternative presented to them. Administration of morphine appears to bring estimations closer to the proportions actually presented.

The previously described conditional emotional procedure (CER) for screening potent analgesics and testing theory of the actions of such drugs employed two different tones as conditioned stimuli. Since there was some question of the range of auditory acuity of the rat, systematic studies were undertaken to investigate perception of the tones under both no-drug and drug conditions. Using the higher of the tones (slightly squared 523 cps) it was demonstrated that neither morphine nor LSD–25 produced a significant increase in errors while employing a T-maze and a discriminative escape procedure in a T-maze. Pentobarbital and amphetamine produced marked impairment. However, in using a conditioned avoidance procedure (CAR) with this same tone none of these drugs produced a significant reduction of conditioned responses except in the first few trials of the first session. Nevertheless, morphine produced a decrease in latency in the CAR and a 100-percent increase in a secondarily conditioned (spontaneous) response which consists of crossing from one conditioning compartment to another between presentations of the tone.

The Mode of Action of Central Nervous System Depressants

Investigation of the basic pathophysiological processes responsible for the abstinence syndrome seen in animals physically dependent on morphine has been continued, and observations reported in previous years have been extended. Studies on the relationship between acute physical dependence

and tolerance induced by infusing morphine at a rate of 3 mg/kg/hr (Martin and Eades, J. Pharmacol. Exper. Therap., *133:* 262–270, 1961) and chronic physical dependence and tolerance in long surviving high (C–5 to 6) and low (T–10 to 12) spinal dogs have revealed the following facts:

(1) In confirmation of the findings of Houde and Wikler (J. Pharmacol. Exper. Therap., *103*: 236–242, 1951) the depressant effect of morphine on the skin-twitch reflex is markedly reduced in animals in which the spinal cord has been transected (C–5 to 6) above the level over which this reflex is mediated. Although tolerance develops rapidly to the marked depression of the skin-twitch reflex produced by morphine in the intact dog, no rapidly developing tolerance is seen to the slight depressant action of morphine on the skin-twitch reflex of the high spinal dog. In addition, during infusion of morphine in the intact dog, depression of the hindlimb reflex is maximal within 3 hours and becomes progressively less thereafter. In contrast, depression of the hindlimb flexor reflex of the spinal dog becomes profound during a 7- or 8-hour infusion of morphine. These results strongly suggest that tolerance to the effects of morphine develops more rapidly in supra-spinal structures than it does in the spinal cord.

(2) Although tolerance develops more slowly in the spinal cord, the capacity of the spinal cord to develop tolerance to morphine is very great. Thus, in a low spinal dog chronically addicted to 20 mg/kg/day of morphine, approximately 36 times as much morphine is required to produce the same degree of depression as is required in the nontolerant spinal dog. In comparison, approximately 4.3 times as much morphine is required to produce the same degree of miosis in the acutely tolerant dog as in the nontolerant dog, whereas 8.1 times as much morphine is required in the chronically addicted dog. The disparity of the rates at which the spinal cord and forebrain develop tolerance suggests that more than one mechanism may be involved in the development of tolerance to opiates.

(3) Comparable observations have been made with regard to physical dependence. Thus, following a 7- to 8-hour infusion of morphine to spinal dogs, large doses of nalorphine precipitate a violent abstinence syndrome above the level of transection, but only minimal signs of abstinence below the level of transection. However, when spinal dogs are chronically addicted to 20 mg/kg/day of morphine, small doses of nalorphine precipitate violent signs of abstinence in structure innervated by nerves both above and below the level of transection.

(4) As previously reported (Martin and Eisenman, J. Pharmacol. Exper. Therap., *138:* 113–119, 1962) one factor that may be responsible for the development of acute physical dependence is a shifting of the level of function of homeostatic regulatory systems by morphine. When nalorphine is administered to an animal markedly depressed by morphine, the homeostatic regulatory mechanisms are rapidly resensitized and are strongly stimulated by the disordered internal environment. It is possible that a similar mechanism may be operating in the chronically physically dependent animal, except that due to the recruitment of adaptive mechanisms tolerance develops, and, as a consequence, homeostatic levels of regulatory mechanisms that can be resensitized to morphine are set at higher levels than they are in unaddicted animals.

I–K–1 [1-(p-chlorophenethyl)-2-methyl-6,7-dimethoxy - 1,2,3,4-tetrahydroisoquinoline], codeine and morphine, intravenously administered, have been compared with regard to their effects on autonomic function and spinal reflex activity in the chronic spinal dob. The overall effects of I–K–1 (2.5 and 5.0 mg/kg), codeine (2.5 and 5.0 mg/kg) and morphine (0.25 and 0.5 mg/kg) were qualitatively similar in that these agents produce some depression of body temperature, constrict pupils, slow heart rate, produce an initial but transient acceleration of respiration and depress both the crossed extensor and ipsilateral flexor reflexes. The relative potencies of I–K–1, codeine and morphine in depressing the ipsilateral flexor reflux were determined using the area under a 5-hour segment of the time action curve. A four point assay was employed and all statistical requirements for a valid assay were satisfied. One mg/kg of morphine was equivalent to 13.8 (9.6–20.0) mg/kg of I–K–1 and 18.2 (9.3–35.5) mg/kg of codeine. Both I–K–1 and codeine had a shorter duration of action than morphine. The chronic spinal dog is a very useful preparation for assessing addictiveness of the morphine type, since it is sensitive, gives a definite qualitative pattern, and yields relative potency ratios approximating those obtained

in man. In addition, the ease with which autonomic effects of morphine-like agents can be assessed in the spinal dog allows a relatively complete comparison between drugs.

Conditioning Factors in Addiction and Habituation (Relapse)

1. In the annual report for 1961, experiments then still in progress were described in which, after continuous forced drinking of a 5 mcg/ml aqueous solution of etonitazene (formerly designated as "I–G–2"), tagged with anise-flavor (hereinafter designated as Fl ETZ^5) for 22 successive days, six otherwise untreated rats were subjected to a schedule of continuous forced drinking of Fl ETZ^5 on weekends (3 successive days) and testing for "choice" between distilled water and Fl ETZ^5 during 8 hours of each of the intervening days (16 hours fluid deprivation daily). As noted, these rats developed a marked "preference" for Fl ETZ^5 during the first 8 weeks after the initial 22-day period of forced drinking of Fl ETZ^5, in contrast to the marked aversion to the flavored drug solution which they had exhibited before that 22-day period. However, on continuing this schedule for an additional 10 weeks, the rats' "preference" for Fl ETZ^5 changed to "aversion," and this "choice" pattern persisted even after the concentration of the drug solution was doubled (Fl ETZ^{10}). Following a final 5-day continuous period of forced drinking of Fl ETZ^{10}, all six rats were removed from the "linear mazes" and placed in home cages with tap water (and food) *ad libitum*. In the home cages the rats exhibited an ETZ-abstinence syndrome characterized by loss of weight and increased "wet dog" frequencies (mean counts in 15 minutes), both maximal during the first 24 hours after withdrawal of Fl ETZ^{10}. Body weight began to increase over the prewithdrawal value by the fifth day of abstinence, but "wet dog" frequencies remained at values slightly higher than in control rats for over 79 days. On the first "relapse" test (free choice between distilled water and Fl ETZ^5 for 3 continuous days in the "linear mazes") conducted 14 days after drug withdrawal, the rats consumed 36.5% of the total fluids in the form of Fl ETZ^5 (compared with 8.2% during the initial pretraining period). On the second "relapse" test, 44 days after drug withdrawal, they consumed 24.8%, and on the third "relapse" test, 56 days after drug withdrawal, 25.8% of total fluids in the form of Fl ETZ^5. During and immediately following continuous 6-day "extinction" period (free choice between distilled water and anise-flavored distilled water) the rats consumed a mean of 26.6% of fluids in the form of anise-flavored water.

Concurrently, a control experiment was conducted on six other rats which were subjected to exactly the same forced drinking and choice-testing schedule as described above, except that anise-flavored distilled water (Fl H_2O) was used instead of Fl ETZ. During the training-testing period the proportion of total fluids consumed in the form of FlH_2O increased from a pretraining value of 17.4% to as much as 27.7%. These rats exhibited no "abstinence" syndrome on transfer from the "linear mazes" to the home cages. On three "relapse" tests conducted in a manner analogous to those described above, 14, 30 and 50 days after "withdrawal" of Fl H_2O, the proportion of total fluids consumed in the form of Fl H_2O ranged from 19.1% to 27.2% (contrasting with 44.2% consumed from the same tube when it, like the other tube, contained distilled water).

From these data, it appeared that in the experimental group the long-term effects of training were to eliminate the rats' initial aversion to the etonitazene content of the anise-flavored drug solution, but not to induce a "preference" for it (except possibly on the first "relapse" test, 14 days after withdrawal of Fl ETZ^{10}). These results, rather equivocal from the standpoint of the hypothesis under investigation, may have been due to lack of control over the experimental rats' levels of physical dependence on etonitazene, which under the conditions of this study varied as a function of the animals' performance on the four "free choice" days of each week during the training period. Therefore, it was decided to redesign the experimental approach, using morphine (by intraperitoneal injection) as the "addicting" drug and etonitazene in aqueous solution as the "reinforcing" drug, as in the earlier "pilot" studies described in the annual report for 1961, except that fluid-deprivation would be eliminated. However, before commencing such a study it was decided to carry out systematic quantitative studies on the morphine-addiction cycle in the rat and on the effects of *ad libitum* drinking of etonitazene solution upon the morphine abstinence syndrome in this species, in order to determine the most ap-

propriate schedules of morphine-injection and etonitazene-reinforcement for testing the "two-factor learning" theory of relapse (classical conditioning of physical dependence and reinforcement of drug-acquisitive behavior through reduction of morphine-abstinence induced drive).

2. Chronic Morphine Intoxication and Morphine Abstinence Syndrome in the Rat

In conjunction with the study on rats addicted to 320 mg/kg/day of morphine described elsewhere in this annual report, the characteristics of chronic morphine intoxication and "primary" morphine abstinence were investigated on a group of eight experimental rats maintained on single daily intraperitoneal injections of morphine, 200 mg/kg, which were always compared with a group of four control rats receiving volumetrically equivalent intraperitoneal injections of normal saline. On this schedule the experimental rats displayed hyperactivity, absence of "wet dogs," increased oxygen consumption rates, and increased colonic temperatures when these variables were measured 1–4 hours after the daily injection of morphine. In contrast, these rats showed significantly increased "wet dog" frequencies and hypothermia 22–25 hours after the daily injection of morphine, as well as copious soft stools, polyuria and hostility at this time. These "primary" abstinence phenomena were abolished not only by injection of morphine but also by intraperitoneal injection of etonitazene, the additional effects of which (increased activity, oxygen consumption rates and colonic temperatures) were qualitatively indistinguishable from those of morphine. After substituting daily injections of etonitazene (final dose level, 300 mcg/kg, i.p.) for morphine, and then after a period of "stabilization," terminating these injections abruptly, a "primary" etonitazene abstinence syndrome was observed which was qualitively similar to, but less intense than the morphine abstinence syndrome. Also, during the 43-day period over which these rats continued to be observed, their daily consumption of water was consistently greater than that of control rats, indicative of a "secondary" abstinence syndrome.

These experiments confirmed certain assumptions drawn from earlier less systematic experiments, which are basic for the design of new conditioning procedure envisaged (see below)—namely, that rats maintained on single daily injections of morphine (200 mg/kg i.p.) every morning are acutely abstinent every evening; that "wet dog" frequencies are a reliable measure of the intensity of such abstinence; and that, at least when given parenterally, the pharmacological effects of etonitazene are qualitatively the same as those of morphine.

3. Factors Relating Oral Consumption of Etonitazene Solution in Morphine-Addicted Rats

This study was designed to test systematically another and very crucial assumption involved in the "conditioning" designs used previously and planned for the future; namely, that by drinking etonitazene in an adequately concentrated aqueous solution in the late afternoon and evening, rats maintained on single daily intraperitonel injections of morphine, 200 mg/kg, can suppress the morphine-abstinence phenomena that would otherwise supervene between injections. The same rats described in *2* above were used in this study. Morphine injections (200 mg/kg i.p.) were given daily in the morning at 0730 to the experimental rats (N=8) and saline at the same time to the control rats (N=4). Both groups were offered water *ad libitum* from 0800 to 1500, and the control rats were always offered water also from 1500 to 0800 next morning. For the experimental rats, water was offered from 1500 to 0800 on some days, and on other days water was replaced by various concentrations of etonitazene (unflavored) in aqueous solution. Measurements of water intake during the two periods each day revealed a marked and characteristic difference in drinking patterns: experimental rats drank significantly more water than controls from 0800 to 1500, and significantly less water than controls from 1500 to 0800. In contrast, the experimental rats drank significantly more of a 5- or 10-mcg/ml concentration of etonitazene from 1500 to 0800 than, and as much of a 20- or 40-mcg/ml concentration of etonitazene as control rats drank of water in the same period. In the experimental rats, drinking all concentrations of etonitazene suppressed "wet dog" responses and hypothermia (morphine abstinence signs) but activity was decreased (and soft stools and polyruria were present) when the concentration of etonitazene was 5 mcg/ml, and colonic temperature was elevated above normal when the concentration of etonitazene was 40 mcg/ml. In a different group of normal rats, fluid consumption

was not changed by substituting etonitazene solution (5 or 10 mcg/ml) from 1500 to 0800, but activity was decreased and one of the 4 rats died while drinking the 10-mcg/ml concentration of etonitazene.

These experiments demonstrated that morphine-addicted rats could "normalize" themselves completely by drinking a 10 (or 20) mcg/ml concentration of etonitazene during the late afternoon and evening, when they would otherwise display morphine abstinence phenomena (when on one dose of morphine, 20 mg/kg i.p. in the morning). Also the enormous amounts of the 5- or 10- mcg/ml concentrations of etonitazene "voluntarily" consumed by the experimental rats from 1500 to 0800 contrasted with the much lower amounts of water consumed by them during the same period, and the absence of such differences in normal rats strongly suggests that under conditions of morphine abstinence etonitazene in aqueous solution is positively reinforcing ("rewarding"). Furthermore, the sharp decrease in "voluntary" intake of the 20- or 40-mcg/ml concentration of etonitazene by the experimental rats from 1500 to 0800 suggests that such rats try to achieve "normalization" rather than morphinelike intoxication under these conditions.

4. Conditioning of Physical Dependence and Reinforcement of Opioid (Etonitazene) Drinking Behavior in Morphine-Addicted Rats

Utilizing the information derived from *2* and *3* above, this new conditioning study was designed as follows. Fourteen rats maintained on single injections of morphine, 200 mg/kg i.p. at 0800 daily, and an equal number of control rats receiving saline i.p. on the same schedule were housed in individual "linear mazes" provided with food bars *ad libitum* and drinking tubes at each end. After first determining the "preferred" and "non-preferred" ends for each rat when both tubes contained distilled water (continuous *ad libitum* drinking), the morphine addicted rats were randomly assigned to two numerically equal subgroups, "experimental, trained" (ET) and "experimental, nontrained" (ENT), and the control rats to analogous subgroups, "control, trained" (CT) and "control, nontrained" (CNT). In the linear mazes, all rats were locked out of the "non-preferred" end-compartment from 1400 to 0800 each day except on three nights each week, when they were locked in the "non-preferred" end-compartment from 2000 to 0800. The drinking tube in the "preferred" end-compartment always contained distilled water. The tube in the "non-preferred" compartment contained distilled water from 0800 to 1400 daily, but from 2000 to 0800 on the three nights of each week mentioned the tube for the ET group contained Fl ETZ^{10}, that for the ENT group Fl H_2O, for the CT group Fl ETZ^5, and for the CNT group Fl H_2O. This arrangement was intended to ensure both spatial ("Non-preferred" end-compartment) and gustatory (anise-flavor) cues for discriminative learning (morphine abstinence and its relief by drinking Fl ETZ^{10} for the ET group; abstinence without relief by drinking Fl H_2O for the ENT group; no abstinence but possible aversive effects from drinking Fl ETZ^5 or Fl H_2O for the CT and CNT groups respectively).

After 14 of such nocturnal training sessions over a 6-week period, all injections were terminated, and after a final 15th training session from the 36th to 48th hour of "withdrawal," all rats were removed to individual home cages. Thereafter "relapse" tests were conducted as follows on the 9th, 23d, 44th, 58th and 72d days following cessation of injections: At 0800 "wet dog" frequencies were measured both in the home cage and in the linear maze for each rat, the order of testing being randomized and balanced. Then each rat was returned to the "preferred" end-compartment of its linear maze and allowed to drink water and eat food *ad libitum* there until 2000, when the gate to the "non-preferred" end-compartment, provided with a tube of Fl ETZ^5 (all rats) was also opened, thus allowing each rat to "choose" between water and Fl ETZ^5. At 0800 next morning all rats were again removed to their home cages where they remained until the next "relapse" test. At all times, tap water and food were provided *ad libitum* in the home cages.

The most important findings to date are: (1) On each "relapse" test, mean "wet dog" frequencies *in the linear mazes* have been greater for the combined ET+ENT ("post-addict") groups than for the combined CT+CNT ("nonaddict") groups, even after "wet dog" frequencies of the ET+ENT groups *in the home cages* had declined to "normal" levels following withdrawal of morphine. This result lends support to the hypothe-

sis that morphine abstinence phenomena can become conditioned to temporally contiguous stimuli (linear mazes) in the classical Pavlovian manner. However, further studies are needed to determine whether "conditioned wet dogs" are specific for morphine abstinence or for aversive events in general (2). On all relapse tests, the "free choice" intake of Fl ETZ^5 has been greater for the combined ET+ENT groups than for the combined CT+CNT groups, even though "free choice" water intake was about the same for both groups. However, contrary to expectations, the ENT subgroup has consumed greater volumes of Fl ETZ^5 than the ET subgroup in the "free choice" trials from the second "relapse" test on, the order of Fl ETZ^5 intake on each of these four relapse tests being ENT>ET>CT>CNT. The very low volumes of Fl ETZ^5 consumed by the CT and CNT subgroups (with the exception of one rat which consistently drank relatively large amounts) on the "relapse" tests suggests that for control rats at least Fl ETZ^5 is definitely aversive. It is possible that during the 15 forced drinking training sessions, reinforcement with Fl ETZ^{10} was both appetitive (abstinence-reducing *and* aversive for the ET subgroup, the latter effect emerging progressively in successive relapse tests as "residual tolerance" for the etonitazene content of Fl ETZ^5 has declined. Presumably, however, loss of "residual tolerance" by the ENT subgroup has proceeded at a rate equal to that of the ET subgroup and yet the mean intake of Fl ETZ^5 by the ENT subgroup did not decline at all from the first (9 days abstinent) to the second (23 days abstinent) "relapse" tests (17.6 and 17.8 ml respectively), and stabilized at a slightly lower, but still relatively high level (15.2, 11.4 and 14.0 ml) over the next three "relapse" tests (44, 58 and 72 days abstinent). Tentatively, therefore, it is postulated that for the ENT subgroup, which had been trained on Fl H_2O, appetitive reinforcement (reduction of "conditioned abstinence" by Fl ETZ^5) has been stronger than aversive reinforcement in each of the five "free choice" relapse tests. Alternatively, it may be postulated that the greater number of unrelieved periods of abstinence in the linear mazes during the 6-week training period (42 late afternoons and evenings for the ENT subgroups, compared with 27 for the ET subgroup) resulted in stronger "conditioned abstinence" for the ENT subgroup in the "relapse" tests; however, conditioned "wet dog" frequencies (in the linear mazes) on the five "relapse" tests have been approximately the same for the ET and ENT subgroups.

Currently, experimental extinction (first, drug-extinction and then, flavor-extinction) is being carried out, with occasional additional "relapse" tests and widely separated tests on forced drinking of Fl ETZ^5 by all subgroups in the linear mazes. It is hoped that the results of these further studies will help clarify the interpretation of the data obtained to date. Tentatively, it may be concluded that the results so far are consistent with the "two factor learning" theory of relapse, but support the first of these postulated factors (classical conditioning of morphine abstinence phenomena) more than the second (reinforcement of instrumental drug-seeking behavior through reduction of morphine abstinence).

Psychophysical Studies

During the year work has progressed on four experiments: (1) effects of chronic administration of opiates on electrodermal responses to discrete stimuli, (2) effects of single doses of opiates on electrodermal responses to discrete and conditioned stimuli, (3) effects of chronic opiate administration on psychomotor performance, and (4) effects of chronic administration of opiates on coding tests.

1. The pilot study of the effects of chronic morphine administration (240 mg/day) on the electrodermal response to electric shock, light and tone stimuli was completed during the year. This study showed marked increases in the basal level of conductance across the skin and increased in absolute magnitude of the electrodermal response to electric shock, light and tone stimuli in patients receiving morphine chronically as compared with their preaddiction controls. If the basal level of conductance is taken as an indication of autonomic activity, these results suggest an increased level of activation during mild stress under chronic morphine administration. The increased size of the electrodermal response (EDR) to discrete stimuli has to be interpreted cautiously however, since it is well established that the size of a response is highly and positively correlated with the basal level from which the response begins. Thus, when the responses were analyzed with correction for the changes in basal level, they were found to be of the same magnitude in the predrug and

chronic drug periods. This finding was contrary to the prediction that the responses to the more noxious stimulus (electric shock) would be reduced under chronic morphine conditions relative to responses to the less noxious stimuli (light and tone). These predictions were based on theoretical grounds as well as pertinent experimental and clinical observations.

The results of two additional analyses of the data are interesting. In one, the mean differences between the EDR to light and to shock during the predrug and chronic morphine conditions were used as an adjustment for individual differences and changes in basal level. When this was done, the difference between the EDR to shock and light was significantly larger during the morphine than during the predrug period. In both this and the previous analysis, there was no indication that the EDR to shock was reduced during the chronic morphine period. However, when a comparison was made of the relative increase in size of the EDR from the predrug to the morphine condition for the shock, light and tone stimuli, it was found that the response to tone was increased 55 percent, that to light 42 percent, and that to shock only 28 percent. In this comparison the basal levels of conductance were the same for the three types of stimuli.

In summary, the study indicated that during the chronic morphine period as compared with the predrug period: (1) there is a marked increase in basal conductance level, (2) the absolute size of the EDR to shock is unchanged or possibly larger, and (3) the stimulus which was presumably the most noxious (shock) showed relatively the least increase in EDR.

2. In a related study the effects of single doses of 8 and 16 mg/70 kg of morphine subcutaneously, pentobarbital and placebo are being compared. In addition to the EDR, electromyographic and skin temperature responses were measured during conditioning of a response to tone by pairing with electric shock. Each of the four drug treatment groups was divided into two subgroups of eight patients each. In each treatment group one of the subgroups was presented with tones paired with electric shock, while the other subgroup received tones and shocks separately. We have predicted that the morphine-treated subjects should show less conditioning and generally reduced tonic and phasic responses in this presumably anxiety producing situation.

Only a small portion of the analysis of the data from this study has been completed at this time. Thus far there are marked trends showing appropriate dose responses for basal conductance levels. There is, however, a great deal of interindividual variability in the data, such that the statistical significance of the differences has not been satisfactorily determined. It does seem relatively certain, however, that single doses of morphine reduce rather than increase the basal level of conductance as was found in the chronic morphine study. Whether conditioned responses were established and what effects the drugs had on them will have to be presented in subsequent reports.

3. During the past year, in conjunction with the opiate and barbiturate unit, a study of pursuit rotor performance during a cycle of opiate addiction was made. The pursuit rotor was used as an index of psychomotor performance and was intended as a measure of motivation. Other investigators have reported no impairment of psychomotor performance from single doses of opiates. The study done here predicted that there also would not be an impairment of psychomotor skills during chronic addiction, but that reduced levels of motivation during addiction would be reflected in reduced performance on the pursuit rotor and that these effects would be reversible under increased incentive. There was not, however, any significant change on daily performance on the pursuit rotor during the addiction cycle. While other measures of general physical activity showed declines, psychomotor performance remained stable.

4. In the latter part of the current year studies of performance on a coding test during an addiction cycle were begun. This test required that the subject give close attention to a clerical type of task for a period of approximately 8 to 12 minutes. While this study is still in progress, to date it appears that there will not be any significant acute or chronic drug effects.

Social Science

The Section was established and began work on 1 July 1962. Activities have focused on (1) the recruiting of personnel, (2) the orientation of these personnel to the Addiction Research Center,

the hospital, to addicts, and to the literature on addiction, (3) the obtaining of equipment, and (4) the planning of a program.

The section took over the direction of a followup study of Kentucky patients, which had been started by the Social Work Service of the PHS hospital under an NIMH grant. Field trips have by now accounted for almost two-thirds of the subjects, by locating their death certificates, by interviewing informants, or by interviewing the subjects. Since it is to be expected that more time and effort will be required to locate the remaining subjects, it is now estimated that the field work will continue until the Spring of 1963, with the terminal date depending largely on how much field work will be permitted by weather conditions in the winter of 1962–63.

Plans were completed for the replication of this followup study in Puerto Rico, and for related studies of addiction in this different cultural setting. A Puerto Rican investigator has been recruited for these studies, and her orientation and the completion of administrative arrangements for her work should permit these studies to begin in early 1963.

A beginning has been made on arrangements to transfer to IBM cards a large amount of data on past admissions to the Lexington and Fort Worth hospitals, so that these data will be usable for research purposes.

The staff of the section are continuing to visit programs in other cities, and to use consultants in an exploration of other possible studies.

LABORATORY OF CELLULAR PHARMACOLOGY

As in earlier years, the work of the Laboratory has continued along four main topics: (1) mechanism and pathways of protein biosynthesis; (2) biological methylation; (3) biological oxygenation; and (4) alkaloid biosynthesis. The protein synthesis project may be regarded as the main project of the Laboratory at the present time.

1962 has been a year which has seen great advances in the work of the Laboratory and many of the projects have come to fruition in the course of the year which has been our most successful so far.

During the past year, the research efforts in the Section on Alkaloid Biosynthesis have continued along the lines described last year. Some of the positive results which have been attained in each project are as follows: (a) *Methionine activating enzyme.* The mechanism of action of this enzyme has now been worked out. The biosynthesis of S-adenosylmethionine proceeds by a condensation of ATP and methionine to form S-adenosylmethionine and inorganic tripolyphosphate. At the time of discovery, this was the only known instance of such a split of ATP. Another instance of such reaction has recently been found to occur in the synthesis of coenzyme B_{12}. In the case of S-adenosylmethionine synthesis, the tripolyphosphate remains bound to the site on the enzyme where it arises and is hydrolyzed there. A detailed analysis of the energy changes in these steps has shown that the binding of tripolyphosphate plays an important role in allowing the cell to accumulate S-adenosylmethionine for use in transmethylation reactions. (b) *Hordenine biosynthesis.* It has been shown that the enzymes which form hordenine in germinating barley are themselves synthesized *de novo* at this stage of development of the organism. The level of the alkaloid is at first a reflection of the amount of enzyme available. The factors which initiate and terminate enzyme synthesis are now under investigation. Use of isolated embryos has enabled us to demonstrate that one or more plant hormones play a role while nutrition may be varied within wide limits without effect. It will be possible to put these findings in better perspective when we have some idea of their specificity. Investigation of this aspect is now proceeding. (c) *Plant tissue culture.* An interesting tool is now available in the cactus callus culture developed during the past year. Since this tissue has been taken from a plant which forms hordenine and related compounds, it should be possible to gain more insight into the metabolic role of these alkaloids by studying this tissue. In addition, the tissue shows a requirement for light for growth. Preliminary results suggest that this light requirement is *not* connected with photosynthesis, but is more closely related to one or more of the photoreactive systems which play a major role in determining growth, differentiation and morphogenesis in plants. The response is in some manner also related to the plant hormone giberellic acid. Further work is planned to elucidate these

relationships. Such a system would offer a tool for investigating metabolic regulatory phenomena at a biochemical level.

The Section on Cellular Regulatory Mechanisms continued its fundamental work on the mechanism of action of hydroxylating enzymes. One interesting result has been the demonstration that both the phenylalanine hydroxylase and the DOPamine-β-hydroxylase-catalyzed reactions utilize molecular oxygen. This proves that these enzymes are of the oxygenase type. The results rule out any mechanism in which the hydroxyl group is derived from water and provide strong support for the generalization made earlier by Kaufman who predicted that molecular O_2 would be the source of the hydroxyl group in any hydroxylating system in which both O_2 and an external electron donor are required.

As has been detailed in earlier reports, phenylalanine hydroxylation requires the cofactor originally discovered in this Laboratory by Dr. Kaufman and extensively purified and characterized chemically by him. Chemical studies of the phenylalanine-hydroxylation cofactor have progressed to the stage where a partial structure can be written for the compound; it is a 2-amino-4-hydroxypteridine bearing a substituent on carbon atom number 6. This great advance has been made possible by the finding that a pteridine, found in the eyes of *Drosophila* containing non-sepia pteridine, possessed very high activity in the phenylalanine hydroxylating system. It is fair to say that this breakthrough was a happy by-product of Dr. Kaufman's sabbatical in Prof. Hadorn's Laboratory at the University of Zurich. It would have probably taken many years of hard work and substantial financial expenditure to achieve the same results, since the sepia pteridine which can be extracted from the *Drosophila* eye is 1,000 times as active as the crude liver extracts and possessed a structure which has been fairly well characterized chemically. The high cofactor activity of sepia pteridine and of reduced biopterin, together with all of the other evidence available indicates that the cofactor is an unconjugated pteridine, closely related to biopterin. Its participation in the enzymatic hydroxylation of phenylalanine represents the first, and until now, the only known metabolic role for an unconjugated pteridine.

The role of ascorbic acid in the formation of noradrenalin and from DOPamine has been documented by studies *in vivo* by Kaufman and Friedman. While these results are yet preliminary they confirm earlier results which ascribed to ascorbic acid a role in the formation of noradrenalin. It should be emphasized that this is the only well documented biological role of ascorbic acid.

The work of the Section on Proteins was devoted, in large part, to a study of the structure of S–RNA. With McCully, we have succeeded in constructing a base sequence model of rabbit liver S–RNA based on base sequence frequencies determined by analysis of enzymatic hydrolysates of S–RNA. The model sequence consists of a polynucleotide chain of length 70, doubled back upon itself; about 25 contiguous bases on the one limb are specifically paired with 25 bases on the other limb in a double helix. The base-paired limbs are joined by a loop structure in the center of the chain, containing the minor components and, presumably, the site for transfer specificity. The model is consistent with a number of observations of the biological and physical properties of S–RNA.

A model which in its general features is similar has been also proposed by Wilkins and his collaborators on the basis of a study of X-ray diffraction pattern of crystalline S–RNA. It has been very interesting, and probably significant, that the same general model has been reached independently by two different approaches, namely, the one through X-ray study and the other from biochemical analysis, and that these two models have lead our group and Dr. Wilkins' group to similar suggestions and speculations.

Independent evidence for some of the features of our proposed model has been obtained by Nihei and Cantoni and further work is now underway to substantiate it further.

Important progress has been made in the direction of resolving S–RNA which is a mixture of molecular species each specific for a different amino acid into the various component species. This progress has been due to the development of a new technique of partition chromatography on Sephadex which was developed in this Laboratory by Dr. K. Tanaka, G. L. Cantoni, and Mr. Henry Richards. Preliminary results indicate that we

have obtained one of the amino acid specific S–RNA in near pure form and are now in the process of attempting to determine its base sequence.

The availability of amino acid specific S–RNA's in pure, or near pure, form opens up a large area of investigation which this Laboratory intends to develop and exploit as fully as our resources and brain power permits.

Among the areas which appear particularly promising are: (1) the full development of techniques of base sequence analysis. This development would parallel in its significance Sanger's development of the amino acid sequence in insulin; (2) a study of specific interaction between proteins and nucleic acids. Exploration in this latter direction is underway as Dr. Makman is attempting a purification of amino acid activating enzymes which specifically react with S–RNA. Specific interactions of this type are undobutedly of paramount importance in cell biology and must lie at the basis of biochemical genetics, cellular differentiation and morphogenesis; and (3) comparative structural chemistry of S–RNA.

This summary does not cover all of the projects which have been actively pursued by the members of the Laboratory, but only highlights the most important developments of the past year.

We have continued to benefit from our association with Dr. Maxine Singer, NIAMD, Dr. Samuel Luborsky, NCI, Dr. T. Nihei, Visiting Scientist from Japan in our Laboratory and now with NIAMD, Dr. K. Tanaka and Dr. H. Ishikura, Visiting Scientists from Japan. I am developing a very beneficial collaboration with Dr. V. Luzzati of the Centre de Recherches, Strasbourg, France, and Dr. S. Pontremoli, Head of the Department of Biochemistry, University of Ferrara, Italy.

LABORATORY OF NEUROBIOLOGY

Scientific Program

1. Physical Analysis of Excitability

Excitability is one of the most fundamental and general characteristics of living systems. All plant and animal cells share certain features of membrane excitability which govern the flux of ions, nutrients and cell products. The entire neuromuscular and neuroglandular apparatus of behavior depends upon mechanisms intrinsic to cellular excitability. The objective of Dr. Tasaki and his colleagues is to clarify the physico-chemical nature of the excitation processes as it takes place within the nerve membrane. During the last year, a new powerful method of investigating physico-chemical properties of the squid giant axon was developed. It was found possible to perfuse the interior of the axon with various artificial saline solutions without eliminating excitability of the membrane. During the past year, various difficulties associated with the problem of insertion of cannulae and maintenance of the flow were overcome and a large variety of new results were secured. Secondly, a mechanism for exquisite time resolution for radioisotope fractionation during passage of the nerve impulse was also utilized for analysis of ion transport.

The following results were obtained: Intracellular perfusion with isotonic potassium chloride or sulfate does not reduce the resting or action potention as it should according to contemporary theory. Conduction along the perfused portion of the cell can be maintained for hours. Furthermore, a dramatic change in concentration of intracellular potassium brought about by diluting the perfusion fluid with isotonic sucrose solution does not alter the resting or action potential appreciably. These findings contradict the widely accepted concept that the resting potential is determined by the ratio of concentration of potassium ions across the membrane. These findings lend further support to hypotheses as to the nature of the excitable membrane which have been developed in Dr. Tasaki's group. The resting potential is recognized to be not simply a diffusion potential, as presently supposed, but mainly a phase-boundary potential. The action potential is visualized as an exchange of cations attracted to fixed negative charges borne within the membrane itself.

It is possible to maintain conduction of nerve impulses for about an hour under continuous intracellular perfusion of solutions made up of sodium chloride or sodium sulfate and containing no potassium. The concentration of calcium or magnesium salts in the surrounding medium then becomes critical and must be increased. This finding indicates that intracellular potassium is not essential for production of action potentials, and that differences between sodium and potassium as

regards excitable membranes may be more quantitative than qualitative. Tetraethylammonium and other quaternary ammonium ions, quanidine, choline, rubidium, cesium and other salts may be substituted for potassium in intracellular perfusion. Conduction along the perfused zone can be maintained for about an hour providing that the divalent ion concentration in the external medium is properly adjusted.

The method of intracellular perfusion offers a unique opportunity for measuring isotope fluxes during a time-independent state. Influxes and effluxes of Na^{22}, Na^{24}, K^{42}, Ca^{45}, Mg^{28}, Br^{82}, etc., were determined during the summers of 1961 and 1962. The results of these measurements show that the permeability of the axon to sodium ion is not very different from that to potassium ion in the resting state. This indicates that the resting potential is not maintained by a difference in permeability of the two cations, Na and K. This further stresses the importance of the fixed negative charge and the two stable configurations of the axon which are the basis for the theory advanced in recent years by Dr. Tasaki and his colleagues.

Dr. Spyropoulos in collaboration with Dr. J. R. Segal of Boston conducted an extended series of observations on the electro-osmotic properties of single glass tube orifices. This was motivated by the success of Teorell's "electrohydraulic" analogue of electrophysiological activity. The advantage of the single pore is that many complications of geometry known to exist in porous membranes, living and artificial, are thereby eliminated. The model provides an explicit experimental check on Teorell's mathematical treatment of the fixed charge membrane which assumes such an idealized pore. Drs. Spyropoulos and Segal were able to study the effect of building several tubes in parallel so as to more closely imitate "membranes" and yet permit the examination of individual "quantum parts" separately.

Using tubes of about 2 micra internal diameter, in contact with solutions of potassium chloride of selected concentration, Dr. Spyropoulos and Dr. Segal detected rectification such that conductance increased when the concentrated side was made positive. This is consistent with a negatively charged glass wall. Transition from low to high conductance occurs rapidly, but after a latent period. This latency is nonlinear. At constant concentration ratios, the latency is unaffected until the concentrated solution reaches 0.01 M, above which it quickly increases. Hydrostatic pressure applied to the concentrated side decreases the latency; if such pressure is applied to the dilute side, the latency is increased. After pretreatment of the glass with concentrated thorium, which apparently binds the negative charge sites in the glass, this rapid transition in conduction is eliminated. When 100 or more tubes are placed in parallel, a smooth sigmoid curve in conductance transition is observed, as in Teorell's model. Slight diameter variations should lead to a spectrum of latencies; the coupling time constant between adjacent tubes may allow appreciable local currents. Altogether, this work on a model of what are presumed to be key parts of a membrane suggests that the contemporary theoretical treatment of the kinetics of conductance variation in membranes should be reexamined. Two theoretical physicists, Professor D. Moore and Professor E. Spiegel of New York University, have undertaken such a theoretical investigation as related to the experimental system devised by Drs. Spyropoulos and Segal.

Perfusion experiments were extended to cells of the plant Nitella and to sepia (cuttlefish) axons. Some headway was made in obtaining fractionation of *in*fluxes of radioisotopes. The action potential of the sepia can be prolonged to more than a minute, sufficient time to allow fractionation of Na^{24} and K^{42} during the action potential. Use of a newly devised platinum-platinum black microelectrode of low resistance and high current stability for examining extremely localized activity in squid giant axons yielded apparently random local lowering of resistance which increased in frequency with increasing strength of stimulating current. Partly in collaboration with Professor E. Belloni of Genoa, Dr. Spyropoulos studied the concentration profiles of radioisotopes as a function of streaming potentials: these could be related to membrane potentials and transmembrane concentration ratios during current flow. Work with Professor Teorell on nerve membrane potentials as a function of extracellular electrolyte concentration was completed by Dr. Spyropoulos.

2. Mathematical Analysis of Visual Perception

Mrs. Marimont continued her investigation of visual mechanisms from the viewpoint of physical theory, mathematics and formal logic. She made substantial progress in the formulation of a mathematical model which fits various aspects of visual

perception. Her model for brightness discrimination has now been extended by attempting to combine the space-dependent model she developed with the time-dependent models of Dr. D. H. Kelly and others. Even in the investigation of an obviously nonlinear system, Mrs. Marimont feels that a careful assay of the applicability of methods of linear analysis is necessary for an evaluation of current experimental results and deductions drawn from them. Mrs. Marimont hopes to be able to convey the implications of this mathematical treatment in such a way that they can be understood by nonmathematically trained biologists eager to use new concepts and techniques but uncertain respecting their application.

The present model for brightness discrimination includes an averager (eventually incorporating both space and time), a differencer and a variable gain amplifier. The basic model is improved by addition of a local averager, which yields edge effects, and a coupling capacitor which yields the effect of instability of the retinal image. Further progress has been realized in applying this model to physiological systems involved in vision, e.g., the local averager probably corresponds to a neural configuration extensive enough to include a central excitatory and surrounding inhibitory region.

3. Modification of Activity in Sensory and Non-Sensory Corticipetal Pathways

Dr. Gabriel Frommer devised experimental means to compare responses conveyed along a classical sensory pathway (the trigeminal, which conveys facial sense data from brainstem nuclei to cortex) with responses taking place along a nonsensory pathway (cerebella nuclei projecting to cortex). Each of these pathways relays through the thalamus, by way of different nuclei; they project into neighboring regions of sensorimotor cortex. The operational comparison is designed to give insight into whether sensory pathways are modulated independently and differentially from other projections.

Recent experimental work here and elsewhere has demonstrated that sensory activity in response to standard sensory stimuli can be modified by stimulation of the mesencephalic reticular formation, portions of the cerebral cortex and cerebellar cortex, among other sites. Further, activity in sensory pathways is functionally modifiable in parallel with the state of the EEG and behavioral arousal, and in accordance with previous experience and expectations of the animal in respect to the stimuli being tested. Therefore, it is important to establish whether these two distinctly different functional systems may be subject to interdependent and perhaps generalized controls or whether the modulation of sensory signals is a primarily sensory control phenomenon.

Single and repetitive test shocks were delivered to the cerebellar nuclei projecting to cortex and to the relay nucleus, ventralis lateralis, in the thalamus. Similarly, shocks were delivered to the bulbar trigeminal nucleus and to the trigeminal relay nucleus, ventralis posteromedialis, in the thalamus. The effects on cortically evoked responses from these four subcortical sites during different levels of arousal, as defined behaviorally and by the EEG, were analyzed. In the quiet alert cat the cortical response to cerebellar stimulation consists of a short latency biphasic response followed by slow waves. Greater arousal, or movement, are associated with slight attenuation of this response. Drowsiness and large amplitude EEG waves are associated with an increase in latency and decrease in amplitude to the point of complete disappearance of the late components and fluctuations in amplitude and often complete disappearance of the early biphasic response. In contrast, the trigeminal response consisting of a short latency monophasic wave in the quiet alert cat tends to disappear during arousal and to increase during drowsiness and sleep. One can observe these opposite effects taking place simultaneously in the same animal when both the cerebellar and trigeminal circuits are implanted.

Results indicate that plastic modifications do take place in both the sensory and the nonsensory pathways in association with different levels of arousal. The two functionally different pathways are both subject to central control, but the effects are essentially the opposite. The nature of these differences in central integrative control may provide insight into the functional significance of brain events relating to perceptual experience and sensorimotor coordination.

4. Longitudinal Organization of Sensorimotor Coordination

Although considerable is known from an electrophysiological point of view about segmental re-

flexes, much less is known about reflexes which link together different levels of the neuraxis. Although a long descending propriospinal pathway was recognized more than 20 years ago, studies in this Laboratory, under the leadership of Dr. Bo Gernandt, pioneered in demonstrating that there also exists an ascending propriospinal reflex system. This is weaker than the descending projection and requires either strychnine or the facilitative influences of the brainstem reticular formation to be manifest. Both the descending and ascending propriospinal systems are diffusely projecting, that is, they influence both sides of the spinal cord and provide impulses that cross, recross and may re-recross, decussating abundantly at each level of the cord.

Another long intersegmental sensorimotor coordination system discovered in this Laboratory involves reflexes which ascent the spinal cord to the bulbar brainstem from whence they reenter the spinal cord. This latter spinal control system involves a supraspinal loop which passes through the bulbar reticular formation. This system can be activated from every sensory level of the spinal cord. Impulses cross at the level of sensory input and ascend on both sides of the spinal cord without emitting motor responses during their entire upward trajectory. The upward path of this supraspinal loop circuit is much more rapid in conduction than is the direct ascending propriospinal pathway. Thus, impulses from lower spinal segments, after relay in the bulb, descend to exert motor effects at cervical spinal levels at the same time that motor effects are being expressed under the influence of the direct ascending interlimb reflex system. Also, sensory signals from the hind limbs can modulate forelimb reflexes simultaneously via both the direct propriospinal system and the supraspinal loop projection. Thus, events taking place in each of the pairs of limbs will find expression in the other pair of limbs via both direct and indirect projections simultaneously. The importance of these two sensorimotor coordination systems in the control of walking, running, jumping, and balance is obvious.

This work was initiated by Dr. Gernandt and further extended by Dr. Muneo Shimamura. Dr. Shimamura demonstrated for the first time the interrelationships between cranial sensorimotor and spinal sensorimotor mechanisms as relating to the bulbar relay. Thus, direct and indirect ascending influences of spinal origin reach cranial motor outflows, the indirect pathway being relayed in the bulbar reticular formation. Similarly, impulses of cranial sensory origin have both direct and indirect access to spinal motor mechanisms, the indirect control being exercised through the same bulbar center. These findings provide neurophysiological foundations for the classical cranial nerve reflex effects on spinal motor activity: visual and vestibular righting reflexes, head and neck righting reflexes, auditory startle reflexes, etc. Reverse effects, namely, spinal sensory effects on cranial motor outflow, are also evidently accommodated by these projections. At last the head and body are reflexly linked together neurophysiologically.

Professor Konrad Akert, recently named Director of the Brain Research Institute in Zürich, elected to spend his first 5 months with that new title by assigning himself to the Laboratory of Neurobiology while his new Institute was under construction. Professor Akert and Dr. Shimamura collaborated on a thorough analysis of the relationship of both propriospinal and spino-bulbo-spinal reflexes to their specific peripheral nerve inputs and outputs. They investigated cutaneous and muscular afferent inputs and efferent connections to intrafusal and extrafusal motor units in both flexor and extensor muscles. Spino-bulbo-spinal reflexes are characterized as primarily cutaneous afferent in origin, propriospinal primarily motor afferent in origin. Propriospinal projections relay impulses to both flexor and extensor motor units bilaterally, whereas spino-bulbo-spinal reflexes of cutaneous origin involve flexor muscles and spino-bulbo-spinal reflexes of extensor muscle afferent origin involve extensor muscle activation. The excitability of both reflexes is systematically increased and decreased by neck proprioceptor activation and by activation of stretch receptors in the limb muscles.

Taken together, we now have a very much clearer notion of how the nervous system is stitched together in its input-central-and-output relations, not simply at the segmental level but all the way between highest and lowest sensorimotor components. It is now possible to identify functionally distinct central projection and relay centers which will account for longitudinal sensorimotor reflex coordination all along the neuraxis and which involve characteristic differential input and output relations.

5. Somatic and Autonomic Convergence on Vagal Outflow

It is abundantly clear from studies conducted by Drs. Gernandt, Shimamura, and Akert that the bulbar reticular formation is the focus for convergence of impulses from many directions and that it constitutes a synaptic nexus for elaborate central controls relating to both spinal and cranial sensorimotor coordination. Since the bulbar neural structures thus implicated include the respiratory and cardiovascular "vital centers," it is only natural to inquire how autonomic mechanisms enter into this coordination, both as affectors and effectors.

Professor Akert collaborated with Dr. Gernandt in a fascinating analysis of the hierarchy of somatic and autonomic convergence on vagal motor outflow. They discovered that a most powerful influence is exerted by the respiratory center, less by vestibular and limbic structures, and least by vagal and trigeminal inputs. By using vestibular, trigeminal, vagal, and limbic stimulation, and double combinations of these in any order, and by recording spontaneous and evoked vagal efferent discharges, they were able to visualize activity of the respiratory center in relation to these other variables in the competition for access to vagal motor outflow. Many of these mechanisms are implicated in very elaborately integrated and little understood syndromes such as motion sickness. Interactions between vestibular and limbic influences on vagal outflow may occur in the brainstem reticular formation, in some region above the inferior colliculus but probably not in the hippocampus itself, and in cerebral and possibly also the cerebellar cortex.

6. Electrical Activity of Hypothalamic Feeding Center

Techniques developed in this Laboratory, largely through the efforts of Dr. Arnold Starr and Mr. Ron Sandlin, make it possible to observe quantitative changes in nervous activity over long periods of observation. This permits the search for "tides" of nervous activity which may correspond to changes in level of appetite and satiety, thirst and satiety, sexual drive, and many other functions relating to appetite and motivation. Heretofore such mechanisms have had to be analyzed through either gross stimulation or destruction. Previous methods of recording have thus far proven equivocal. Now, using recently developed techniques, it may be possible to design far less disturbing and more physiological experiments for recording during feeding, etc., and during sessions involving conditioning, extinction, etc., where expectations and purposes as well as learning and memory can be deliberately analyzed. This enterprise is in line with long-standing mutual interests shared between this Laboratory and that of Professor Jerzy Konorski at the Nencki Institute in Warsaw. Fortunately, Professor Konorski was able to arrange that one of his ablest collaborators, Dr. Wanda Wyrwicka, spend a few months in the Laboratory of Neurobiology.

Dr. Wyrwicka implanted a number of cats with bipolar electrodes directed to the lateral hypothalamus in the region of the feeding center. For purposes of identifying the feeding center more exactly, electrical stimulation is applied in this area in completely satiated animals. In appropriate loci even weak stimulation will impel the animals to eat again during stimulation. Following such identification, electrical activity of the feeding center is recorded using integrative techniques developed here. Dr. Wyrwicka finds that electrical activity increases in the feeding center during spontaneous eating, but this appears to be true also for a number of other regions in addition to the feeding center and, therefore, is likely to be a relatively nonspecific response or activation. She has observed differential effects, however, using classical conditioning techniques. After some repetitions of tone preceding food reinforcement, an increase in activity localized to the feeding center is observed. Inhibitory differentiation is also seen. Since these experiments are still in progress, nothing more can be said than that this method appears useful to combine with classical and instrumental conditioning techniques for analysis of appetitive centers which are so important in the specific and generalized control of somatic and visceral behavior.

7. Action of the Tympanic Muscles

It has been demonstrated long ago that the middle ear muscles have the capability of markedly attenuating sound induced mechanical energies delivered to the cochlea. This muscular control, which is reflexly induced by sound, has been supposed to be essentially a protective

reflex for safeguarding the inner ear from acoustic damage. Dr. Arnold Starr and Dr. Peter Carmel undertook to investigate the attenuation of microphonic responses recorded via a chronically implanted electrode placed against the round window of the cochlea. Dr. Carmel extended these studies by severing either or both of the muscles and the eighth nerve in order to study the role of the individual muscles in both crossed and uncrossed reflexes in response to sounds of various intensities. Later Dr. Carmel devised means to record the electrical activity of each of the muscles (EMG) continuously by means of implanted bipolar electrodes. Dr. Tasaki and Dr. Carmel were able to perform acute experiments using microelectrodes for recording unitary responses of individual muscle fibers within these tiny muscles.

It was immediately apparent that attenuation of the round window response did not depend solely upon high energy sound stimuli. The tympanic muscles were activated by even quite subtle sounds. Moreover, they were active whenever the animal moved, even though there was no change in the sound environment. It appeared as if the middle ear muscles might be stabilizing the fluid columns in the inner ear during head movements, perhaps to preclude the internal induction of false noise, or perhaps to preclude damage to the delicate hair cell apparatus in the cochlea which might presumably result from fluid motion. It also was clear that in the case of sounds generated by the animal's own vocalization, middle ear muscular contraction *preceded* the onset of cochlear microphonics. Even yawning, swallowing and occasionally eye blinking are accompanied by middle ear muscle contraction. Finally, cutaneous stimulation of the pinna and the external auditory canal, even on the side deafened by previous eighth nerve division, results in reflex contraction of the middle ear muscles. Physiological evidence of several kinds indicate that the middle ear muscles have reflex patterns which are similar to those analyzed in larger skeletal muscles known to have muscle spindles. Either the middle ear muscles have spindles or else they have something functionally equivalent to the muscle spindle feedback control loop circuit.

In short, Drs. Carmel and Starr found evidence that the middle ear muscles behave reflexly and also in response to central commands in many ways analogous to other sensorimotor circuits in the spinal cord and brainstem, and not exclusively or even preponderantly as protective devices. Dr. Carmel went on to show that the acoustic reflex could be "conditioned" by acoustic, nonacoustic, and other experiential factors. More recently, the central nervous mechanisms governing these processes have begun to be explored: factors relating to sustained middle ear muscle contractions in response to continuing sound depend, directly or indirectly, on structures lying above the level of intercollicular decerebration; the anterior lobe of the cerebellum is apparently involved in patterning the firing of individual muscular units. All of these results have been published, accepted for publication, or presented before national or international meetings.

8. *Physiological effects of prolonged sensory stimulation*

Dr. Starr developed techniques whereby four or more local components of the entire classical auditory pathway could be simultaneously monitored for "spontaneous" electrical activity and responsivity. The recording system he and Mr. Ron Sandlin developed provides an integrated record during ongoing experiments such as might be produced by elaborate computer techniques. In this case, they continuously integrate the total complex of electrical activity locally picked up by fine electrodes in a system whereby the parameters of integration can be readily selected for optimum physiological differentiation. This is the same system referred to above in the experiments of Dr. Wyrwicka involving recording from the feeding center in the hypothalamus.

Dr. Starr elected to study responses of the auditory pathway before, during, and after prolonged steady sound stimulation. He found that there are distinct differences in magnitude and dynamics of response among the various ganglionic stations along the auditory pathway. There are, moreover, substantial differences in these responses according to whether the animal is awake, asleep, or anesthetized. Background activity is vastly altered as well as responsivity to sound: there is a slight reduction of background level of activity at the cochlear nucleus, but this is more marked in the superior olive and especially in the inferior colliculus; the medial geniculate is not much affected;

the cortex shows an apparent increase in background activity during anesthesia.

There are substantial changes in dynamic response of various auditory stations during and following prolonged sound stimulation. At the round window, cochlear nucleus, superior olive, and inferior colliculus, there is a sharp, large amplitude increase in electrical activity associated with onset of sound, and a sharp decrease associated with discontinuation of sound. Following onset, however, there is a gradual rise in activity during continuing steady stimulation. This rise is *controlled centrally* via the middle ear muscles which gradually reduce the degree of their attenuation of sound during continuation of the same sound stimulation. Following discontinuation of sound, there is a marked depression of electrical activity to below resting (pre-stimulus) levels in each of these lower stations. This depression is under the control of an entirely central circuit which is not completely inactivated by anesthesia. It appears as if the middle ear muscle mechanisms for attenuation of sound energies may be employed initially for quick effects, which are sustained under the direct or indirect control of nervous centers lying above the level of decerebration (see above). A slower, completely central process, however, which is slower to be discontinued at the end of sound stimulation, apparently gradually takes over during the phase of middle ear muscle gradual relaxation.

The work of Dr. Starr was pioneering in two other important general respects: in his utilization of *prolonged stimulation* in contrast with the usual neurophysiological practice of using brief stimuli, and in his attempt to examine the *whole column of a given sensory circuit simultaneously* so that the several parts could be compared with each other in the same animal under the same conditions. These ventures have proven advantageous. In all respects, the generalizations derived from these experiments have borne out the pertinent data provided by Dr. Tasaki, Dr. Galambos, Professor Katsuki, and others on single unit responses in the auditory pathway. Dr. Starr's techniques also powerfully reinforce these data inasmuch as his recordings reflect the activity of larger cellular aggregates. Dr. Mary A. B. Brazier and Dr. W. Ross Adey have recently emphasized the special value of this type of recording from the point of view of averaging purposes.

As in the experiments of Dr. Wyrwicka, this system of stimulation and recording is proving particularly suitable for adaptation to conditioning techniques. Therefore, when Dr. David Galin returned to this Laboratory, and Dr. Starr left to assume a research post with Professor Fritz Buchthal in Copenhagen, Dr. Galin elected to extend Dr. Starr's program in the direction of determining how conditionable the auditory pathway may be.

Dr. Galin has been recording spontaneous and evoked electrical activity along each station of the classical auditory pathway when sound signals are associated neither with punishment nor reward, and when accompanied by either positively or negatively reinforcing stimuli. He is attempting to investigate these mechanisms under conditions of both classical (Type I) and instrumental (Type II) conditioning. There is evidence that the inferior colliculus and the medial geniculate body are different in their reactions to positive reinforcement conditioning as that affects the background activity at these two sites. Ultimately, Dr. Galin intends to record from hypothalamic feeding centers à la Wyrwicka, during conditioning experiments using food reward in association with differentiable acoustic signals.

A principal objective in all of the Laboratory's scientific and academic undertakings has been to attempt to cut across the overwhelming complexity of biological and neurophysiological phenomena. What is already known and what yet awaits disclosure constitutes an immense and virtually inexhaustible source for experimentation and conceptualization. It is certainly not difficult to find worthy research to undertake. What chiefly separates us from our best achievements, however, is our inability to discriminate adequately how best to dole out our resources of energies and of time. What is *most worthwhile* to do is perhaps the most important question to which we might address ourselves. *As a matter of principle, then, we have elected to seek an understanding of a few general and comprehensive control mechanisms, the understanding of which might lead to a more valid picture of how living things operate.*

How does a cell membrane exert its control over the differential ionic compositions inside and outside the cell? What happens within this system

during the nimble transitions of a nerve impulse? What backs up the membrane in the way of metabolic supports for its activities, and what is the coupling between metabolism and action? How do dendrites and cell body differ from axon in excitability and transmission properties? What are the engines of physiological triggering in a single neuron and in a cascade of neurons? How, out of the complexities of organization in ganglionic constellations, arise regularities associated with the balance between excitation and inhibition? How do complex systems operate as goal-seeking mechanisms?—At the level of individual spinal segments?—At the level of longitudinal controls which knit together the lengthy neuraxis?

We have long been accustomed to recognizing goal-seeking principles underlying motor performance; now we are beginning to be confronted with evidence strongly implying that *sensory systems may be similarly goal-oriented.* In this case the goals are defined by previous experiences, expectation, and purposes as to actionable potentialities. Our sense of objects has long been known to be affected by our idiosyncratic experiences and the momentary flux of our aims and anticipations. Now we are granted the opportunity to sort out the physiological processes involved. Operations on sense data are begun at the farthest outposts of sensory reception and evidently take place all along the sensory pathway. The modification of sense data does not wait until the sense data have risen within the nervous system, and until they arrive at some lofty and consciously accessible screen, much as we would prefer that to be the case. *Our only contacts with the outside world are by way of dynamic physiological, conditionable systems.* The practical and philosophic consequences of this fact are more far-reaching than we ordinarily suppose.

How does control of ions arise within the organization of membranes? How does control of limbs arise within the organization of spinal segments? How does control of interlimb and spinal and cranial sensorimotor patterns arise within the longitudinal organization of the neuraxis? How does control arise within that peculiar tympanic motor system which so flexibly manipulates our acoustic world? How does control arise within the organization of sensory channels and appetitive centers? How does experience exert its predictable effects on behavior and, internally expressed, on controls operating on sensory channels and appetitive centers as well as motor patterns?

Administration of the Laboratory

Late in the year 1962 Dr. Frederick L. Stone extended a most cordial invitation to join his staff in the extramural program of the NIH. This report, therefore, is the last tally, completing my official associations with the Laboratory of Neurobiology. The hard-working and happy supporting staff is made up of individuals each of whom has vastly perfected his contributory skills. Each has consistently acted as if it were a privilege to take on more and more responsibilities for the shared intellectual and experimental adventures undertaken. I never met such a high spirited and idealistic group: Mrs. Marie Davies, Mrs. Ina Lee Kamp, Mrs. Zelda Wolk, Mrs. Lydia Nobuko Tasaki, Miss Mary Fran Roark, Miss Patricia Kenny, Miss Joyce Stichman, Mr. James Washington, and Mr. Ron Sandlin, individually, and as a devoted team, made possible the accomplishments of the scientific staff.

Now, I pass along the baton of administration to a staunch friend and respected colleague, Dr. Ichiji Tasaki, and extend to him and to the entire lab family all best wishes.

LABORATORY OF NEUROCHEMISTRY

Physical Chemistry Section

In 1962 the work of the section continued to emphasize optical, physical and biological properties of biopolymers and optical properties of dye-polymer complexes. With the transfer of two staff members to another Institute and the acquiring of new staff members, several projects carried on in previous years were discontinued and some new projects were begun.

For some years the section has studied the striking metachromatic shift of the absorption band of a cationic dye toward longer wavelengths (blue shift) when the dye molecules aggregate in solution of bind to a polyanion such as a polynucleotide or a polysaccharide. This blue shift is the basis of the metachromatic staining of tissues. The anomalous optical rotatory dispersion (Cotton effect) of a dye-polyanion complex is also of great interest. There is a good chance that the experimental and theoretical knowledge being gained will soon make it possible to have a comprehensive

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understanding, in quantum mechanical terms, of the basic physical mechanisms of the metachromasy and anomalous rotatory dispersion phenomena and of the relation of these phenomena to the biopolymer secondary structure which induces them.

Experimentally it was established that in a series of polyribonucleotide oligomers of increasing chain length, in which the monomer unit is either adenylic or cytidylic acid, the metachromasy of oligomer-bound acridine orange dye first becomes significant when the number of monomer units in the oligomer reaches five. The theory of metachromasy, on the assumption of strong coupling exciton interaction between adjacent dye molecules, was advanced to the point where distances between nearest neighbors can be computed by simple formulae from the observed blue shift. A new theory of the blue shift was developed in which weak coupling (rather than strong coupling) interaction between dye molecules causes absorption intensity to be transferred from one electronic band to another in the visible spectrum of the dye. New theoretical approaches were also made to a related problem, the dependence of the absorption intensity of a chromophore on the molecular environment (solvent or other chromophores).

Information about dye-polymer interactions and the structures of some biopolymers was obtained from dye-polymer complexes by means of absorption spectra, emission (fluorescence) spectra, optical rotatory dispersion and X-ray diffraction. A complex between the colored antibiotic actinomycin D and DNA was observed from a change in the absorption spectrum of the actinomycin; the direction of the spectral shift (toward the red), the ratio of actinomycin bound per DNA base pair (1:5) and the lack of a Cotton effect are all consistent with the absence of dye-dye optical interactions and the presence of dye-polymer interactions. Changes in the absorption spectra were observed when colored tryptophane derivatives were allowed to bind to the active enzymic site of α-chymotrypsin. The intensity, wavelength maxima and depolarization of the fluorescence of dyes bound to acid polysaccharides were studied at different dye to polymer ratios and the results were interpreted in terms of a varying amount of dye-dye interaction.

For the first time, an ordered secondary structure was demonstrated for a natural acid polysaccharide, λ-carrageenate, by observing a Cotton effect in the optical rotatory dispersion spectrum of dye complexes of the polysaccharide; this secondary structure (at present unknown) is probably important for the biological functioning of polysaccharides and mucopolysaccharides as it is known to be important for nucleic acids and proteins. An investigation by X-ray diffraction of fibers of DNA complexed with the dyes proflavine or acridine orange indicated that the dye molecules bind to the outside of the DNA double helix under the conditions of the experiment rather than, as others have suggested, becoming intercalated between the base pairs of the DNA; this finding means that the mechanism whereby proflavine is able to cause mutagenesis in bacteriophages may not be a simple physical replacement of a DNA base pair as believed by some workers.

Two new projects are concerned with understanding certain optical and physical properties of nucleic acids through experiments on model compounds. One project is to measure the emission from optically excited molecules of mono- and oligo-Nucleotides to provide information about the nature of the singlet and triplet excited states of nucleotides and nucleic acids. It was found that both the fluorescence (short duration emission from singlet states) and phosphorescence (long duration emission, probably from triplet states) of 5′-adenylic acid have characteristic excitation and emission spectra which shift in wavelength with quenching of the emission on going to polyadenylic acid oligomers. The other project is to test the hypothesis advanced by others that the native double helix secondary structure of DNA is stabilized, perhaps to a dominant extent, by so-called "hydrophobic bonds". The denaturation temperature of the helical complex of polyadenylic acid with polyuridylic acid was measured in the presence of anionic, cationic and nonionic detergents which should be able to break "hydrophobic bonds". Little effect of the detergents upon the denaturation temperature was found, casting doubt upon the presence of significant amounts of hydrophobic bond stabilization in helical polynucleotides.

Two immunological projects have been initiated in collaboration with investigators in NIAID and

NHI. The first is a beginning step toward understanding the chemical basis of immunology by identifying and analyzing the specific polypeptide chains of gamma globulin which are responsible for a given allotype. To do this, the gamma globulin or rabbits of known allotype was degraded chemically and enzymically into its constituent polypeptide chains which were then individually tested for their ability to precipitate with antiserum directed against the allotype present in the original gamma globulin. It appears that a given allotype may be localized on one kind of polypeptide chain of the gamma globulin macromolecule.

The second immunological project gives hope of leading to a treatment of "autoimmune" diseases in man, against which there has been little therapeutic success up to now. The disease investigated in the laboratory was antikidney serum nephrosis which occurs when rabbit antiserum against rat kidney is injected into a rat. It was found that a fraction of the gamma globulin of the antiserum with a sedimentation coefficient of 7S is responsible for producing the nephrosis in the rat. However, univalent or divalent fragments which were produced enzymically from the 7S fraction of the antiserum, while they do fix to kidney tissue, produce at most only a transitory nephrosis in the rat. Furthermore, these fragments are known to be unable to bind complement. It therefore seems that in addition to antiserum, the disrupting effect of complement may be necessary to induce nephrosis. If this is so, it may be possible to treat an autoimmune disease by temporarily lowering or removing complement from an animal or person. This exciting possibility is now being investigated in the model rat system.

LABORATORY OF NEUROPHYSIOLOGY

The Limbic System program has continued at a vigorous pace, using the squirrel monkey as its chief subject. Photic stimulation of the eye has been found to be followed by electrical signs of activity in the hippocampus in a preparation under chloralose. Unitary analysis suggests that the activation is through complex relay systems via the cingulum and entorhinal cortex. There is good reason to believe that the limbic system is importantly involved in simple conditioning and these observations suggest pathways.

Another project was aimed at elucidating mechanisms in the brain that are intimately concerned with regulation of blood pressure and cardiac rate. The carotid sinus response to occusion and stretch was observed while electrically stimulating and after removal of various regions of the brain. The results indicate that the blood pressure and heart rate reflexes from sinus stretch and decompression are under the tonic influence of structures lying within the diencephalon, mesencephalon and cerebellum. The depressor response to sinus stretch appears to depend on the integrity of the rostral pons in which the nucleus reticularis tegmenti pontis is possibly an essential element. The excitability of the pressor component of the reflex may be altered phasically by central neural stimulation independently of change of systemic blood pressure. This, and the findings with respect to various lesions indicate that the two aspects of the reflex, pressor and depressor, are not reciprocally related but, on the contrary, may vary independently. Hence it is probable that at least two systems of neurons regulate this reflex.

The medial frontal lobe of the squirrel monkey has been mapped for functional control of heart rate in concurrent observations on the penile erection studies. The majority of points at which electrical stimulations result in marked parasympathetic effects on the heart fall in the same region of the precallosal cingulate gyrus in which stimulation elicits parasympathetic vascular changes in the genital organ.

As part of a general interest in the histochemistry of nerve cells of the brain, the squirrel monkey brain has been surveyed with aldehyde-fuchsin stain. It has been found that groups of cells within a number of phylogenetically ancient nuclei of the brain contain densely packed granules that stain an intense purple with aldehyde-fuchsin. These cell groups stand out distinctly from surrounding neurones and thus make possible a further subdivision of the nuclei in which they are found. Preliminary findings with special stains suggest that the positive staining granules contain a glycolipoprotein.

A systematic study has been done on the role of the amygdala in the regulation of heart rate. This study has added further information about the powerful influence of this region on vagal function and has demonstrated that vagal excita-

tion may occur without the reciprocal sympathetic inhibition. It represents the first time that the amygdala has been systematically explored in regard to cardiac changes and has also shown for the first time that cardiac extrasystoles, often lasting for periods of minutes, may result from stimulation in this region.

Prior to the work begun in this Section, the literature contained little evidence that brain stimulation could elicit overt sexual responses. A recent study on sensory mechanisms in conjunction with this Section's previous findings on effector systems has suggested for the first time sensorimotor pathways that are essential for certain autonomic aspects of procreational behavior. In addition this work has indicated that certain nuclei of the thalamus heretofore looked upon as "nonspecific" in function, represent respective parts of the body and may participate in the appreciation of such primitive sensations as tickle, itch and pain. The brain mapping has also suggested how these primitive somatosensory nuclei—if they may be regarded as such—are linked to the hypothalamus. Finally, this work together with the Section's behavioral studies on small groups of squirrel monkeys has relevance to such unresolved questions as the evolution of visual influences in psychosexual functions and the origin of a sense of modesty in dress.

This Section has carefully worked up a very useful stereotaxic atlas of the brain of squirrel monkey which will be valued by investigators throughout the world. It is in press at the Government Printing Office.

The program of the Section on Membrane Physiology is directed in the general area of the ubiquitous excitable membrane and is currently investigating muscle fibers. The surface membranes of both nerve and muscle fibers are electrically excitable, i.e., a wave of self-propagating electrical activity travels along it. Ionic currents carried by sodium and potassium movements are responsible for this activity and the mechanism of electrical excitability has many features common to nerve and muscle. In muscle, however, there is an additional mechanism for the passage of potassium into or out of the cell. This mechanism is not necessary for the production of electrical excitation. An explanation of the additional passage of potassium that evokes the submicroscopic internal tubular structure of muscle is in accord with many of the experimental observations. The tubules (endoplasmic reticulum) could be the pathway for the additional passage for potassium. Such a system could relay the electrical activity from the surface membrane of a muscle fiber to the internal trigger areas for contraction. Some mechanism of this sort is necessary to activate the internal contractile process with only a short delay after the electrical activity at the surface. The one considered here is admirably suited for this purpose.

It has been found that hypertonic solutions cause a prolonged afterpotential which can be explained by an increase in volume of the endoplasmic reticulum and mathematical models computed in collaboration with the Biometrics Branch provide concordant support. Electronmicroscopy also shows enlarged endoplasmic space after the fibers are exposed to hypertonic solutions, a remarkable confirmation for this model.

This Section is also conducting an analysis of the relatively large erythrocytes of Amphiuma, an amphibious Mississippi River eel. The electrical properties of the cell membrane and the nuclear membranes are being investigated.

Two years ago a project was launched by the Section on General Neurophysiology to determine if, as had been previously reported in the literature, there was a significant change in pH of the cortex during the passage of a wave of spreading cortical depression. This was carefully worked up and answered in the negative. It was also determined that in anoxic conditions there was little change in pH values as long as circulation could be maintained, even though the cortical surface drifted steadily negative. The surface of the cortex commonly reads electrically negative for many hours after death. This provided additional inference that the long-lasting negativity after death was essentially neuronal in source and not due to restrictions of H ion transport across a pia-glial boundary.

This work immediately led into an examination of the Gesell phenomenon and the use of this phenomenon for some concise tests on mechanism of the blood brain barrier. Since the original observations by Ehrlich in 1885 it has been known that there appeared to be a striking difference between the CNS and all other regions of the body in the transport from blood to tissue of many kinds of dyes, chemical agents, charged ions, toxic agents and drugs. Despite the fundamental and practical

importance of this phenomenon the question of the existence or nonexistence of a unique and identifiable barrier is still considered by many to be a controversial matter. There is certainly no agreement about the location of the boundary. The barrier has been located at, or in, the capillary endothelium, attributed to the low extracellular space of the CNS, to the glia system, and the ground substance. There is controversy about any structural differences between CNS capillaries and other capillary systems. Electromicroscopists deny the existence of the ground substance. Others contend that the barrier is largely illusional on basis of selective affinity of dyes and test substances for the blood proteins or the selective metabolic demands of the CNS neurons.

Accordingly the system for measuring cortical pH changes to 0.02 of a unit developed in this Laboratory was applied to study the Gesell phenomenon. This is an anomalous acid shift of the cerebral spinal fluid in the subarachnoid space which occurs if sodium bicarbonate is injected into the blood stream. The blood moves alkaline and the C.S.F. moves acid. This is presumed to be caused by the fact that the blood brain barrier restrains bicarbonate and allows CO_2 to pass freely from capillary into the extracellular space. The Gesell phenomenon was first thoroughly confirmed. (Since discovery of this phenomenon in 1926 by Gesell and Hiertzman it has been denied by several investigators right up to the present time.) A great deal of work was done which supported the hypothesis that the effect was due to differential penetration of CO_2 and HCO_3.

A most important feature of this work is the clear proof that the Gesell phenomenon is one case of an unequivocal blood brain barrier mechanism.

The effect of various agents known or suspected to be effective in breaking down the blood brain barrier were then systematically applied to the cortex and the Gesell phenomenon used as a test for the efficacy of these agents. It was found that the alcohols from 1 to 4 were very effective in the ascending order, alcohols of higher number showed a progressive decrease in effectiveness. Certain surface active agents were also very effective, as were also hypertonic solutions of simple salts. Massive carbonic anhydrase inhibition did not affect the reaction. Anoxia, hypercapnia and very strong metabolic inhibitors were relatively or entirely ineffective. These results strongly support the hypothesis that some membrane system was physically altered and that metabolic pathways are not concerned in this particular example of blood brain barrier. These results furnish strong support for the hypothesis that the barrier for the bicarbonate ion is in the capillary wall.

A correlative finding was that spreading cortical depression did not transiently alter blood brain barrier to bicarbonate ion.

The project on synaptic transactions in the lateral geniculate nucleus of the cat is continuing. Several years ago investigators in this Laboratory discovered and identified the phenomenon of second subnormality and determined that it was an anesthetic effect. This has been denied by another laboratory so the phenomenon was reexamined using implanted electrode preparations. Our original conclusion was thoroughly confirmed and the error was shown to be due to inadequate tests for viability of brain stem sectioned preparations on the part of the challenging laboratory.

Work is continuing on this project in a two-phase attack. One is to attempt to secure electrical impedance measurements which may reveal to what extent this is associated with changes of extracellular space, changes of which would indicate volume changes of dendrites, teleodendrons or glial cells. Another phase of the work involves unitary recording to determine if there is a change in excitatory postsynaptic potentials associated with second subnormality. Solution of this fascinating question would significantly extend knowledge of synaptic mechanisms.

During this year the technical staff has developed a new electronic programmer accurate to 10 msec on a switch stop system which is conveniently set and read. A photographic method for constructing circuit boards has been worked out to a very practical operation.

CLINICAL NEUROPHARMACOLOGY RESEARCH CENTER

1. Further Developments in the Clinical Program

The past year has seen a further encouraging evolution of the St. Elizabeths-NIMH program towards its stated goal of providing, within a typical public mental hospital, an advanced research center in Clinical Psychiatry, and in some

relevant areas of the Basic Sciences. Last year's report noted the conversion of a Chronic Care facility into an Acute Admission Service. This process continues. The William A. White Building now houses comprehensive clinical facilities for hospitalized and ambulatory patients. It comprises some 200 beds, a Followup (and Out-Patient clinic); and a Day Care facility for the management of transitional cases. Hopefully, the various clinical services can now begin to serve the long-term goals of a program of clinical investigation. The studies so far envisioned fall into eight major areas as outlined below; they can be expected to mature into detailed projects within the coming year.

(*a*) *Clinical Psychopathology*

Nosological diagnoses are not only controversial in terms of etiology and epidemiology, but manifest considerable difficulties in the execution of clinical research. It has long been apparent that sample selection cannot be based on diagnostic entities alone. Most of the major diagnostic categories contain subgroups characterized by behavioral and symptomatic polarities: e.g., manic as well as depressive syndromes within the affective disorders; retarded and agitated depression within the group of the endogenous depressions; apathetic and withdrawn as against excited and ecstatic patients, both belonging to the catatonic group; intensely introverted "simple" schizophrenics as against the extroverted "histrionic" hebephrenics. Neither on logic nor empirical grounds can it be assumed that these patients do, or in fact could, respond in a uniform manner to the same treatment which is believed to attack an identical, though hypothetical, disease process. While it would seem unjustified to dispense with diagnostic categories, a "double bookkeeping" method has been used in the William A. White Service to complement these categories over the past year. This method maintains a carefully formulated diagnostic category, and adds an elaborate psychopathological profile, listing the specific constellation of behavioral, experiential and somatic manifestations of a particular clinical state. Such a psychopathologically oriented approach would seem to promise a more realistic basis for correlating pathological phenomena with therapeutic and prognostic response patterns. The concept of "target symptoms" was originally introduced to study the effects of psychoactive drugs in an effort to determine what constitutes drug-treatable psychopathology. It is now apparent that this method may lend itself to a more general application in the investigation of the modifiability of symptoms and dysfunctions in both psychotic and nonpsychotic disorders.

The development of comprehensive scales of psychopathological variables for recording on IBM cards has begun. In this scheme the conventional "mental status" examination has been replaced by a psychopathological profile which will, it is hoped, help the admitting physician to focus more clearly on the individual aspects of symptomatology and personality structure. This study is being related to another study on the relevancy of psychological test material to clinical observations, and the validity of psychological test predictions in terms of immediate and long term therapeutic prognoses. Another aspect concerns the influence of the psychologist's report on the psychiatrist's attitude toward making a diagnosis and formulating a therapeutic plan.

(*b*) *Clinical Psychopharmacology*

The study of new compounds, the therapeutic assessment of various established neuroleptic and antidepressant drugs (used singly and in combinations), the search for individuality differentials relevant to response patterns, and finally the development of immediate and long term criteria for evaluating the therapeutic impact of drug treatment form major objectives of studies in this area.

Because of the lack of depressed patients, the investigation of the antidepressant effects of Desmethylimipramine has thus far been limited to 12 patients. This metabolite of imipramine had, in experiments carried out at the National Heart Institute, exhibited a strong stimulant effect in various animal species. Similarly early clinical trials in Switzerland had suggested that it might exert its antidepressant effect earlier than its parent compound, Imipramine. The present studies (of necessity too limited in terms of number of patients) failed to reveal any significant differences between the metabolite and Imipramine. While some of the somatic effects, such as profuse sweating and tremor, were found less marked and less frequent than with Imipramine, the overall therapeutic results did not support the

claims for the superiority of this compound. These results are therefore at variance with other reports.

Another study is concerned with the psychoactive effects of indolyethylpiperazine (WIN 18, 501–2). This compound had been reported to exhibit depressant as well as stimulant effects in experimental animals. A study in the Laboratory of Clinical Science, CI, IR, NIMH at the Clinical Center, Bethesda, had suggested favorable effects on withdrawn, catatonic patients. For the purposes of the present study patients were selectecd with a view toward diversity of psychopathological syndromes, in the hope of recognizing possible differential effects. Plasma levels were noted, despite similar dosage. The preliminary evidence so far available suggests that this compound may perhaps have activating or disinhibiting qualities which may be useful in patients manifesting states of psychomotor retardation or stupor.

Other studies in their preliminary phase involve Methotrimeprazine and a study of the disinhibiting effects of some antiparkinsonian agents. While the latter drugs are usually administered for their primary effects, some also may have stimulant properties. A brief state of euphoria following intravenous injection has been noted in some patients. This may prove ueful in exploratory interviews.

(*c*) *Differential Values of Somatic Therapies*

Since the introduction of treatment with psychoactive drugs, there has been a marked decline in the use of other somatic treatments, notably insulin coma and electroconvulsive therapy. At St. Elizabeths Hospital, somatic treatments other than drug treatment were discontinued some years ago. Electroshock has been only sporadically used on one or two services. Allowing for fluctuation of treatment preferences with time, it has become clearly apparent that only selected groups of patients respond to the pharmacotherapies.

A study of the differential value of the various somatic treatments (including the pharmacotherapies) is therefore timely. It is the aim of this program to develop differential criteria for, and to conduct comparative studies of the various somatic therapies. A small insulin coma unit has been established. Equally, a study of the dysmnestic syndrome, indiced by ECT, has been initiated; this is being related to serial EEG studies.

(*d*) *Psychotherapy*

Because of the recent organization of a psychotherapeutic service, no actual studies have as yet been initiated. However, plans have been completed for a systematic study of the motivations which prompt individual physicians to recommend a given patient for psychotherapy. The belief is often expressed that only the more sophisticated patient is suitable for psychotherapy. This preconceived idea begs the question of adequate and effective communication. There is, in fact, growing evidence in the literature that education and social status are of only minor significance in the person's potential responsiveness to purely psychological measures. Considerable interest will therefore focus on the development and practice of short term methods of psychotherapy. The reason for this approach is not primarily an empirical attempt to help large numbers of patients in need of guidance and support. Rather, it rests on the assumption that good therapy often depends on the elucidation of crucial conflicts and attitudes evolving as key targets in the process of psychotherapy. These may become manifest in the absence of full insight; and their consequences will vary with basic personality structure and the realities of a particular social aspiration. Such considerations are of special relevance to the type of patient seen in the mental hospital ward and clinic.

(*e*) *Psychosocial Modes of Treatment*

With the emergence of social psychiatry, goals of treatment and functions of therapists from different disciplines have been subjected to much change. Conceptual differences and interprofessional rivalry have often contributed to considerable confusion. In the William A. White Clinic and Services, the position has been taken that psychosocial modes of treatment differ from conventional psychotherapy by focusing on the adaptational dynamics relative to family relationships and to life-situational elements. In order to avoid competition and overlapping between psychotherapy and other approaches complementary to it, psychosocial treatment has been established as a specific treatment modality. By defining the goals of treatment, the therapist is chosen on the basis of particular skills and knowledge concerning the patient's family and relevant social prob-

lems. Senior social workers are actively engaged in individual and group treatment. Social workers also undertake collateral treatment of husbands, wives and, especially, the parents of hospitalized patients. This has proved highly effective in producing a favorable climate for the patient's social rehabilitation. The Chief Social Worker is now engaged in developing an index of social-pathological syndromes which could serve as therapeutic guideposts and prognostic indicators in the management of a particular case. There is reason to believe that there are as yet few valid correlations between socioeconomic status, family attitudes and the adaptational patterns of psychiatric patients. Rather than utilizing the questionnaire type of approach in assessing domestic circumstances, the use of multiple observers is envisioned. In this context, home visits by social workers, the clinic nurse and physicians have produced information of much promise.

(*f*) *Family Studies*

A study of patients' records at St. Elizabeths Hospital has revealed evidence of high incidence of psychiatric disorders in given families. So far 85 "Index Cases" have been culled from the records of the William A. White Service. These are patients who are being, or who have previously been treated in the William A. White Building, and who have one or several members in their families suffering from various psychiatric disorders. Involved are 129 relatives of whom more than 50 have also been patients in St. Elizabeths Hospital. A breakdown of degree of relationship indicates 2 children, 31 parents, 53 siblings, 8 grandparents and 35 classified as "other relationship." The number of mentally ill relatives varies from one to seven members in a given family. This material will be of value in a number of genetic and sociological studies.

(*g*) *Catamnestic Followup Studies*

Every patient discharged from the William A. White Building becomes automatically a subject for a long term catamnestic investigation. Longitudinal observations and evaluations are carried out by the William A. White Social Service. Since clinical and social prognoses are formulated at the time of termination of treatment, this study is expected to contribute to assessment and validation of prognostic criteria. Certain phases of these studies are to be carried out in close collaboration between social worker and psychiatrist. Patients and families will be seen singly or jointly, in the Clinic or at the home whenever crises or complications necessitating decisions on further treatment call for such interviews. The role of family attitudes as precipitating factors leading to relapse will be a particular object of investigation. Several other studies focusing on family attitudes as well as social class aspects are contemplated, but will of necessity depend on additional personnel.

(*h*) *On the Concept and Functions of a Model Psychiatric Clinic in a Mental Hospital Setting*

Recent therapeutic advances and changes in the public attitude toward mental hospitals have greatly accentuated the importance of psychiatric clinic, functioning as part of a comprehensive community-oriented hospital service. The William A. White Clinic was established with the following aims: (1) to investigate the range of criteria which qualify patients for the ambulatory therapies; (2) to evaluate the impact of ambulatory therapies on the immediate and future course of the illness; (3) to modify the outmoded team-concept of psychiatrist, psychologists, and social workers by experimenting with a realignment of selected disciplines, utilizing particular skills for specific assignments; and (4) to develop new concepts regarding function and structure of a model psychiatric clinic. There are now over 130 patients attending the William A. White Clinic, nearly matching the number of patients now hospitalized in the wards. The services offered include: (a) individual and group psychotherapy for patients and their families; (b) pharmacotherapy and ambulatory ECT; (c) rehabilitation programs with community participation; and (d) a Day Clinic facility for limited periods of intensive treatment. The latter serves not only as a much needed additional therapeutic service, but also as an instrument for studying the preventive effects of intensive treatment in patients showing evidence of decompensation. The great advantage which the Day Clinic offers from the point of view of research is the opportunity for continuous observation. These observations will center on the identification of the kind and severity of clinical and social symptomatology and dysfunction; the con-

ceptualization of therapeutic targets which serve as bases for assessing change; and the assessment of various therapeutic modalities used singly, and in combination.

2. Some Aspects of Information Processing in Man

As evidenced in earlier reports, problems of Neurocommunication (i.e., the application of communication theory to an understanding of the detection, transmission and coding of information by the nevous system) have formed a long standing interest of the program of the Center. The recently created small Unit on Neurocommunication in the Office of the Chief has centered on three problems. These are: (a) Central nervous system processing of complex auditory information; (b) the experimental analysis of motor control systems in man; (c) utilization of sensory feedback in diseases of the nervous system characterized by abnormal movements.

(*a*) *Central Nervous System Processing of Complex Auditory Information*

This program aims at an understanding of the role played by some regions of the central nervous system in the processing of complex acoustic stimuli, particularly as related to the perception of intelligible speech. Most of the patients in this study have documented intracranial lesions. After initial evaluation with routine pure-tone audiometric techniques, special speech intelligibility tests are employed. The stimulus materials consist of phonetically balanced words, presented at various gains and under different conditions of filtering, to the two ears separately. Additional tests involve presentation of the stimulus word to one ear while competing messages are presented to the contralateral ear. Such tests permit the study of possible "filtering mechanisms" in the processing of acoustic information. The patients are required to repeat or write down the test words they hear, and the number of correct words constitutes a speech intelligibility score. Several patients who have documented temporal lobe lesions, including surgical ablations, demonstrate marked impairment of speech intelligibility when the test words are presented to the ear contralateral to their lesions. Preliminary correlation of functional and anatomical findings suggest that lesions involving Heschel's gyrus are more likely to impair speech intelligibility than are other lesions of the temporal lobe. One is however reminded that such simple correlations do not serve as a sufficient explanation of impairment of speech perception by the finding of normal speech intelligibility scores for *both* ears in an adolescent girl who had undergone almost total unilateral temporal lobectomy.

(*b*) *The Experimental Analysis of Motor Control System in Man*

The major concern in this project area is the definition of patterns of utilization of sensory feedback required for the control of voluntary motor activity in man. Studies are proceeding on input-output relationships of the control systems for voluntary movements of the hand in man by providing subjects with a compensatory tracking task. The subject is required to maintain his index finger at a point in space which is represented by a fixed beam at the midposition of an oscilloscope screen. A second beam is activated by the motion transducer linked to the subject's finger, and provides an analog signal related to the position of the index finger in space. The task becomes one of maintaining the two beams of the oscilloscope superimposed as nearly as possible by holding the index finger in the reference position in space. By altering the form in which the error signal is presented, it is possible to synthesize specific information patterns and observe the type of motor control which results when they are utilized for monitoring purposes. Four basic forms of error signal can be provided; namely, proportional, integral, logarithmic and derivative. Each basic form of the error signal has been found to be associated with a corresponding characteristic pattern of finger movement. These patterns are stable and characteristic of the normal nervous system when operating upon the specific feedback signal. The fact that the pattern of motor activity under study is so sensitively linked to the pattern of the visual information provided for monitoring, establishes this system as a powerful analytic tool for exploring relationships between the form of sensory feedback information and the pattern of motor control; and thereby provides the basic groundwork for inference about central processing functions underlying the motor control.

Extensive studies on the role of increasing the amplification of a proportional error signal on

motor control have also been undertaken. As this is increased, normal subjects demonstrate progressive increases in the accuracy with which they are able to maintain the index finger in a fixed position in space. As the subject utilizes higher amplification signals to obtain finer control, he shifts from a pattern of low-frequency, high-amplitude movement to one of high frequency, high-amplitude movement. The frequency of finger movement under the high amplification feedback conditions is so high that point-to-point voluntary control must be doubted. These observations suggest that an important shift in the mode of voluntary motor control may occur as a function of the amplification of sensory feedback information. These relationships (between pattern of motor control and the amplification of the feedback signals) are characteristic of the normal central nervous system. No exceptions to these findings have been found in the study of over 50 control subjects.

In addition to an interest in the *form* of sensory feedback information required for optimal control of motor activity, the *amount* of sensory feedback information required for motor control is also being investigated. The visual feedback signal can be provided as a discrete pulse of information. The on-time of this pulse and the off-time (over the range 1 to 1,000 millisecond) can be controlled. Although the basic concern of the study is with the temporal parameters of central information processing functions, it is felt that the temporal structuring of the stimulus provides a reasonable initial phase for the exploration of these issues. The question of how much information the nervous system requires for control of information is being presented. It has been observed that subjects maintain very accurate control of finger position when operating upon a visual feedback signal that is presented as a 50 millisecond pulse with a thousand milliseconds of off-time before the 50 millisecond pulse is represented. These observations have been made when a pure proportional or a pure integral error signal is used for monitoring. When integral and proportional signals are mixed, motor control shows marked impairment under most blanking conditions, even those in which a long on-time is combined with very short off-times. It would appear therefore that the central processing of this mixed signal is markedly impaired by discontinuity in the sensory presentation.

(*c*) *Utilization of Sensory Feedback in Diseases of the Nervous System Characterized by Abnormal Movements*

The studies outlined in the previous section are being pursued in parallel in a number of patient groups comprising cerebellar lesions and basal ganglia lesions. The expectation is that the comparison of sensory input and motor output correlations for normal subjects, and these patient groups, will illuminate some of the complex issues concerning the information processing functions underlying normal motor control, as well as the mechanisms of abnormal movements.

A number of patients with unilateral Parkinson's disease have been examined before and after unilateral thalamotomy. It has been found that these patients can utilize visual feedback signals of increasing amplification to obtain increasing accuracy of control of finger position in a manner similar to normal subjects. This is the case, however, only for low amplification feedback signals. At higher amplification, these patients no longer realize any additional improvement in motor control. Their tremor remains unmodified, and is the limiting factor in their ability to maintain a fixed position in space. The fact that the patient with Parkinson's disease is able to utilize increasing amplification of a proportional error signal to eliminate high amplitude, low-frequency error characteristic of low amplification feedback conditions, indicates that he does not have a global and irreversible deficit of functional operations necessary for the sensory-feedback control of movement. It would rather seem that such patients are able to process sensory feedback information in a normal fashion, and can organize appropriate motor control commands to the anterior horn cells of the spinal cord; but that these may compete unfavorably with the motor control commands underlying tremor activity. The source of the latter commands remains unclear.

Patients with cerebellar disease respond to visual feedback signals at increasing amplification in a manner similar to patients with Parkinson's disease. The most striking difference so far observed between such patients and other groups (including normal subjects) consists in their abil-

ity to attenuate their intention tremor by weight-loading of the index finger. Under such circumstances the patient is able to operate upon a variety of visual feedback signals to obtain exquisitely fine voluntary motor control in a manner virtually indistinguishable from that observed for normal subjects. This finding emphasizes the role of peripheral feedback factors in the mechanisms of abnormal voluntary movement in cerebellar disease, and particularly the possible role of muscle receptors, and the gamma efferent system.

The high relevance of the above studies to an understanding of the drug-induced sensori-motor disorders in man, and the effect of drugs upon established disturbances of motor control, is self-evident.

3. Some Aspects of Catecholamine Metabolism in the Experimental Animal, and in Man

In keeping with previous interests, the program of the Section on Neurochemistry has continued to center on various aspects of the storage and disposition of catecholamines in mammalian tissues and their estimation in body fluids, in the experimental animal, and in man.

(*a*) *Release of epinephrine from adrenomedullary particles in vitro*

The retention of catecholamine in specific granules, and the mechanism of their release are of importance for an understanding of the normal function of these hormones and the action of many drugs. The effect of reserpine and other drugs on the release of epinephrine from adrenomedullary particles prepared from rabbit glands was, therefore, studied *in vitro*. Reserpine, at 10^{-4}M, caused practically complete release of particle-bound epinephrine within 30 minutes at 25°. Chlorpromazine had a qualitatively and quantitatively similar effect. Of other phenothiazines, those with potent tranquillizer activity also had strong releasing ability, while others less active as transquillizers, were found less active as agents of release. Imipramine (which is structurally related to phenothiazines) was found active, but to a smaller degree than chlorpromazine.

Derivatives of chlorpromazine, promazine and imipramine (representing either actual metabolites, or analogues of metabolites), showed varying degrees of releasing activity which in most cases correlated reasonably well with their pharmacological activity *in vivo*.

Out of over 50 drugs tested, only 3 were found to have releasing activity comparable to that of reserpine and the phenothiazines: These were *p*-chloromercuribenzoate, sodium deoxycholate and suramin sodium. It would seem likely that, in these instances, the releasing effects were the results of nonspecific physicochemical effects (i.e., denaturation or lysis).

Various combinations of releasing drugs with each other, or with inactive substances, were tested. No instances of potentiation or inhibition were observed. In particular, it was not possible to duplicate, *in vitro*, the inhibition of reserpine-induced release by monoamine oxidase inhibitors observed *in vivo*. Studies in this area continue.

(*b*) *Catecholamine Excretion Patterns in Personnel Subjected to Space Flight Training and Space Flight*

The analysis of urinary catecholamines and their metabolites in personnel subjected to space flight training, and actual orbital flight (originally undertaken at the request of the National Aeronautical Space Administration) has continued. Base-line values were established for each member of the group by studying the diurnal variation during periods free from imposed stress. The mean excretion rates of several metabolites were significantly increased as a result of stressful tests, such as centrifuge trials. Although marked stress responses, affecting most (or all) of the parameters measured, were observed in some instances after a test performance, or flight, such results were sporadic, and did not indicate a direct relationship between the response and the physical stresses involved. It is therefore assumed that they are more related to attitudinal and emotional factors. In keeping with this supposition, elevated values in some instances were found to persist for 48 hours following a flight.

The excretion of epinephrine, although representing less than 2% of the total discharge of the hormone, proved to be the most sensitive and efficient index of stress. Vanillyl mandelic acid (VMA) seemed the next best indicator. The excretion of Dopamine, was found to be another useful stress index.

(c) Urinary Excretion of Catecholamines and Their Metabolites During a Course of Morphine Addiction and Withdrawal in Man

In view of the interaction between morphine and the adrenomedullary system, and the symptoms of adreno-sympathetic stimulation resulting from rapid morphine withdrawal, a study of catecholamine metabolism during addiction and withdrawal was initiated in collaboration with the Addiction Research Center, Lexington. A former addict volunteer was studied during a preliminary control period, a period of a gradually increasing schedule of morphine injections, and, finally, a period of withdrawal and recovery.

It was observed that the excretion of catecholamines, and catecholamine metabolites rose during the addiction period, especially while the dosage of morphine was being increased. During the plateau phase of addiction, the excretion rates again approached their normal level. During withdrawal and recovery the excretion of catecholamines increased or remained normal. The excretion of most catecholamines *metabolites* was diminished, a trend which persisted throughout the recovery period. In this study, however, the interpretation of the results was complicated by the fact that creatinine output and diuresis were lower during the periods of withdrawal and recovery than during the preceding periods, suggesting an incomplete collection of urine. However, even when related to these lower creatinine excretions, the excretion of some metabolites was found significantly below the control level. The experiment is now being repeated with a second subject. If confirmed, the results would indicate an inhibition of catecholamine metabolism during the withdrawal and recovery phases of morphine addiction.

(d) The Estimation of Catecholamine Metabolites in Rat Urine

In view of the obvious limitation of human experiment, methods for the estimation of catecholamine metabolites in rat urine are desirable. By and large, the methods developed for human urine are unsuitable when applied to rat urine, two of the principal difficulties being the small amounts of urine available, and a high concentration of pigments and other interfering materials, resulting in high blank readings. To overcome these difficulties, methods based on isotope dilution techniques are now being developed in the Section on Neurochemistry. This has involved the preparation of highly purified samples of radioactive metabolites, mainly by enzymatic reactions. After adding these metabolites to a sample of rat urine, they, and their preformed nonradioactive counterparts, are isolated and the activities determined. It is hoped to develop, by these means, reliable methods for the estimation of metabolites in rat urine.

4. Psychoactive Tryptamine Derivatives

The intermediate metabolism of psychoactive tryptamine derivatives has, as heretofore, continued to form a central theme in the Section on Psychopharmacology. In view of the variation in the metabolism (6-Hydroxylation) of Diethyltryptamine previously encountered, the metabolism of this substance has been reexamined from a comparative point of view. Considerable species differences were shown to exist in the rate of 6-hydroxylation of this compound, ranging from 1.6% (guinea pigs) to 51.5% (mice), with humans occupying a low medium range (4–5%). It was also shown that administered DET disappears from the rat brain very rapidly, and more gradually from brain of the rabbit; while in the guinea pig the substance was found to linger without significant decrease for at least 3 hours. The rate of disappearance was found to correlate closely with the varying ability of the three species to hydroxylate this compound.

A program of synthesis of several new psychoactive derivatives of tryptamine has moved forward with the collaboration of a Visiting Scientist. These compounds are useful guideposts in the study of the relationship of intermediate metabolism to behavioral effects. The operant conditioning technique (using rats), was found to be of limited value only, since the animals were found to respond with deterioration to both hallucinogenic compounds and some psycholeptic tranquillizers (like Chlorpromazine). Furthermore, rats were found to be very sensitive to a-Methyltryptamine (*a*-MT) in the shock avoidance situation; several trained animals were lost at low 3 mg/kg level of the drug. In spontaneous motility studies, d,l-*a*-Methyltryptamine produced considerable hyperactivity, and the 6-OH-metabolite a statistically significant ef-

fect in the same direction. Also, using the same test, the 6-hydroxy derivatives of Dimethyltryptamine (DMT) and Diethyltryptamine (DET) were found to be more active than the parent compounds. 6-Hydroxy-α-MT was slightly less active than α-MT. Indolyacetone, another metabolite, was found completely inactive in this test, even in high doses.

A number of compounds were examined in regard to their effects on reserpine-induced ptosis, and on general motor activity. In the case of α-MT, ptosis reversal was found to start after a few minutes reaching a maximum score within 25 minutes. The effect of the drug on motor activity, on the other hand, was found to begin at 30 minutes, and gradually to reach its maximum after 2 hours. This delayed activity curve is typical for α-MT; and it is of interest that other activating compounds (such as amphetamine), suchowed a parallel (rather than a staggered) effect on ptosis and motor activity. This separation suggests that the α-MT effect on Ptosis may be more directly related to the parent substance (α-MT) whereas the delayed effect on motor activity may be due to the formation of an active metabolite from α-MT. Some possible metabolites are being tested at present; their structural resemblance to LSD–25 continues to intrigue.

A further area of work has been initiated on the effects of psychoactive drugs on the regional distribution of serotonin in the brain. Serotonin levels in various areas of rabbit and guinea pig brains were determined at the time of the peak effect of a particular drug. All simple alkylated derivatives of tryptamine (which are hallucinogenic in man) were found to increase serotonin content in the hypothalamus, while leaving the amygdala-hippocampal areas unaffected. LSD–25 however did not follow this rule, presumably because of a difference in the mechanism of action. Such a difference is also borne out by the difference in effective dose level.

In a preliminary clinical study, carried out in conjunction with the Vienna Psychiatric Clinic, Austria, a Fluoro analog of DET (6–FDET) designed to block the 6-hydroxylation, was found to produce principally autonomic changes, without the sensory and affective components characteristic of DET administration in man. It is hoped that 6-FDET may prove useful as an "active placebo" in comparative human studies on this group of compounds.

5. Studies on Nicotinamide Adenine Dinucleotides (NAD) in Rat Liver

Theories have been proposed (see, e.g., H. Laborit, Presse Medical 69, 2428, 1961) according to which certain drugs act by shifting the ratios NAD+/$NADH_2$ (i.e., Nicotinamide Adenine Dinucleotides/Reduced Nicotinamide Adenine Dinucleotides) and NADP+/$NAPH_2$ (i.e., Phospho-Nicotinamide Adenine Dinucleotides/Reduced Phospho-Nicotinamide Adenine Dinucleotides) with far-reaching consequences for the metabolism of the cell, and the organism as a whole. Since these theories lack adequate experimental foundation, an examination of this problem was undertaken by a visiting scientist working within the Section on Neurochemistry.

Existing methods for the estimation of these compounds were found to have some serious drawbacks; these were successfully overcome by suitable modifications.

The interval between death and fixation of the tissue was found to be of great consequence in these estimations. Fixation in liquid Nitrogen resulted in much higher values of NADP+ and NADPH concentrations than those reported by previous investigators. When the fixation of the tissue was delayed the following changes were observed: dephosphorylation of NADP+ to NAD+, reduction of NAD+ to NADH, and finally hydrolysis of NAD+ and NADP+ by NADase.

Coenzyme levels were found to depend on age; they were lower in very young and very old rats than in those of 4 months of age. The NAD+/NADH ratio was also higher in 4-month-old rats than in either younger or older rats.

Hypoxia, glutathione, ethanol and several CNS depressants led to a reduction of NAD+ to NADH, while administration of CNS stimulants and also LSD–25, had the opposite effect. The level of total coenzymes was decreased by the injection of metabolic poisons (such as p-chloromercuribenzoate, 2,4,-dinitrophenol and malonic acid). Evidence has been obtained that p-chloromercuribenzoate, administered *in vivo*, detaches NAD-coenzymes from their protein binding sites.

6. The Intermediate Metabolism of Chlorpromazine and Related Compounds

(b) The Metabolism of Chlorpromazine in Man

The study of changes undergone by Chlorpromazine and related compounds in the mammalian body continues to be an important area of investigation in the Section on Neurochemistry.

In order to determine the possible relationship between the metabolic handling of chlorpromazine in individual patients, and clinical responsiveness to the drug, a method for the quantitative estimation of a number of metabolites in human urine was developed. This involves the estimation, by quantitative paper chromatography, of individual spots obtained from three types of urinary extracts. As a result, the following metabolites have now been identified in human urine: chlorpromazine-N-oxide, the primary, secondary and tertiary amines related to chlorpromazine sulfoxide; the primary, secondary and tertiary amines related to a monophenol of chlorpromazine; a neutral monophenol; and a diphenolic compound. The phenolic compounds were excreted almost completely as the glucuronide conjugates. The monophenols most probably possess the 2-chloro-3-hydroxy or 2-chloro-7-hydroxy ring structures. Hydroxylation of promazine appears to occur at the 3-position. Several additional unidentified spots were also noted.

Demethylation was found to be extensive, the demethylated forms predominating over the nondemethylated forms in the cases of both nonphenolic and phenolic metabolites. In general, phenolic metabolites appear to predominate over nonphenolic metabolites. Unchanged chlorpromazine, chlorpromazine sulfoxide and chlorpromazine-N-oxide were found to be minor metabolites. The ratios of the various metabolites, were quite uniform in nine patients. There was one exception to this general rule. Also, in the case of two individuals receiving single doses of chlorpromazine, phenolic metabolites were essentially absent.

(b) Pharmacological and Behavioral Effect of Model and Authentic Metabolites of Chlorpromazine and Promazine

Studies initiated last year to evaluate the pharmacological and behavioral effects of model (or actual) metabolites of chlorpromazine and promazine have continued with a view of learning more about the structure-activity relationship of this series. A number of related compounds were compared, using various simple behavioral tests. It was found that 1- and 2-hydroxypromazine were about equally active, but less active than promazine in both the potentiation of hexobarbital sleeping time and so-called rotating rod ('rota rod') test. Neither 7-methoxychlorpromazine or chlorpromazine sulfoxide prolonged hexobarbital sleeping time. 7-methoxychlorpromazine however, did show a graded dose-response effect using the rotating rod test; this was greater than that of chlorpromazine sulfoxide. 7-methoxylchlorpromazine and 1- and 2-hydroxypromazine were lethal at higher dose levels, whereas chlorpromazine sulfoxide was not. Other compounds will be tested as and when they are received.

(c) The Validity and Usefulness of the Forrest and Forrest "FPN" Test for Urinary Phenothiazines

In confirmation of studies begun last year, it was again found that although the chances of this test detecting relatively high doses of drugs were reasonably good, correct results at the 1+ level of the chart (i.e., 70 mg of phenothiazines per day, or less, the dose range for which the test was mainly designed) were more tenuous. Thus, over a 5-day consecutive period, the urine of about 20 patients gave, on the average, only 34% correct tests for trifluoperazine administration at normal doses (70 mg/day or less) when early morning urines were tested. About 50% correct readings were obtained when urines collected 1½ to 3 hours after drug administration were tested. This time interval is regarded as the peak time of urinary excretion. Treatment with resin, as suggested by Forrest et al., (Am. J. Psychiat. 118: 300, 1961) rather than improving the test, eliminated almost all color development. When data for individual patients were examined, no correlation between dose of drugs and percent correct tests was found. Thus, the urine of some patients receiving the highest doses did not furnish a positive test over a 5-day period, whereas urine of some receiving the lowest doses, (10 mg/day) gave 100% correct

tests. It is concluded that the test is difficult to interpret for drugs administered at low dose (i.e., 1+ chart) levels.

7. The Reversible Isolation of Areas of Brain by Short-Term Cooling

In view of the importance attaching in the program to the regional physiology and pharmacology of the brain (and, particularly, the brain stem), it was deemed advisable to attempt to develop a method for the reversible isolation of areas of the brain in the conscious animal. Cooling of small areas of the braing appeared to offer possibilities in this regard. The method has a long history in the study of conduction along nerve fibres; and, lately, has come into prominence in neurosurgery in man.

The aim of the study was to produce cooling of small areas of the brain tissue in depth so as to reversibly block conduction in a plane, or in a series of planes, which enclose a three dimensional space. The principle of the method is relatively straightforward. If a needle is cooled along its length, a cylinder of lowered temperature is formed in the tissues around it. When cooled needles are inserted in a picket fence (or fork) arrangement, the cylinders of lowered temperature interlock and a wall of cooled tissue blocks conduction along the plane in question. To investigate the feasibility of this principle, a cooling fork of suitable dimensions was made, suitable for placing across the brain-stem of cats at midcollicular levels. Each tine of the fork was made up of a hairpin loop of fine stainless steel tubing. Cooling fluid (heptane) was pumped through the system by specially constructed motor-driven syringe pump. It circulated through a Millipore filter, a helical stainless steel heat-exchanger immersed in a dry-ice/alcohol mixture, entering the intake manifold of the fork, and finally recirculating through the reservoir. The fork is implanted stereotaxically at mid-collicular levels.

When subjected to experiment, such cats were found to move above freely, showing no ill effects. Pumping the cooling fluid through this arrangement resulted, successively in loss of response to pain, changes in posture and muscle reflexes and finally, the appearance of classical decerebrate rigidity. This change was found to take about 2 minutes and the state of decerebration could be held for up to 45 minutes. The change could be reversed within minutes by arrest of cooling. Between such decerebrations (which were repeated up to eight times in the same animal), the animals appeared neurologically and behaviorally normal.

A film of this experiment has been made to document and analyse the various stages of the changes encountered. Histological evidence is not available at the time of writing. Nevertheless, there seems little doubt that reversible cooling of small areas of the brain is technically feasible. Since the area for cooling is only limited by the geometry of the cooling device to be implanted into the brain, the method may well find application in a number of areas. These could include studies on the mode of action of drugs by the reversible exclusion of varions functional areas of the nervous system during the course of action of a particular drug; the study of some long-term processes, such as the consolidation of the memory trace (through reversible blockade of transcallosal connections between hemispheres); and in neurosurgery, where the reversible functional isolation of a particular area, (possibly in conscious man) could conceivably be made to precede surgical removal.

It is hoped that this program will be continued in conjunction with the Department of Pharmacology, Albert Einstein College of Medicine.

8. Microelectrode and Micropipette Studies on the Discharge Patterns of Single Cells in the Spinal Cord and the Brain

Work carried out in the Section on Neurophysiology during the past year has been primarily directed towards the investigation of the organization of spinal cord neurones partaking in the control of respiratory motor activity; the determination of the location, and the functional properties, of neurones partaking in the act of swallowing; and the development of the technique of five-barreled micropipette recording for the study of the pharmacological properties of individual cells in some areas of the brain.

The work on the organization of the spinal cord respiratory neurones has suggested that internuncial respiratory units in the spinal cord may play an important role in the integration of the activity produced by the rhythmic discharge of respiratory neurones in the brain-stem, and the activity originating in peripheral receptor fields. During this study, it also became necessary to in-

vestigate the alterations produced by swallowing upon the patterns of activity of respiratory units in the spinal cord. This subsequently led to a systematic study of the neuronal correlates of swallowing. The patterns of response of brain-stem neurons which change their rate of activity during swallowing (as well as the patterns of the response in single afferent fibers of he laryngeal nerve and of efferent fibers to muscles of the pharynx) were determined. The evidence suggests that prevailing theories regarding the neuronal mechanisms responsible for the act of swallowing may have to be radically reconsidered.

In collaboration with a guest worker, another group of studies has centered on the sensitivity of medullary nerve cells to acetylcholine and related substances, using the technique of electrophoretic application of substances to individual nerve cells through five-barreled micropipette electrodes. Acetylcholine sensitive nerve cells have been identified in the brain stem. Such cells, however, were found to be intermingled with units which were nonresponsive. Also acetylcholine-sensitive units could be classed into two groups, depending on whether their activity was increased or decreased during the application of acetylcholine. In all cases the response was enhanced following electrophoretic administration of physostigmine. In most cases it could be partially or completely blocked by the tropical administerio n dihydro-β-erythroidine or hexamethonium.

In collaboration with the Neurosurgical Branch, National Institute of Neurological Diseases and Blindness, some preliminary studies were also carried out of the changes occurring in unit activity in the somatosensory cortex of the cat during the development of cortical steady potentials.

The above experiments have provided the necessary background of experience for the investigation of possible regional differences in the pharmacological response of individual units in various areas of the brain. A long-term view must obviously be taken of such studies. A study of the response to acetylcholine and noradrenaline of nerve cells in the olfactory bulb of the rabbit is proceeding, and a study of the pharmacological properties of individual nerve cells in the hypothalamus of the cat has also been initiated. Studies on the pharmacological response of nerve cells in various critical areas of the Limbic System are contemplated for the coming year. An organized effort will also be made towards the development of multilead microelectrodes (involving five or more leads). This should enable one to record simultaneously the activity of neighboring groups nerve cells and to carry out a topological analysis of the interaction between them. It is conceivable that such studies could be carried out in conscious man.

9. Miscellaneous Animal Behavior Studies

During the past year work has continued in the area of stimulus generalization, a further analysis of conflict behavior in rats, and a preliminary study of concomitants of fatique in monkeys. In the area of stimulus generalization a study has been made of the extent to which a subject will respond to a stimulus which resembles (but does not exactly correspond) to the original conditioning stimulus. It was shown that monkeys displayed greater visual stimulus generalization in an aversive than in an appetitive situation. The generality of these results is being tested in a variety of different circumstances. It was found that, even after specific training to differentiate one auditory stimulus from another, rats still showed much more generalization of an avoidance response than of an appetitive response. By continued discrimination training, monkeys were eventually brought to the same level of visual stimulus generalization of both appetitive and aversive responses; however, discrimination was acquired much faster for appetitive than for aversive behavior. Pigeons are being used in studies of visual generalization and discrimination; the effects of response extinction, type of visual cue, and reward schedule are being investigated. Results obtained so far have generated further experiments in more complex areas, which might be properly regarded as "perceptual."

A new technique for analyzing conflict behavior in rats (involving intermittent presentation of appetitive and aversive stimuli) has proven sensitive to the effects of several variables among them intensity of punishment and amount of food deprivation.

Also, two types of response inhibition (extinction and punishment) have been analyzed in rats, and the effects of hyoscine and other drugs on these responses have been examined. Hyoscine

was found to lead to a return of behavior suppressed by extinction, but did not reinstate behavior suppressed by punishment. Furthermore, it was also found that animals would work more rapidly in the presence of a rewarded stimulus if this stimulus is alternated with a nonrewarded stimulus, than if the rewarded stimulus is continuously present; this effect is referred to as "behavioral contrast." An analogous situation with avoidance behavior has been examined; no evidence of a "contrast" effect was found in this instance. Further analysis is also being carried out of the specific reasons for this difference between approach and avoidance behavior; and it is possible that this difference may be related to the differences in stimulus generalization discussed above.

In another context, studies have been conducted to examine some metabolic and behavioral concomitants of fatigue. It was found that the resistance of monkeys to fatigue, as measured by long term performance in a continuous shock-avoidance task, improved after repeated exposures to the fatiguing situation; and that monkeys may well learn to "sleep" and simultaneously respond to avoid shock. The effect of drugs on these situations will be of particular interest.

ADULT PSYCHIATRY BRANCH

The program of the Adult Psychiatry Branch includes three major areas of study: (1) the relation of family patterns and transactions to the development and treatment of schizophrenia; (2) normal and disturbed adolescent development; (3) physiologic and biochemical correlates of psychological processes. As certain projects are reaching completion, gradual changes in emphasis in the program are taking place. In general, the Branch program is increasingly focusing upon the determinants of cognitive and integrative personality functions. Biological processes are selected for study when they have quite specific psychological implications or effects and when they provide useful indices of psychologic processes. The previous interest within the Branch upon developmental aspects of psychological processes has heightened during the past year, and the possibilities of using cross-cultural approaches, especially in the study of schizophrenia and adolescent development, are being explored.

Studies of Schizophrenics and Their Families

For some years a major research focus in the Adult Psychiatry Branch has been the study of the families of schizophrenics. This program has included both exploratory, hypothesis-developing clinical observations and systematic, hypothesis-testing research. The clinical work has especially involved family psychotherapy, in which parents, young adult or late adolescent patient-offspring and the siblings of the patient are seen together, usually twice weekly. On the basis of substantial experience with family therapy, the staff is now formulating its ideas about technique, indications, and contraindications for this new and important but complicated treatment method. Individual psychotherapy and case work, family art therapy, and naturalistic home visits have also been important sources of clinical observations in the Family Studies.

A considerable number of specific findings have now been documented in the systematic research of the Family Studies program. This research has now demonstrated that the presence and variety of schizophrenic disorder in a considerable variety of patients, both adults and children, can be deduced from the forms of thinking and patterns of communication found in the rest of the family in which the individual patient has developed. Diagnosis and form of thinking in patients has been predicted successfully using a transactional, psychodynamic interpretation of both projective test data obtained from the other family members and excerpts of interaction of the parents with a psychiatrist, all studied blindly. Secondly, patients have been matched blindly with the families to which they belong. During the past year this work has achieved enhanced significance because it has been shown that the same principles for making the differentiations can be used successfully with the diversed data of projective tests and excerpts of interaction, and because these principles have been extended to the families of three kinds of child patients.

The blind differentiations have been based upon the hypothesis that schizophrenia involves a breakdown or a developmental failure of certain essential psychological capacities which are ordinarily developed in and through the family environment. These capacities include the ability to

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sustain focal attention, to differentiate self and nonself, to use language communicatively, to develop adequate modes of perception, motor activity and interpersonal relating, to develop stabilized ways of deriving meaning from experience, and to delineate fundamental roles for oneself, especially generation and sex roles. These functions are all closely related to the thinking disorders, that is, to the attentional and cognitive disorganization, found in the various forms of schizophrenia. A new classification of the schizophrenias in terms of forms of thinking has been conceptualized in the Family Studies program and has been found to be empirically useful in making predictions from family patterns.

It has been assumed that particular patterns of communicating and thinking in family transactions have helped shape the forms of thinking found in offspring. By looking at the manner in which parents, patient, and "well" siblings could fit together in a family constellation and by making inferences about the developmental phases in which particular maneuvers might be especially important in a child's psychological growth, it has been possible to make the blind predictions with a very high degree of specificity along two dimensions: forms of thinking (four varieties), and degrees of severity of psychotic tendency (five levels). Using this diagnostic schema for individual patients, predictions have been made, both from the projective test and from the interaction-excerpt family data, at a statistically significant level of accuracy ($p=.001$).

As the criteria for the differentiation of young adult schizophrenics became more detailed, it appeared to the investigators that the criteria for the differentiation of the parents of autistic children would not be the same. It seemed reasonable to assume that the developmental process in the parent-child relationships of the autistic children was probably disturbed more drastically and earlier than for the young adult patients. Projective test protocols from the parents of 20 autistic children and 20 matched neurotic children have now been studied and compared with protocols from the parents of young adult patients. (Projective test protocols from the parents of 20 asthmatic children and 20 child medical patients, carefully matched in terms of education, age, and occupation with the autistic and neurotic children, have been obtained during the past year and are currently being studied.) The parents of autistic, "nonorganic" childhood patients show forms of behavior that would rebuff, impair and interfere with the very beginnings of relationships they might have with the child; in contrast, relationships in the families of the young adult schizophrenics are not so totally shattered, but rather the focusing of attention and the achievement of coherent meaning is impaired, blurred and "confused." The details of these findings have now been written up at some length and will be published during the forthcoming year.

The findings with the families of the child patients are especially interesting because they have generated a number of specific hypotheses which in turn have been tested and confirmed. For example, it was predicted that the parents of the autistic children would show more overt hostility and less blurring and fragmentation of thinking than the parents of the young adult schizophrenics. Independent judges rating Rorschach protocols of these parents have found that the frequency and intensity of disorganized forms of thinking is in fact significantly higher in the parents of the young adult schizophrenics. Similarly, using a genetic-level scoring system in terms of degree of psychological differentiation, the parents of the young adult schizophrenics give Rorschach responses of a significantly less differentiated and less integrated quality than found in the other parents. The parents of the autistic children, in contrast, have relatively clear, well-differentiated perceptions, but seem more openly sadistic and often brutally disaffiliating.

In addition, independent ratings of hostile effect in the Rorschachs differentiated the various groups of parents, with the parents of the autistic children showing the highest hostility scores, the parents of the acting-out aggressive children next, and the parents of the young adult schizophrenics and withdrawn children lower. As a further indication of the degree of specificity which is being achieved in these studies, it was hypothesized that parents of the autistic children would introduce hostile or threatening content more commonly in their very first Rorschach response, in accord with the observation that these parents put distance or negative tone into transactions much earlier than other parents including the parents of young

adult schizophrenics. This hypothesis was confirmed at a statistically significant level.

Thus, the systematic aspects of the Family Studies are currently generating a variety of new hypotheses which are amenable to specific confirmation or negation. Some of these hypotheses will be evaluated with new techniques in which family transactions are observed directly under standardized conditions. The Color-Matching Technique, as developed in the Child Research Branch program, and Strodtbeck's Revealed Differences Method are being used in comparative family studies now underway.

Certain of the hypotheses which the previous work has generated call for cross-cultural evaluation. The work with projective tests and family-therapy excerpts has attempted to establish criteria for the differentiation of families which do not hinge upon distinctive cultural or social class characteristics but upon formal matters, particularly related to thinking disorders, which hypothetically can be found and evaluated for the schizophrenics of any culture or social class group. On a small pilot study basis it has been found that the same criteria for evaluating projective test protocols do seem to be applicable to protocols obtained from diverse cultures. As a further approach to this problem, collaborative arrangements have been set up with Dr. Herant Katchadourian at the American University of Beirut in Lebanon. Dr. Katchadourian, who was a member of the Branch staff during the past year, has now returned to Lebanon to conduct family studies of schizophrenics under an extramural NIMH grant.

Other issues relevant to the understanding of the familial and cultural context of characteristic forms of personality functioning are being evaluated by Dr. Stanley Diamond, research anthropologist, who transferred to Adult Psychiatry during the past year. Dr. Diamond has examined processes of personality development comparatively along a continuum of historically evolving forms of cultural organization—primitive, transitional, and contemporary. In field work during the past year among the Allegheny Seneca he has explored the role of familial and extrafamilial kinship ties in a situation of rapid social change and has elaborated earlier conceptions of the junction of primitive rituals as structured outlets for ambivalent feelings.

Studies of Adolescent Development

A major program in the Section on Personality Development has been concerned with evaluating the psychosocial hypotheses of Erik Erikson concerning the crisis of identity in late adolescence. The interactions between the parents and the late adolescent has been selected as the psychosocial field of study.

On an open psychiatric unit and adolescent treatment program has been established in which the focus is family therapy. The adolescent, his parents, the psychiatrist, who also treats the adolescent in individual sessions, and the social worker who also sees the parents separately, meet together in weekly sessions. These family sessions are observed by the research group and are tape-recorded.

The concept of "delineation" is a central concept around which data from family sessions is analyzed. By delineation is meant behavioral interaction between two people which carries in it an explicit or implicit communication of the one person's image of the other person. The focus up to now has been on the relation of parental delineation of the adolescent to adolescent self-delineation in family sessions and to adolescent self-concept as it can be ascertained from research interviews.

The findings have revealed important areas of coercive interaction, where persistent parental delineation of the adolescent in a particular way refuses to acknowledge behavior on the part of the adolescent which in some way contradicts this delineation. Such limiting delineations serve a purpose in the parents' personality. They may have a decisive impact on the adolescent, imposing specific areas of patterning or constriction in his evolving identity formation. Detailed analyses of 15 transcripts of family sessions for each of 3 families have been made, from which parental delineations of the adolescent and adolescent self-delineation have been extracted. These delineations have been related to the events or issues under discussion in the sessions and to adolescent self-concept as it is manifested in research interviews. This approach is now being extended to other families. This work is an effort to isolate one important part of the adolescent's current psychosocial experience and to establish its relationship

to the complex current and genetic factors bringing about identity consolidation or diffusion in late adolescence.

In the next year a group of adolescents and their parents in family group sessions will be studied prior to the separation experience of going away to college. These adolescents will be selected because they evidence difficulty over issues of leaving their families and making the transition to college, in discussion with counselors and advisors in high school. Later, if they develop incapacitating symptoms in this transition they will be hospitalized, treated, and studied further. The delineation data in family sessions prior to breakdown can then be compared to the delineations following breakdown, which will help to clarify further the relation of parental delineation to outcome in adolescence. Where successful adaptation occurs, the data of delineation from family sessions through this period will give material for comparisons with the delineations in families where difficulties have actually developed.

Two studies in the Section on Personality Development are concerned with the self-concept in the late adolescent period. The first deals with psychosocial factors involved in preferences for types of colleges among high school seniors. One of the important determinants in the adolscent's choice of college is his concept of himself and how he feels this will match with his picture of particular types of colleges. Therefore, the college preference is an interesting situation within which to learn more about the relationship between self-concept and an important life decision. A questionnaire for this study has been developed and pretested with two groups of high school seniors. This includes an adjective check list of 120 items which will be used to explore the subject's conscious view of his actual self, as well as his ideal self.

In a second study, the relationship of stability of the self-concept to perceptual style is being explored. Witkin's work suggests the hypothesis that "field-independent" subjects will have a more stable self-concept than "field-dependent" subjects. The stability of the self-concept in normal adolescent subjects and hospitalized patients is also being compared.

Thirty-one normal subjects and ten patients were used in this study. Each subject was asked to rate himself on an adjective checklist, both as he actually saw himself and how he would ideally like to be. Each adjective was assigned a numerical score depending on the subject's self-rating.

After an interval of 3 weeks each subject was asked to rate himself again. For each subject, a difference score between each scale (both for his actual self-concept and for his ideal self-concept) was obtained on the two different occasions when the checklists were administered. These scores were then used as a measure of the stability of the actual self-concept and the ideal self-concept. Measures of field dependence-independence were obtained by giving each subject Witkin's Embedded Figures Test.

The analysis of this data is in progress and is not complete. However, some of the normal subjects were interviewed and the impression was obtained that, for the most part, subjects who show more evidence of identity-diffusion also reveal more instability according to the measures used. Qualitatively, the hospitalized patients revealed much more difficulty in making self-ratings and often would check both extremes in rating themselves in regard to a particular adjective.

In addition, highly competent students who had been previously studied in the transition from high school to college have been followed up. These students are now entering the senior year of college; this material provides useful clinical data about normative patterns of development during the late adolescent and young adult period.

A third research group in the Section on Personality Development has collaborated with the Puerto Rico Institute of Psychiatry and the University of Puerto Rico in order to explore the cross-cultural validity of hypotheses about adolescent development in a society undergoing very rapid economic development and social change. Twenty students selected from the University High School in Puerto Rico for superior performance in academic, extracurricular and social spheres in their high school year are being followed up through their college career. The criteria for the sample selection and methodological procedures in testing and interviewing are comparable to those used in studies previously used in APB research in the United States.

Another collaborative study was based on a quantitative analysis of the Student-TAT data of three groups of Puerto Rican students. The

group Student-TAT is a new projective test consisting of 10 slides depicting college scenes of arrival on campus, dating, taking an exam, calling home, etc. These three Puerto Rican groups of adolescents were rated through "blind" procedures on a five-point scale for three problem-solving variables. Analysis of variance results indicates that the difference between the competent and emotionally disturbed groups of Puerto Rican adolescents is significant at the .01 level. This replication in Puerto Rico using the Student-TAT measures of competence indicates that at the projective test level there is some justification for the cross-cultural use of the competence concept. On the other hand, when qualitative and quantitative studies are taken together, we find important differences in the cultural patterning of adolescent competence in the two societies.

Another collaborative developmental study of normal adolescents was negotiated with Poona University, India, in the spring of 1962, through the cooperation of the Office of International Research of NIH. This is designed as a longitudinal cross-cultural approach to adolescent problems and their resolution in the college years. A key theoretical objective is to sharpen our conceptual tools for identifying psychosocial variables in competence of adolescents in changing cultures, like India, where the dominant modes of family organization differ considerably from those of American society. The methodological objectives include (1) testing the cross-cultural validity of instruments which have been successfully used to differentiate highly competent from vulnerable college freshmen in studies at NIMH and in Puerto Rico, and (2) designing new transcultural procedures and techniques for cross-cultural research.

A sample of 26 competent students has been selected from the preuniversity degree class from the constituent five colleges of Poona University for intensive followup study throughout their college career. The following tests of cross-cultural relevance have been administered: Who Am I Questionnaire, Self-Description, Reference Groups Questionnaire (Harvard), Ways to Live (Morris), Semantic Differential (Osgood), Student-TAT (modified), Expectations Checklist (Harvard), and Autobiography Until the Year 2000 A.D. (Allport-Gillespie-Young).

Experiment in Training Mental Health Counselors

In the spring of 1960 a pilot project was begun in the training of 40-year-old married women as mental health counselors. The object of the experiment was to test the hypothesis that mature intelligent people with a particular psychological gift but without any special education in the mental health field, can be trained within a period of 2 years to function as psychotherapists in supervised settings. The 10 to 20 years of experience in family living and child raising were considered to be an advantage which the young student just out of school cannot possibly have. Eight women, all college graduates, all with children, were carefully selected and trained for four semesters. Recruitment, selection, training, and evaluation after the first two semesters were described in last year's report.

The 2-year training period ended in June 1962. The eight women, selected in June 1960, all completed the course satisfactorily. The program consisted primarily of practical on-the-job training. The work was, in the main, once a week individual psychotherapy such as is usually practiced in community mental hygiene clinics. In addition, all the trainees had some experience in interviewing couples together, and four of them functioned as cotherapists in groups. The work was always carefully supervised. Some of it was done in the NIH outpatient clinic; some in community agencies. During the second year the trainees saw altogether 165 individual patients for 1,573 individual interviews and 4 groups for 141 group sessions. The patients ranged in age from 14 to 60. The diagnoses ranged from psychosis in remission to adjustment reaction of adolescence. Approximately two-thirds of this work took place in community agencies, during the last two semesters.

Blind ratings of tape-recorded interviews provided a favorable evaluation at the end of the first year. At the end of the second year evaluation was carried out primarily by an examination of the trainees by three experts from outside NIH: Dr. Lotte Bernstein, Director of the Child Guidance Clinic and Professor of Psychiatry in Louisville, Kentucky; Dr. Robert Gibson, Clinical

Director at Sheppard Pratt Hospital, Towson, Maryland; and Dr. Julius Seeman, Professor of Psychology at George Peabody Teachers' College, Nashville, Tennessee. They spent 3 days at NIH early in June reading case reports written by the trainees, listening to tape-recorded interviews, and giving each trainee a 1-hour oral examination. The ratings can be summarized by saying that the average rating on a five-point scale with five as the highest score, was four or "good." In a tape-recorded discussion at the end of the period, the examiners stressed the following five points:

(1) That the degree of commitment of the trainees to their work was high, regarded as higher than in the average professional group;

(2) That the trainees showed a lack of defensiveness, which was attributed to the training;

(3) That the training advantageously "kicked over the traditional traces of giving a long string of academic work ahead of time and then building up to a little bit of practice," (this program did it the other way around);

(4) That the women had a strong sense of group identity which might well continue to serve them in place of a "union card;"

(5) That the women had the advantage of not having to relay on this program for their main source of security either economically or psychologically.

All three examiners concurred with the one who said, "I could think of an awful lot of patients that I would like to be able to refer to them and I wouldn't feel badly that they weren't going to see a psychiatrist."

In addition to this examination, the placement supervisors and the NIH staff rated the trainees and also came up with an average of four or "good" on a five-point scale.

With the cooperation of the Testing Services of the National Board of Medical Examiners, the trainees were given two-thirds of the questions of one of the National Board Examinations in Psychiatry. The average raw score on these questions of the National Board candidates was 90.36. The average for our group was 97.09 with no one of our trainees having a score as low as the national average.

The best proof so far of the success of this project is that all eight of the students are now employed and all intend to continue working in this field indefinitely. The employing agencies for this year are four community mental hygiene clinics, one public high school, one college, one state hospital adolescent service, and one experimental hospital word. The salaries are $5,000 a year, a little less than that of a beginning social worker in government. A followup study is being conducted by the University of Maryland under a grant from NIH.

The experiment has significance in three different directions. First, with regard to the manpower situation in the field of mental health, it has demonstrated that it is possible to provide more therapy more cheaply and at just as high a level of competence as we are at present providing. Second, the program deals with the problem of the intelligent middle-aged woman who finds herself out of a job when the children are grown. Third, the experience in training these women has relevance for the training of psychotherapists in general.

Studies on Stress and Anxiety

During the past year, the study of stress and anxiety has been approached from two major directions by the section under Dr. Sheldon Korchin. On the one hand, cognitive mechanisms felt to be important in the understanding of stress-induced performance changes have been studied and, on the other hand, studies of personality and situational determinants of psychoendocrine activity, particularly in the pituitary-adrenal system, have continued. Together with these research activities, Dr. Korchin has been deeply involved in theoretical and integrative analyses, in order to get a broader view of the mechanisms of stress behavior. Some of these theoretical analyses are now in press.

The research program has included both experimental-laboratory and field studies. The experimental work has been concerned largely with the study of the relations between focal and incidental attentional processes. Test tasks and procedures have been developed which require focal attention,

and in which incidental learning can be assessed. Also, certain task determinants and individual differences in cognitive style as related to these measures of attention deployment have been studied. The issue of anxiety has only been of indirect concern in these experiments; the one study directly testing the relation between anxiety and attention deployment has involved "expectant fathers" in the National Naval Medical Center. Aspects of attentional behavior and learning are being assessed in these men in the naturally-anxious state of awaiting the births of their babies.

This experiment is illustrative of the kind of "field experiment" which often makes possible the study of phenomena not easily varied in the Laboratory. A more important example is the study of the Project Mercury Astronauts, which is now in its third year. In collaboration with Drs. George Ruff (University of Pennsylvania), Hans Weil-Malherbe (NIMH) and Kristin Eik-Nes (University of Utah), this study has involved measures of psychological, affective, and psychophysiological (both adrenocortical and adrenomedullary) changes in response to the stresses of training and flight over more than a 2-year period. In order to understand the sources of stress-resistance in these competent men, as well as to serve as a basis for personalistic analyses, the personalities were studied in some depth at the outset and as possible on later occasions, particularly at the time of flights. Thus, this study has provided an unusual opportunity to study the personality changes in mature, competent men over time as they have coped with social and physical stress. So, too, the temporal dimension has allowed for the study of change over time in psychophysiological stress response patterns. By now, five of the original seven astronauts have been studied under flight conditions. Presently, a report is being prepared for NASA, and for subsequent publication.

Other activities in this Section include the completion and report of earlier research, including the studies of affect and somatic experience and of cognitive style differences in Italian psychiatric patients. The findings of these studies, conducted during 1960–61 in Italy, are sufficiently promising to warrant further cross-cultural work. The possibility of further research in collaboration with Italian colleagues is being explored.

In addition to participation in some of the work described above, Dr. Joseph Tecce, a postdoctoral research fellow in the Section on Stress, has been pursuing other related studies. These have involved a study of problem solving as a function of anxiety and task complexity, studies of cognitive and personality correlates of adrenocortical activity under life stress (in collaboration with Drs. Mason and Wolff), and an investigation of the effects of task complexity and stress on paired-associates learning in schizophrenics.

Studies of Sleep and Dreaming

There has been a major revival of interest in the study of sleep over the past decade which stemmed primarily from the discovery that dreaming is associated with a distinctive and regularly recurring physiological pattern. It now appears that dreaming is the psychological concomitant of a very basic biological function, and this reopens for experimental study many longstanding questions concerning the relationship between sleep or dreaming and psychopathology. Dr. Frederick Snyder, of the Section on Stress, has collaborated with Dr. Robert Feinberg and Richard Koresko over the past several years in a study of physiological sleep patterns in schizophrenic patients at Saint Elizabeths Hospital. The progress of that study, which has now become a major focus of effort at CPRC, is described under the report of that unit, while sleep researches in the Adult Psychiatry Branch have tended to concentrate upon other areas. Attempts to study the pathology of sleep quickly lead to vast lacunae in our knowledge of sleep physiology, and it is to certain of these that Dr. Snyder and Dr. Allan Hobson of the Laboratory of Clinical Sciences have turned their attention during the past year.

Foremost among these has been the study of entire nights of undisturbed sleep in normal control subjects by the simultaneous recording of multiple physiological variables. In the recent studies, these have included electroencephalographic patterns, eye movement, respiration, heart rate, blood pressure, basal skin resistance, skin temperature and finger pulse volume. Since there are formidable technical difficulties in this recording procedure the accumulation of data is necessarily slow, and beyond this the task of data processing is extremely tedious and time consuming. Despite this,

a considerable body of data has been collected and processed over the past year, and with the help of Dr. Donald Morrison of the Biometrics Branch this is now being statistically analyzed. Early results indicate that periods of dreaming sleep are distinctly different from the rest in terms of at least three of the variables studied in addition to the EEG and eye movements. Respiration, heart rate and systolic blood pressure seem to differ very very significantly during dreaming sleep both in terms of their average levels and in terms of the sequential variability. In contrast to the rather stable, regularly oscillating autonomic activity of nondreaming sleep, that of dreaming sleep shows marked flux and unpredictable change.

The most obvious explanation for these autonomic changes is that they reflect the changing emotional experiences of the dreamer, though there are reasons to suspect that this may not be the case. A study now in progress is designed to test this question by attempting to relate the patterns of physiological activity to the subject's recall of his dream experiences just prior to awakening.

The measurement of sleep depth is a matter of considerable clinical importance, particularly in relation to psychopathology. A project contemplated for the next year will attempt to contribute to the understanding of this problem. The previously mentioned battery of physiological measures will be recorded in normal subjects under their usual conditions of sleep, and will then be repeated in the same subjects after 36 or 60 hours of sleep deprivation. The various physiological measures will then be compared over these several conditions, on the assumption that any consistent differences resulting may reflect varying degrees of sleep depth.

While these systematic studies are in progress, it is hoped that a series of longitudinal studies of physiological sleep patterns in selected patients, chosen on the basis of a history of marked fluctuations in their clinical course, will be built up.

Study of Parents of Leukemic Children

In the past year the study of parents of leukemic children has been focused on the relationship between the individual parent's psychological pattern of adaptation to stress and his mean 17-hydroxycorticosteroid excretion rate during that period of stress.

In the previous 2 years of study, it was established that in this stressful situation of the threatened and impending loss of a child with leukemia, individual parents show the following phenomena: Each parent has a characteristic mean 17-hydroxycorticosteroid excretion rate; the day-to-day and week-to-week variation in steroid excretion rates of each parent is relatively small. In addition, the parents may be arbitrarily divided into three major groups. There are those whose means fall in the "high" range (High Reactors), in the "middle" range, (Middle Reactors), and in the "low" range (Low Reactors). Parents in the Low Reactor group rarely, if ever, show any single 24-hour 17-hydroxycorticosteroid excretion in the High Reactor range and vice versa.

The hypothesis was derived from clinical impression that the mean 17-hydroxycorticosteroid excretion rate of a given parent is negatively correlated with the effectiveness with which that parent defends against the threat represented by the impending loss of his child.

During the past year this hypothesis has been tested through the use of a blind predictive study with the psychiatric interview as the predictive tool. In addition, a correlation study of Rorschach responses of these parents with their cronic mean steroid excretion rates has been done.

In the predictive study the psychiatric interview was used (1) to delineate the long term and relatively basic and unchanging ego operations called into play in the attempt to adapt to the threatened loss of a child, and (2) to evaluate their effectiveness by using as criteria of ineffectiveness clinical evaluation of inner distress and impairment of ego functioning. Basic to this evaluation is the concept that the steroid mean of an individual will be proportional to the degree of painful arousal or, in psychoanalytic terms, psychic tension of unpleasure; and that clinically observed distress and ego impariment will reflect this hypothesized state of tension.

Assessments of ego operations and degree of distress and ego impairment were made for each parent on the basis of two interviews totaling 3–4 hours of interviewing. On the basis of this evaluation, prediction of the steroid mean was made

in a blind fashion, without access to either the steroid data or other psychiatric, psychological or nursing observation data on the parent.

The major methodological problem has been the way of evaluating and discounting transient ego states either existent at the time of interviewing or actually provoked by the interviews themselves. This has been particularly true because at the time of the study many parents had already lost their children and returned for the interviews 6 months after the loss of the child.

Preliminary results show a correlation between predicted means and actual means for both male and female parents. For eight men $r=.87$ ($p<.005$). For 11 women, $r=.57$ ($p<.025$).

Work now in progress is directed at enlarging the predictive sample, refining the hypothesis and the criteria of evaluation, and revising the methodology to minimize the "transient ego state problem." In addition, a validation study of the prediction criteria through independent assessment of parents is being attempted. A third major focus at present is the more intensive study of selected parents, representative of the High and Low Reactor groups.

The work with the Rorschach protocols of the parents has tentatively established significant correlations between various Rorschach category scores and mean steroid levels in the parents. These data are now being analyzed further.

Study of Depression

A study of behavioral and endocrinological aspects of depression has been in progress during the past 2 years. Interest has centered on studying patients during periods of crisis in an attempt to answer the question, "Do endocrine changes precede or follow behavioral changes?" The natural course of depressive illnesses has been studied and a psychodynamic theory of depression formulated, based in part on observations during periods of crisis and findings concerning the function of "precipitating" factors. During the past 2 years about 150 depressed patients have been seen and 38 have been hospitalized for intensive study.

A search for a biochemical which fluctuates with changes in mental illness has long been underway. After some preliminary investigation, the urinary 17-hydroxycorticosteroids level was selected for the following reasons: (1) It has been reported by one group that they are high in depression. (2) They represent an important breakdown product of an adrenal cortical hormone. (3) They can be measured accurately. (4) They have a major role in body metabolism.

Three general methodological desiderata contributed to the research design: (1) to collect biochemical and behavioral data independently, (2) to collect data on a continual, longitudinal basis, allowing for the retrospective analysis of any day that might be important theoretically, without contaminating the independence of biochemical or behavioral data, (3) to study periods of change, which offered methodological advantage in that during these times of stress there are dramatic shifts in the defensive structure of the individuals and in the biochemical homeostatic mechanism. To implement the study a research metabolic ward was developed, rating scales to measure mood and behavior were devised, and reliability of these scales with a trained nursing research team was tested. Data is also collected concerning the patients from tape-recordings of biweekly psychotherapeutic sessions, from patients' diaries and from the psychiatric social worker's weekly interviews with the closest relative.

In summary, the findings in this study are as follows: Fluctuations in 17-hydroxycorticosteroid levels correlate at a .0001 level with ratings of depression in certain groups of depressed patients. Depressed patients as a group have levels clearly outside of the range of normal or of the range of individuals under chronic stress. Levels of 17-hydroxycorticosteroid seem specifically sensitive to certain aspects of human behavior and that depression is one important factor. However, when one looks at individual cases, a few severely depressed patients with normal levels are discovered.

These observations led to an attempt to identify more specifically the behavioral factors which elevate or lower 17-hydroxycorticosteroids. Those cases were analyzed in detail which high and low 17-hydroxycorticosteroid levels and five factors were identified which are associated with increased levels and four factors which are associated with lower levels.

In addition, it appears from the total experience in studying over 250 17-hydroxycorticosteroid determinations and the corresponding behavioral data that the study of 17-hydroxycorticosteroid levels during period of stress is beginning to open a new dimension for the understanding of defen-

sive mechanisms of psychiatric patients. For example, high levels two to five times normal, are associated with a breakdown in defenses and with intensive concern over the emergence of ego-alien affect, thoughts and actions, while low levels have been associated with mania in a number of patients. Psychoanalytic theory has hypothesized that mania is a defense against the pain of depression. The endocrine data offers an interesting biochemical confirmation of this theory. One patient fluctuated between mania and depression every 48 hours. On 14 consecutive days the 17-hydroxycorticosteroid levels were lower without exception on the agitated, wild, manic days while on the depressed quiet days the levels are higher. The evidence suggests that the running of urinary 17-hydroxycorticosteroid levels in psychiatric patients may become a most useful measure for the estimation of "psychic pain" and the state of the defensive organization during stress. It also appears that depressed patients with very high 17-hydroxycorticosteroid levels are increased suicidal risks.

In 35 patients, depressive crises occured in 7. These were studied intensively from a biochemical and behavioral point of view, with interest focused on the understanding of the environmental factors preceding the crisis. Similarities were found between events preceding the original onset of the illness and events occurring prior to the crisis in the hospital. The precipitating events seem to function as specific disruptions of the patient's effort to ward off conflict areas.

Temporal analysis showed that in all but one of the cases the 17-hydroxycorticosteroid changes occurred on the same day as the behavioral changes rather than preceding them. Precipitating events occurred on the same day as the behavioral changes or 1 to 2 days prior to them. Also, a patient who has a major period of crisis and behavioral change without concomitant increases in 17-hydroxycorticosteroid levels is likely to have a poor long-term prognosis. Thus, study of the factors affecting 17-hydroxycorticosteroid levels is increasing our understanding of stress and defensive organization.

Studies of Neurochemical Factors in Behavior

During the past year neurochemical work in the Adult Psychiatry Branch has focussed on (a) neurochemical differences between inbred strains of mice and (b) the development of the McIlwain technique as a test system for the study of the effects of plasma from schizophrenic subjects upon certain neurochemical events. (Basically the McIlwain technique is an *in vitro* method of electrically stimulating slices of brain cortex and thus inducing metabolic events akin to those seen *in vivo*.)

The inbred strains of mice used were the BALB/cJ and the C57BL/10J. These strains are of particular interest because of clearly established behavioral differences—i.e., the C57BL/10J strain is characterized by greater exploratory activity and less fearfulness than the BALB/cJ strain. Work to date indicates the following: (1) the mice presently being tested have retained the behavioral differences noted earlier; (2) the serotonin content of a portion of brain consisting of pons, mesencephalon and diencephalon is significantly greater ($P = <0.01$) in the BALB/cJ mice in comparison with the C57BL/10J mice; (3) the norepinephrine content of this same portion of brain is the same for both strains; (4) the increased serotonin found in the BALB/cJ strain is located in the pons, mesencephalon and diencephalon and that total serotonin in the remainder of the brain is equal in the two strains; and that (5) studies with reserpine and Parnaet, an MAO inhibitor, indicate that the serotonin differential is more likely due to a difference in intracellular binding mechanisms than to differences in production or degradation of this amine.

Present work is concerned with determination of brain serotonin in other strains as well as the development of finer behavioral measures as a preliminary to attempts at establishing what relation, if any, exists between the serotonin and behavioral differences.

The McIlwain technique is now sufficiently developed that the examination of the effects of schizophrenic plasma on neurochemical events has begun and will be used during the coming year with patients studied with some of the diagnostic instruments that have proved useful in the Family Studies of schizophrenia.

CHILD RESEARCH BRANCH

Program Summary

The general aims and strategy of the longitudinal program, the conceptual orientation to-

ward developmental problems, the methods employed, the samples of subjects studied and the expected timetable of specific projects is available by consulting the annual reports for 1960 and for 1961, and by reference to planning documents and publications available on request.

The past year, our third, has been one of data gathering. At the present stage of theory and methodology in the behavioral sciences, concerns with *developmental channels in the infant*, *stage-specific aspects of marital interaction and patterns of parent-infant interaction* involve the investigator with new methods, insufficiently tested concepts and time consuming data gathering and data analysis procedures. Nevertheless, on the basis of this third year of experience in operation of the program, the scientists in the Child Research Branch continue to be encouraged at the apparent soundness of the strategy of the program. Results from the analysis of data from this first phase of the longitudinal program will be available over the course of the next 2 years.

At the end of calendar year 1962, data has been gathered on approximately 90 male infants, 80 female infants, 60 male 2-year-olds, 80 female 2-year-olds, and 40 husband-wife pairs married for 3 months. Data is in process of being gathered on 20 husband-wife pairs at the seventh month of pregnancy, 20 matched husband-wife pairs married a similar length of time but not pregnant, and 20 mother-father-first born infant triads during the first 3 months of life. Data gathering is continuing to augment our samples of male and female infants, mother-father-infant triads and 2½-year-olds.

On the basis of experience over the past 3 years, it appears wise to modify our original strategy of gathering a large number of subjects at a given phase in as short a time as possible, and rather to build an ongoing supply operation of young married couples who can constantly feed into our studies of the later stages but at a slower rate than has been true in the past operation of the program. Such a strategy makes the program more adaptable to the diverse developmental timetables of our subjects and leads to less loss of longitudinal data. In order to facilitate later cooperation with the nursery school phase of the program, only newlywed couples who live within a half-hour driving radius of the NIH are accepted for the longitudinal study.

Concurrent with the stabilization of the program, it has seemed appropriate to establish a formal administrative structure to reflect the actual working roles which have emerged. The new organization of the Child Research Branch will place those whose primary efforts are directed at developmental channels in the infant in the Section on Infant Development and those whose work is concerned with stage-specific family interaction patterns in the Section on Family Development. Staff members of the Branch whose work overlaps both areas will be assigned to the Office of the Chief.

Exploration of Behavior Continuities From Infancy to Age 3

The work directed at defining congenital behavior patterns and at exploring their biosocial correlates has depended upon data gathered at three points in the infants development from birth to age 3. These are at 3 to 4 days of life, at 1 month of life and between age 2 years and 3 months and age 2 years and 9 months.

Between 72 and 84 hours of age, detailed observation of the child's behavior is carried out in a carefully controlled environment by means of time sampling or continuous recording of behavior. This approach has been used in the local obstetrical hospitals (Naval Medical Center, Suburban Hospital, Georgetown University Hospital, George Washington University Hospital, Providence Hospital, Washington Sanitarium, Columbia Hospital and elsewhere) with the cooperation of local pediatricians and obstetricians.

At 1 month the data consist of parental ratings of a variety of infant behavior attributes which have been observed over the course of the first month of life and compared by the parents with a same-sex sibling in the same family. This retrospective set of ratings of neonatal behavior patterns is also obtained at age 2½ years. Factor analyses have indicated certain patterns of ratings which may correspond to congenital types. To date we have no adequate prospective sample of infants on whom these ratings have been carried out both at one month and at age 2½ years. We expect to be able to report on such data by 1964.

The other approach to assessing congenital behavior patterns is direct observations at age 2½ years in a seminaturalistic, nursery school laboratory in Building T–4 on the grounds of the NIH. The children are studied in groups of six for 4-

week periods, attending the school 1½ hours each morning. Separate observation schedules have been developed for each of the separate geographical-social settings within the morning session. Males and females have required separate observation schedules because of the disparity of social behavior and play patterns between boys and girls of this age.

Over the course of the past 3 years, these assessment techniques have been considerably explored and refined. Samples of infants observed between 72 and 84 hours of life and on whom the 1 month parental questionnaire was filled out are now beginning to be seen in the nursery school. A completely different set of data analysis procedures has been required in the nursery school for each sex, so different is the usual behavior patterning at age 2½ for each sex. This, and other considerations, has led us over the past 3 years to study at age 2½ about 60 males and 80 females on whom we have only the parental retrospective ratings at this age but no prior data. Such a cross sectional (nonlongitudinal) sample is of great value in beginning statistically and conceptually to explore for behavioral criteria to differentiate types of children at this age.

At this time we have identified in a tentative fashion several behavioral syndromes at age 2½ for each sex and are exploring the relations between these patterns and the patterns of ratings obtained from the parents. In the data now being processed we have for the first time all four types of data: direct observations at age 4 days, parental ratings at age 1 month, parental ratings at age 2½ years (of the recalled behavior at age 1 month) and direct observations of nursery school behavior at age 2½ years. After they have been accumulated and analyzed these data will provide a more solid basis for exploaration of congenital infant patterns.

All of this work is a necessary precursor to the planned search for family interaction patterns during the first 3 months of life and at age 2½ years. During 1964 and 1965 we will see the first pilot families, who were studied as recently married couples and who have subsequently had infants under our observation, come into the nursery school laboratory for asessment of the child. It appears that we must anticipate a minimum of 4 to 5 years for subjects to traverse the longitudinal program in this fashion.

The work on observation of infants, which was originated in the Laboratory of Psychology in 1956, is the most advanced. Here we can be reasonably certain that *muscle strength*, *skin sensitivity* and *effectiveness of feeding* represent independent behavioral apparatuses which can reliably be assessed at 4 days of life. Interestingly, provided one restricts testing to the more sensitive body surface areas, the factor of skin threshold to touch and pressure stimulation continues to show the sex difference at this early age. Whether this is a hormonal, other metabolic or anatomical effect is unknown; there are data on adults to suggest that it exists later on in the life cycle as well.

Over the past 3 years from the work of this laboratory as well as work going on in 3–4 other locations it has become abundantly clear that in order to establish meaningful behavioral syndromes in the first 2 weeks of life correlated observations of organic, physiological and behavioral factors are required. Data are now in process exploring the interaction of our *effectiveness of feeding*, *skin sensitivity* and *muscle strength* variables with antenatal drugs given prior to delivery, sex of the infant, arousal level at the time of assessment of the variables, whether the mother feeds by bottle or breast and whether there are minimal signs of possible brain damage or morphological immaturity. (Infant subjects with any medical history or examination even indirectly suggestive of known sources of brain damage have been eliminated from cross sectional samples prior to study; nevertheless, careful observational assessment suggests that there may still be some infants included in the sample with minimal damage.) During the past year one of our senior investigators has carried out a study of related problems in a nonhuman species and has spent time with Prechtl learning new techniques for neurological judgements. These activities have strengthened the laboratory's potential in this difficult area of research.

The analysis of observations of males at the age of 2½ is completed and now merely requires cross-validation, which is underway. It is of interest that on the retrospective questionnaire the parents report a factor which seems conceptually congruent with our observed effectiveness of feeding pattern. Males who are rated by their parents as high on this effectiveness of feeding infant pattern show play adaptation in the nursery school charac-

terized by frequent falls and accidents, not being easily blocked by animate or nonanimate obstacles, persistence in goal-seeking, and not requiring much help from the teacher. These data suggest the possibility that there is some relationship between the type of feeding pattern at birth and the presence of autonomy and persistence of play in the nursery school at age 2½ years.

Suggestive evidence exists for the hypothesis that certain types of motor and visual behavior in the nursery school may indicate subclinical types of brain damage or morphological immaturity; explorations are now in progress to check this out on the direct observation of 4-day-old neonates. A number of other such possible developmental links between the immediate postnatal period and the age of 2½ are also being explored. Findings on these studies will be available in 1963 and 1964.

Exploration of Initial Marital Patterns of Adaptation

Just as the work with infants has been toward clearer definition and identification of adaptationally relevant, congenital behavior patterns, so has the work with husband-wife pairs been toward a clearer definition and identification of adaptationally relevant, interpersonal patterns of behavior. Since the specific foci and methods of this area of the program have been described previously, and since subsequent annual reports will focus on findings, the annual report this year will outline the theory which has seemed appropriate to the problem.

In formulating the problem of effectiveness or ineffectiveness of adaptation to the stages of initial marriage (and of initial parenthood), three entities are central: developmental task, family role and ego identity. Each of these concepts was drawn from a separate area of psychosocial research: *task* from developmental theory, *role* from sociological theory and *identity* from psychoanalytic theory. These concepts can be related meaningfully to one another through our conception of *the developmental transaction*, the basic unit of change at a developmental stage in the family system.

The change processes characteristic of a given developmental stage may be supposed to occur at all levels of functioning—cultural, psychological and biological. Each new stage is initiated by a single event, such as the marriage ceremony for the initial stage of marital adaptation and the birth of the first child for the stage of initial parenthood. These events carry with them culturally-defined pressures for the husband and wife to change in specified directions so as to fulfill certain new developmental tasks within the social and interpersonal context of new family roles. These special developmental turning points we refer to as *developmental transition events.*

Embedded within the complex of pressures exerted upon husband and wife are qualitatively new potentials for relatedness and subjective experience. It is assumed that to the degree these new forms of interpersonal and subjective experience can be learned or adopted, to that degree the new developmental tasks will be observed to be carried out with minimal stress and disorganization of behavior or personality. And with husband-wife pairs who are unable to work out ways of adapting to these new pressures, signs of disturbance are predicted in the new *task situations.* This press acting on the couple (or family) to change in specific directions defined by the new roles and tasks is referred to as the *developmental issue* characteristic of a given developmental stage. The developmental issue characteristic of initial marriage has been called by Erikson *intimacy*, and the issue central to adaptation in the stage of initial parenthood we, following Erikson, have called *initial generativity.* These global concepts, intimacy and generativity (which are not adequately explicated in this report), form the larger context within which specific hypotheses are related one to another.

In order to relate our data on specific tasks and situations encountered by our couples to the larger concept of coping with the stage of intimacy, we work within a hierachy of units. The smallest unit is the single act. The next larger is the husband-wife interaction sequence. More inclusive units follow in the hierarchy so that a sampling of husband-wife interactional behaviors in situations which contain stage-specific issues can be considered to represent the issues of the life stage, provided the sample meets appropriate tests for representativeness.

The work over the past 2 years has been to observe in structured and semistructured situations husband-wife behavior, and as well to gather interview and questionnaire reports of the develop-

ment of the marriage and of the individual development of each adult since infancy. In the pilot project 44 couples have been studied; 20 of these will be studied during the first pregnancy and during the first 3 neonatal months; 20 others will be studied as controls for the pregnancy phase, these being couples who are nonpregnant but married an equal length of time since our initial appraisal early in marriage.

In analyzing the husband-wife patterns it is important to separate for evaluative purposes the role aspects of behavior from those aspects which are more an expression of unique or special psychological characteristics of individuals. For example, considerable data is accumulating on the process of husband-wife conflict resolution. Criteria are being developed which distinguish between effective and ineffective patterns of husband-wife conflict resolution. Lack of clarity in interpersonal communication and attempts to manipulate the other are two examples of types of behavior which are readily quantified and assumed to be nonadaptive behaviors in the situation of interpersonal conflict-resolution. In evaluating these data, attention is being directed to separating patterns characteristic of the role of husband and the role of wife in this middle class group from patterns which appear to reflect personal or idiosyncratic behaviors.

To leave the more idiosyncratic aspects of the marital relationship and to consider the question of adaptation to situation-specific role activities, here data is accumulating on seven activities for the initial stage of marriage: setting up a household, food arrangements, settling on mutual friends, working out new relationships with relatives, establishing an occupational-economic base, working out an initial sexual pattern, and planning for parenthood. These areas can be ordered as to relative importance with regard to the developmental issue of intimacy. For example, activities related to sexual or parental adaptation would be assumed to be directly related to the forms of husband-wife interactions. Occupational activities, on the other hand, would be assumed to be of less direct significance.

Certain of the developmental tasks and roles of initial marriage require for most couples less psychological change and less adaptational work than others. For most, the areas of food preparation and household management require less real change and are less stressful than dealing with relatives or working out sexual patterns. Conversely husband-wife pairs who demonstrate difficulties in coping with such areas as food or household management—as do certain of our subjects—would be predicted to have greater overall tendency to disturbed patterns of family adaptation.

To conclude this brief presentation of the concerns which guide our studies of interpersonal functioning in the initial stages of the family, it should be made explicit that this kind of program is based upon notion that there are a continuous series of potentially definable relationships between individual mental health, successful adaptation to a family stage, effectiveness of family interaction patterns in developmentally salient situations, identification of individual members of the family with their family roles and each individual's personally-evolved sense of identity.

Exploration of Initial Parent-Infant Patterns of Adaptation

The aims of the program in this area to identify primary parent-infant interaction patterns and to determine how these patterns are related to innate behavior tendencies of the neonate, to the preparental relationship of the parents and to the mother's own salient developmental preparatory experiences in her own childhood and adolescence. While these latter areas are studied in the work outlined in previous sections of this report, the work in the parent-infant area has been designed to achieve maximum comparability. The methodology follows the strategy used in the study of initial marriage and of the individual infant and 2½-year-old, namely a diversity of approaches—interview, direct naturalistic observation, special experimental situations, and questionnaires—to similar variables.

Observations in the home are made for 3-hour periods at 7, 14, 35, 41, 85 and 90 days of life; 45 categories of maternal or infant behavior are recorded continuously in an exploratory fashion for these 3-hour periods. The sample includes natural events such as feedings, bathing, vocalizations and affective exchanges between mother and infant. Eight hour home observations are also made when the baby is 21 and 90 days old; an 8-hour laboratory session involving mother, father and infant is carried out when the infant is 7 weeks of age at

the NIH. Sequences of parent-infant interaction will be analyzed to determine the information yield of various events in differentiating between mother-infant pairs.

This past year, the first for this new area of work within the Child Research Branch, has been spent tooling up, exploring methods and concepts, and processing the first few longitudinal families which have provided newborns for us. In addition, special supplementary families have been studied to add to our experience with the new methods. We have learned that the particular situation for study here is a good deal more stressful than is true of the research on newly married couples or in the nursery school. For some young women to serve as subjects while performing novel and emotionally laden maternal tasks requires supportive preparation during the pregnancy. What is perhaps more surprising is how very many families cannot only tolerate, but even welcome such study. The program's success in maintaining a long term relationship with a stable sample of families depends on a careful analysis of the sources of stress inherent in the role of the volunteer research family.

Timetable for the Longitudinal Program of the Child Research Branch, CI, NIMH

Year	Relationship in the 4th month of marriage	Relationship in the 7th month of pregnancy	Congenital characteristics of infants	Mother-father-infant relationship in 1st 3 months of life	Developmental characteristics of child in 3rd year
1959	Planning initiated.		Project transferred from Laboratory of Psychology.		Planning and data gathering initiated.
1960	Pretest couples.		Third sample initiated.		Males studied.
1961	Pilot study initiated.	Planning initiated.		Planning initiated.	Females studied.
1962	Data analysis on pilot couples.	1961 pilot couples initiated.	Infants born to 1961 pilot couples.	Followup of 1961 pilot couples.	Females studied.
1963	Initial findings of pilot study.	Pilot study completed.	Findings reported.		Males and females studied initial findings.
1964		Findings reported.		Pilot data finished; initial findings reported.	Followup of infants of 1961 pilot couples.

NATIONAL INSTITUTE OF NEUROLOGICAL DISEASES AND BLINDNESS

REPORT OF THE CLINICAL DIRECTOR

Since the last report, 799 patients were admitted to the Clinical Center by the National Institute of Neurological Diseases and Blindness. There were 2,049 outpatient examinations, and 1,366 patients were examined at the request of other Institutes. 1,873 electroencephalograms and 17 electrocorticograms were done. There were 172 surgical procedures. This represents a total of 22,237 patient days.

During the past year, intra-Institute clinical committees on Technical Development, Pharmacology, and Clinical Safety functioned as advisory groups to the Clinical Director. These committees are composed of physicians with patient care responsibility, scientists whose skills are relevant to patient care affairs, and senior nurses.

On behalf of the Institute, the Clinical Director gratefully acknowledges the cordial and skillful support of the Director of the Clinical Center and his staff. The efforts of this administrator make our patient care responsibilities easier and make our clinical investigations feasible. Once more, it is a pleasure to thank Mrs. Louise Baker and the staff of the Neurology Nursing Service for their dedicated effort during the period of this report.

MEDICAL NEUROLOGY BRANCH

Introduction

The investigations of the Medical Neurology Branch have been concentrated in three areas: (1) multidimensional studies of patients with neuromuscular disease; (2) pharmacologic investigations of neuromuscular junction and muscle cell excitation phenomena; and (3) integrated neuroradiologic studies of normal and abnormal aspects of patients who have neurologic disease. The essentiality of a multidimensional attack on the chosen target diseases, or disease categories, is to be emphasized. The necessity of adding to the armamentarium of this Branch laboratory facilities for the applied techniques of tissue culture, biochemistory, immunology, histology, and electron microscopy is readily apparent.

For the clinical studies, 375 patients were admitted for a total of 7,114 patient-days, and 225 outpatients were seen.

The only major change in the permanent staff has been the loss of Dr. G. Milton Shy, former Branch Chief (and Associate Director for Intramural Research), who became Professor of Neurology at the University of Pennsylvania. More than being the first Branch Chief of Medical Neurology and the first Clinical Director of NINDB, Dr. Shy constantly provided stimulation, encouragement, and aid to the research of each of us, in both a scientific and personal manner. Although his own research is now at the University of Pennsylvania, the imprint of his scientific leadership will persist at the NINDB in many ways for a long time to come.

Neuromuscular Disease

The investigation of selected types of neuromuscular disorders in this Branch by Drs. Engel and Shy is based upon an integrated study by multiple clinical and basic techniques, in order to analyse many facets of each patient's disease. Particular emphasis is being placed upon applying these techniques to muscle obtained by biopsy, because this tissue is the one severely involved in these disorders; however, in the "primary" diseases of muscle or the neuromuscular junction it provides us with the tissue which bears the brunt of the disease process.

One technique which has been further developed and emphasized in this Branch is the *enzyme histo- and cyto-chemistry* of biopsied human skeletal muscle. The cytochemical techniques are suffi-

ciently refined so that they may be used to demonstrate the functional activity of fine structural components of the muscle cell. With the histo- and cyto-chemical techniques, two fundamental types of abnormalities in skeletal muscle are detectable: (a) a generalized change in a given reaction, for example, loss of phosphorylase activity and (b) a localized change of activity confined either to a specific histochemical type of muscle fiber (as were the mitochondrial aggregates in a case of myotonia congenita) or to a certain region of individual muscle fibers (as in target fibers and central core disease). In normal human muscle, it has been found that specific enzymatic techniques accurately display the loci of mitochondria. The distinct histochemical characteristics of different types of skeletal muscle fibers have been confirmed and the histochemical profiles of Type I and Type II fibers have been extended by the present studies. Because the major site of abnormality in some of the neuromuscular diseases, such as amyotrophic lateral sclerosis (ALS), is considered to be in the lower motor neurons of the spinal cord, the cytochemical techniques which have been developed in regard to muscle are now being applied with Dr. Camp to normal spinal cord material obtained at autopsy to provide a basis for study of cords from patients with ALS and other neuronal degenerative diseases.

To add additional dimensions to our investigations, study of *enzymatic, other biochemical, and immunologic properties* of the biopsied skeletal muscle and body fluids from patients with neuromuscular disease has been undertaken. Enzyme electrophoretic studies have been started by Dr. Brody. Quantitative assay of enzymes associated with glycogen synthesis and degradation in muscle are being done by Dr. B. I. Brown of Washington University. Assay of properties of the lactate dehydrogeneases in muscle from patients with muscular dystrophy is being done by Dr. N. O. Kaplan at Brandeis University, seeking molecular abnormalities in human dystrophy similar to those found by him in chicken muscular dystrophy. It is hoped that the number of biochemical techniques available will be expanded and that a biochemist interested in applied biochemistry of neuromuscular disease will be added to this Branch to work specifically on these studies.

In a patient presenting with a proximal myopathy beginning at age 49, routine histochemical investigations disclosed a complete absence of skeletal muscle phosphorylase. Subsequent studies demonstrated that the propositus and one of her brothers appeared to have a new familial variety of *skeletal muscle phosphorylase defect*, as determined by its clinical onset in later life (fifth decade), in contrast to previous cases of "McArdle's disease" with symptoms beginning in childhood. In the late-onset type, there were both a completely and a partially affected individual. The former had severe muscular weakness and wasting without muscular cramps and in her skeletal muscle there was no phosphorylase activity. The latter had postexercise muscular cramps without weakness or wasting and in his skeletal muscle the total $(a+b)$ phosphorylase activity was about 35% or normal. (The biochemical assays of phosphorylase were done by Dr. Williams of NIAMD).

A detailed clinical, histological, and electromyographic analysis of 131 patients with *late-onset myopathy* has been made by Dr. Shy. It was found in the group of patients with proximal muscle weakness occurring sporadically after the age of 30 that when their age was less than 50 the most common underlying condition associated with the muscle disease (myopathy) was one of the collagen diseases, for both males and females. In men over the age of 50, neoplasia was the most common associated disease (in more than 90% of the cases). In women over the age of 50, there was no common underlying condition and, in fact, many of these women had no demonstrable associated diseases. A review by Drs. Humphrey and Shy of diagnostic *electromyographic examinations* of 159 patients with many forms of neuromuscular indicated that agreement between the clinical diagnosis, the pathological changes in the muscle biopsy, and the electromyographic findings was complete in 85% of the cases.

In the histo- and cyto-chemical studies of biopsied muscle from patients with various myopathies, several interesting features were demonstrated. In a patient with dominantly inherited *facioscapulohumeral muscular dystrophy*, it was found that in a minimally involved muscle the earliest detectable changes occurred exclusively in fibers of histochemical type I, indicating an increased susceptibility of the type I fibers to the disease process. Although the detailed analysis of the histochemistry done on muscle biopsies from

50 patients with *progressive muscular dystrophy* has not yet been completed, it has been shown that an increased phosphatase activity of the connective tissue toward high-energy phosphate compounds was *not* present in any of the cases of progressive muscular dystrophy, in contrast to such findings by other authors. In *myotonia dystrophica*, cytochemical studies revealed that a striated annulet is a subunit of a muscle fiber disoriented *en bloc* with preservation of the relationship of the components (myofibrils, sarcoplasm, and mitochondria) within it. The sarcoplasmic masses were found to lack myofibrillar A-band ATPase activity. They consisted of diffuse, disorganized intermyofibrillar material, and therefore seemed to be regions of the muscle fiber in which the myofibrillar degeneration was greater than that of the intermyofibrillar material. In one case of *myotonia congenita*, unique "mitochondrial aggregates" were discovered, and they occurred only in the histochemical type II fibers. Furthermore, the aggregated mitochondria appeared to lack a flavoprotein associated with succinate oxidation. Typical muscle target fibers have been found in three of five patients having *hypokalemic familial periodic paralysis* and two of three patients have periodic paralysis associated with *paramyotonia congenita*, especially during the paralytic attack. Previously, it had been suggested from this Branch that the target appearance represented a reaction of the muscle fiber to denervation and that it was often an early reaction because it could appear in fibers of nearly normal diameter. With the presence of target fibers in the periodic paralysis patients, it must be considered that they may also be due to denervation, functional if not anatomic, in those conditions. During an attack of flaccid paralysis in a patient with paramyotonia congenita, "vacuoles" in the muscle fibers were found, as may also occur in the hypokalemic familial periodic paralysis. By means of cytochemical techniques, abnormal structures termed "*cytoplasmic bodies*" have been found in pathologic human skeletal muscle and chick embryo skeletal muscle fibers grown in tissue culture. The cytoplasmic bodies are evidence of muscle fiber abnormality but are not of specific diagnostic significance; they are not indicative of a viral infection.

Studies have been continued to more clearly delineate the various conditions which present as muscular weakness in childhood (including *floppy babies*) by means of the more usual diagnostic methods as well as by use of specific histochemical, biochemical, and electron microscopic investigations of these disorders. By use of the various techniques and especially by careful study of the muscle biopsy material, two new varieties of muscle disease have been discovered. One was "nemaline myopathy", found in a child of four years who presented with an apparently nonprogressive type of muscle weakness. Biopsy disclosed the unique appearance of short rod-shaped structures in many of the muscle fibers. Histochemical studies revealed that these rods were not mitochondrial or sarcoplasmic reticulum in character but that they resembled myofibrillar material which had lost its normal myosin antigenicity and its myosin ATPase activity. Electron microscopy by Dr. Wanko of the Ophthalmology Branch disclosed that the rods had a semicrystalline structure with a repetitive longitudinal periodicity of 145 A, indicating that they were composed of the protein myosin or one of its subunits. Recently, the diagnosis of nemaline myopathy was made in a second patient, the slides of a muscle biopsy having been sent here for diagnosis. Subsequently, the patient was admitted and underwent the same diagnostic investigative studies with the same results being obtained. Because the second patient was 16 years old, we now have a longitudinal view in time of the nature of this disease, which has proven to be slowly but clearly progressive. The other new disease, which may be called "central core disease, type II," was first found during the past year in an 8-year old girl who presented with a rather nonprogressive muscle weakness of longstanding, which was not familial. In the muscle biopsy it was shown that virtually all of the muscle fibers contained a central abnormal region, superficially reminiscent of central core disease, type I. However, the details of the histochemical analysis of these abnormal core regions show that they were not like the cores in any of the cases of central core disease, type I.

In patients with *infantile spinal muscular atrophy*, analysis of histochemically stained muscle biopsy material by Dr. Fenichel revealed a specific pattern of muscle fiber atrophy and hypertrophy. The propensity of one fiber type to be affected to a greater degree than the other in this

disease possibly may indicate that the two histochemical types of fibers are a reflection of differences in innervating neurons.

Patients with *amyotrophic lateral sclerosis* (ALS) and other lower motor neuron diseases are being studied to learn more about the pathogenesis of these conditions. The current approach includes a careful evaluation of underlying conditions associated with these diseases. In denervated muscle (due to ALS on other diseases affecting motor nerves), cytochemical studies revealed two basic types of muscle cell reaction. The first is a concentric change represented by the target fiber and targetoid fiber, while the second is a diffuse change. To obtain *animal models* of some of these diseases, Dr. Hogenhuis is denervating muscle by nerve section and by experimental allergic neuritis, with the histochemical and cytochemical findings in the denervated muscle being compared to their counterparts in human disease. Of particular interest is the fact that in none of the experimentally denervated animals have target fibers been produced.

The histology of biopsied skeletal muscle from 37 patients with myasthenia gravis has been reviewed by Dr. Fenichel. A pattern of muscle tissue alteration was noted in relation to duration of symptoms. Nonspecific inflammatory changes occurred early in the disease and then resolved. The inflammatory phase was followed by tissue alteration which was more frequently of the neuropathic type. The results suggest that the presynaptic portion of the neuromuscular junction may be the site of dysfunction in myasthenia gravis.

The neurologic complications of Wegener's granulomatosis were studied by Dr. David Drachman and found to be caused in three ways, by (1) contiguous granulomatous invasion, by (2) remote granulomatous invasion, and (3) vasculitis of the nervous system. Drs. Silberberg and David Drachman have analysed the myopathy associated with Sjogren's syndrome and emphasized that myopathy is often the presenting feature. Drs. Daniel Drachman and Gumnit (of the Electroencephalography Branch) studied the recurrent sleep-waking cycles and periodic respirations in a patient with the Pickwickian syndrome. It was concluded that oxygen lack was the predominant factor in driving the patient's respiration and awakening her from the somnolent state. Weight reduction resulted in significant improvement in the clinical and laboratory findings.

Pharmacologic Investigations

The studies of Dr. Irwin are primarily concerned with the basic mechanisms of pharmacologic activity and muscle cell function, especially at cellular and subcellular levels, to provide a basis for understanding how such functions might be deranged in the pathophysiology of neuromuscular disease. When significant leads occur, they are followed with a view toward developing pharmacologic preparations for clinical application in the treatment of neuromuscular disease, as was done with the lycoramine derivatives.

With Dr. Trams (Neurochemistry Laboratory), the *interaction between macromolecules and pharmacologically active chemicals* has been analysed. The majority of chemicals which alter biological function do so by interaction with cellular components. These components are commonly referred to us "receptor sites" and have been shown to have a high degree of specificity. Almost nothing is known of the chemical composition of the sites, either as to which cellular macromolecules are involved or what molecular or submolecular force is responsible for the interaction. It has been found that large dye molecules, utilized as tissue receptor models, interact with pharmacologically active diquaternary substances, similar in some ways to native tissue receptors. The dye molecules thus may be used as models of tissue receptors for theoretical studies relating to the interaction of pharmacologically active compounds and tissue receptors. These studies may make predictable the design of new therapeutic agents in the treatment of neurological disorders and may lead to a greater understanding of cell function in neurologic diseases.

The basis of the *electromechanical coupling and drug activity in muscle* has been studied to determine the means by which the electrical events of the muscle membrane induce sliding or coiling of protein molecules to produce shortening or tension. Using the slow skeletal muscle fibers of frogs, it was found that the efflux of calcium produces a contractile event which apparently is not related to nerve or muscle membrane activity. It is believed that the movement of calcium outward is a primary event initiating contraction. Although loss of calcium does produce inexcitable

muscles, it does not activate contractile mechanisms which can be shown to be intact by their response to several drugs.

In a comparative *pharmacologic study of fast and slow dog* muscles, it was found that muscles which contract slower have a lower threshold and longer duration of potentiation to several drugs which facilitate muscle contraction. During the course of this study, it became evident that the collection and evaluation of data was the rate-limiting factor in obtaining information from the experiments. An automated instrument system whereby data will be obtained as analogue DC voltages is being developed.

The search for compounds with pharmacologic properties which suggest their clinical use in the treatment of myasthenia gravis has continued, as one aspect of the analysis of the action between cholinesterase and its inhibitors in regard to transmission and conduction processes and blockade by drugs. From these studies, two *lycoramine derivatives* were developed and have received a clinical trial in the diagnosis and treatment of myasthenia gravis. Drs. Somers, Shy, and Irwin have studied the clinical effect of lycoramine derivatives in myasthenia gravis. It was concluded that the long-acting oral form (deoxy-demethyl-dimethyl lycoramine carbamate hydrochloride) was of potential therapeutic usefulness, and the short-acting intravenous form (deoxy-demethyl lycoramine methiodide) was of potential diagnostic use in myasthenia gravis. There is a continued interest in the possible role of the *thymus gland* regarding the pathogenesis of myasthenia gravis. With Dr. Mohamed, extracts of myasthenic human thymus tumor and of normal thymus glands from young calves were found to have biological activity. The extracts potentiated the acetylcholine contracture of test muscles and induced contractures in the absence of acetylcholine stimulation. Inorganic ions were not responsible for this activity.

Because Dr. Galindo of the Surgical Neurology Branch found that during anesthesia the chimpanzee requires an unusually large amount of succinylcholine for the production of paralysis, samples of plasma and erythrocyte cholinesterase from the entire Neurosurgical Branch chimpanzee colony have been examined. Although the *chimpanzee cholinesterase enzymes* were found to have the same substrate specificity as the human ones, the amount of cholinesterase in the plasma of the chimpanzee was much higher than that reported previously for a large number of normal humans. This indicates a high serum enzyme level as a possible factor in drug activity on the neuromuscular junction, in the chimpanzee.

Neuroradiologic Studies

Dr. Di Chiro is emphasizing three aspects of Neuroradiology (1) A good technique is of the utmost importance because in neuroradiology, probably more than in other branches of diagnostic radiology, proper technique and good interpretation are inextricably intermingled. The anatomic structures should, to begin with, be studied by methods capable of revealing them in three dimensions; failure to do so, invites misinterpretations. (2) The neuroradiologic procedures cannot be considered separately; plain X-rays, laminography, pneumography, cerebral angiography, and radioisotope brain scanning should all be integrated with each other. These procedures are the modern, semeiologic tools, each type of study giving information that is not obtainable with other types. Thus, it is not correct to compare the relative "value" of one neuroradiologic technique with another. (3) Only thorough, detailed studies of the normal anatomy and physiology of structures by neuroradiologic techniques can provide the basis for interpretation in pathologic states.

The newly arrived equipment for *axial transverse laminography* (an original technique of Dr. Di Chiro) represents a major technical improvement. This technique gives, for the first time, a true three-dimensional demonstration of brain structures and will be highly accurate in the localization of intracranial space-occupying lesions.

An assessment was made concerning the *reliability of totally integrated neuroradiological procedures* (plain roentgenograms, tomograms, pneumoencephalograms, and angiographic studies). These combined studies have an accuracy of 92% in the detection and localization of intracranial space-occupying lesions and 64% in the assessment of their nature. In the determination of the extent of the lesion, neuroradiology remains somewhat unsatisfactory. With the adjunct of isotope scanning localization, the reliability in the detection of intracranial space-occupying lesions increases, so that all neuroradiologic procedures to-

gether have an accuracy exceeding 95%. Further integration of the several neuroradiologic procedures has been accomplished in studying the *mutual relationships of arteries, veins, and cerbrospinal fluid cavities*, particularly by use of angiographic and fractional pneumoencephalographic techniques. Sometimes changes in the CSF cavities may be inferred from the angiographic studies and vice versa.

Bony Structures

Drs. Di Chiro and Nelson have devised a method for roentgenographic measurement of the *volume of the* sella turcica and, indirectly, of the size of the pituitary gland. By this method, they are able to accurately predict the size of the sella in 83% of the cases and the size of the pituitary in 88% of the cases. Using these findings, a study of the volumetrically small sella turcica has been begun with Dr. Fisher. It is hoped to have an objective method for establishing in which of the various nonturmoral conditions associated with hypopituitarism a small pituitary gland is found. Because little is known of the radiographic changes which may occur in *the clivus*, an anatomical radiographic study of that structure is in progress, including the use of a special projection which has been devised to demonstrate laminographically the clivus in the coronal plane. Some cases of lateralized posterior fossa lesions (such as chordomas) of the clivus are diagnosable only by coronal laminography. Similarly, *the jugular foramen* has been studied with Dr. Nelson by a specially developed tomographic projection allowing bilateral comparison on the same film. This method is important for the differential diagnosis of lesions in this region. With Dr. Fisher, current studies are directed toward measuring *the two hemicrania* and, in particular, to obtain accuracy in measuring the middle fossae. In addition to determining size differences in the normal population, these studies may be important for diagnostic purposes in the field of temporal lobe epilepsy (in which smallness of the middle fossa on the affected side has been previously reported).

Structures Delineated by Intravascular Contrast Material

The angiographic demonstration of the *choroidal plexus of the eye* has been accomplished by means of carotid angiography in 64% of the patients, permitting evaluation of the angiographic position and displacement of the choroidal plexus, *e.g.*, by posterior orbital tumors. In addition, it is also the basis of a method for evaluating the axial length of the living eye, the anterior point being marked by a small X-ray opaque disk placed upon the apex of the cornea.

Soft Tissues

With Dr. Nelson, *soft tissue radiography (STR) of the limbs* has been applied to a large number of patients with neuromuscular disease and the findings correlated with histological observations of muscle biopsy material. In a high percentage of 10- to 20-year-old males with pseudohypertrophic muscular dystrophy, a characteristic pattern of change has been found in the muscle which is sufficiently specific to be suggestive of the diagnosis. While the X-ray is less sensitive than histology in showing the presence of fat in a given area, because it permits wider sampling STR may show fatty infiltration in muscles not examined by biopsy.

Radioisotopes

Localization of brain tumors and other intracranial abnormalities has continued during the past year, using *brain scanning techniques* with RISA and RIAF. A total of 111 RISA brain scans were done on patients admitted to this Branch as brain tumor suspects, of which 26% were positive. An additional 96 diagnostic brain scans were done on patients from other branches, of which 34% were positive. The study of normal structures of the brain demonstrable in the RISA scans is also being pursued. It is now possible to recognize consistently in the brain scan the sylvian fissures and the tentorium, in addition to other large vascular structures which have been demonstrated previously (*i.e.*, the longitudinal and transverse sinuses). The mapping of the normal cranio-cerbro-meningeal vascular structures in the brain isotope scan, *isotope encephalography*, is a new and promising field which will undoubtedly increase the accuracy and diagnostic reliability of brain scanning. There are also plans to demonstrate the cerebrospinal fluid cavities by injecting radioisotopes into them. The *subarachnoid distribution of drugs* following lumbar injections as determined by myelography, autoradiography, and external scanning was studied both in monkeys

and in humans, with Drs. Riesselbach, Freireich, and Rall. In this study, it was shown that for intrathecally injected drugs to attain certain and significant access to the endocranial cerebrospinal fluid compartments (cisterns and ventricles) the volume of the instilled material should be larger than has been assumed previously. Compounds injected intrathecally in volumes smaller than 10% of the cerebrospinal fluid will not reach the basal cisterns in significant concentrations.

SURGICAL NEUROLOGY BRANCH

During the past year, the Branch conducted investigations in epilepsy, involuntary movements, lesions of the visual system, developmental defects, cerebral trauma, language, memory, perception and personality, effects of low temperature on the nervous system, effects of ionizing and radiofrequency energies, relationship of certain anesthetics to internal carotid blood flow, and changes in internal carotid blood flow during craniotomy. Certain technical developments were also undertaken.

Epilepsy

Investigations on epilepsy range from single cell to the human patient. For the first time, the refractory intervals of cortical nerve cells were determined by means of intracellular recordings. They measured between 2.6 and 2.9 msec. This would suggest that the highest frequency of discharge from these cells was at the most 400/sec. It was frequently observed that excitation was produced more effectively by synaptic bombardment than by stimulation applied directly to the neurons. From these observations, it was concluded that in response to direct cortical stimulation the initial surface-negative potential cannot be attributed to direct excitation of the superficial dendrites, but is closely related to synaptic excitation of cortical neurones. The inhibitory mechanism was evoked more readily in cortical neurones of increased excitability than in neurones of normal excitability. It had a higher threshold and longer latency than the excitatory mechanism, but once set in motion it was more powerful than the excitatory mechanism.

The fact that the cortical inhibitory mechanism was more readily evoked when the cortical mechanisms were in a state of increased excitability suggests a cause for the high incidence of nocturnal epileptic seizures in some patients and a possible mechanism with which some of the epileptic manifestations can be aborted by a startling or strong afferent stimulus.

In a study of cortical spreading depression, it was noted that there was a definite relationship between unit spike discharges and cortical spreading depression. The discharges were accelerated when the cortical spreading depression developed. This observation clearly indicated that spreading depression potential is closely related to excessive depolarization of nerve cells in the cortex.

As an outgrowth of these experiments, a method for simultaneous recording from several single nerve cells has been developed. This array consists of a platform secured on the head of the subject which carries two or four high input impedance transistorized preamplifiers and micromanipulated units. The latter carries two or four microelectrodes capable of recording electrical activity of individual nerve cells. It has been tested in the monkey and is being developed for application to patients.

Paroxysmal discharges as a function of experimental penicillin lesions have been related to the cerebral deposition of dilantin at low temperatures. Thus, low therapeutic doses of dilantin given by vein while the cerebral temperature ranges below 25° C. have been followed by subsidence of the epileptiform abnormality as recorded in the EEG. These results which were derived from experimental animals have served as a basis for clinical application, with some success, in four patients. Paradoxically, the effects of low temperature on paroxysmal discharge without coincident medication are not exaggerative, and while the epileptiform discharge continues it is either somewhat reduced in amplitude and frequency, or unaltered.

Depth electrode studies in the human have provided an opportunity for comparison of abdominal sensation thus produced and the epileptic epigastric aura, to further define the nature and mechanism of the abdominal aura in epilepsy. Stimulation of the mesial temporal region and the basal ganglia in eight epileptics and six nonepileptics caused them to report sensations closely resembling spontaneous epileptic abdominal aurae. Recordings of gastric motor activity during spontaneous abdominal aurae and those induced by Metrazol activation and depth stimulation usually

showed no effect upon gastromotor function. The most mesial abdominal sensations were obtained from the centrum medianum thalami. The frequent association of abdominal aurae with disturbances of the highest level of intellectual function is of interest since it implies a spatial or functional contiguity which may be of significance.

Membrane electrodes have been successfully implanted along both parietotemporal cortices in the same patient, and thus recordings of a bitemporal nature have been obtained, outside the operating room, from surface structures.

A group of 110 children in whom epileptic attacks were first investigated under 2 years of age were followed up by means of clinical and laboratory examinations for a period of over 4 years. This evaluation was an attempt to relate mental development to the various characteristics of the seizures. Sixty out of these 110 cases were mentally retarded. The group is divided into those with infrequent generalized seizures (46), and those with frequent minor attacks (64). Statistical evaluation shows a significantly higher incidence of retardation in children with frequent minor seizures as compared to those with less frequent major attacks.

Status epilepticus was successfully treated by a novel combination of topical cooling and anticonvulsant medication in two infants during the past year. It seems that reduction of cerebral temperature in combination with dilantin has a place in emergency treatment of status epilepticus which does not respond to more orthodox methods.

Involuntary Movements

Activity of single nerve cells in the motor cortex in cats was recorded with glass micropipette electrodes while stimulation was applied to the cerebral peduncle, medullary pyramid, red nucleus, globus pallidum, putamen, caudate nucleus and zona incerta. Over 1,000 cells have been studied. All the structures stimulated were found to have synaptic connections with the neurones in the motor cortex. This study is of basic importance in understanding of interaction between pyramidal and extra-pyramidal systems and thus fundamental in the study of involuntary movements.

Instrumentation for better recording of motor performance is being developed. A more meaningful evaluation will be achieved through the use of the Bode Plot Method (gain-frequency interrelationships). In addition, an electrophysiological study of basal ganglia interrelationships is being undertaken in conjunction with the Branch of Electroencephalography. The details of this study are reported under the aegis of that Branch.

Developmental Defects

The pathological morphology and pathogenesis of dysrhaphic states failure of closure of the neural crest and anencephaly have been investigated using human embryological material. Cell counts and measurements of the surface area of the neuraxis at determined levels reveal that the numbers of neural cells are about the same in the early stages of dysrhaphic and normal embryos. Thus in a porportion of anencephalies and also in partial cranial or spinal dysrhaphism, the primary defect is nonclosure of the neural tube. Furthermore, it is concluded that the presence of normally developed eyes in most of human anencephalic specimens relates to the fact that the closure of the anterior neuropore proceeds from both ventral and dorsal regions. Other genetic instabilities were studied through analysis of chromosomal patterns and congenital malformation in mongoloids. All of 14 mongoloids studied had an abnormal number of chromosomes, while only 8 of the mongoloids had classical trisomy for chromosome 21 as the sole abnormality, and the chromesomes of their parents were normal. This instability is of importance in furthering our understanding of nondysjunction. Forty cases of severe cerebral malformations (about 10 per 1,000 pregnancies) have been correlated with complications during gestation and the perinatal stage. The significance of this correlation seems clear and indicates the need for more studies of the etiology of teratogenesis. Finally, in order to provide some norms for study of a particular kind of craniostenosis called trigonocephaly, systematic measurements of the orbital indices have been made during early childhood and tabulated.

Cerebral Edema

The thiocyanate space of the brain has been studied through the production of a cold lesion on the cortex of the cat, following which sodium thiocyanate was given by vein. The thiocyanate content of the two halves of the brain was compared with each other and with the plasma level

of the ion. There was increase in the volume of the hemisphere containing the lesion. This was due to an accumulation of fluid of the same magnitude as the increase in the thiocyanate space which is predominantly extracellular. Similarly, the volume of distribution of fluorescent protein and inulin in incubated brain slices was measured and its histological distribution inferred from a comparison of these findings with the distribution of fluorescen-labeled albumin in slices visualized with ultraviolet microscopy. Fluorescence microscopy revealed that the distribution of fluorescein-labeled protein was limited to the cut surfaces of the brain slice. Since the volumes of distribution of protein conjugated with lissamine rhodamine B 200 and inulin were similar to each other but were presumably limited to the injured edematous surfaces of the brain slice, the results support the concept of a relatively small extracellular space in the brain. Also, serum proteins labeled with fluorescein isothiocyanate were incubated with chick or rabbit choroid plexuses. The chick choroid plexes revealed intense passage of fluorescein labeled albumin (FLA) into the stroma, but when fluorescein-labeled globulin was used, no such passage was observed. It is concluded that the stronal uptake of the FLA is an energy-dependent process with certain features of an active transport.

Cerebral Trauma

Using high sensitivity double accelerometers implanted in the skull of a rhesus monkey and simultaneous EEG recording, the results of trauma as evidenced by coincident recording of various vital functions have been correlated with the degree of acceleration and electrical disturbance produced. A method of rapid serial carotid amgiography in the same animal has also been developed, thus permitting some investigation of the cerebral vessels immediately after trauma. The techniques for cerebral angiography have been successful and preliminary results indicate that in the immediate posttraumatic state in the monkey, there is a marked generalized constriction of cerebral blood vessels, both arterial and venous.

The Effects of Low Temperatures

The relationship of cerebral permeability and reduction in temperature has been pursued with the introduction of d-tubocurarine and dimethyl penicillin into the brain substance in significant quantities. Ordinarily these drugs do not pass into the brain. The one used with some frequency in anesthesia and occasionally in the preparation of hypothermic cases, and the other well known as staphcillin and in frequent usage in the therapy of staphylococcal infections can now be introduced in relatively predictable amounts. Introduction of curare is deleterious and should be prevented, while introduction of the antibiotic may be beneficial in the treatment of cerebral infections.

Topical cooling of the central nervous system has proved an efficient method of achieving selective temperature reduction of the brain in both animals and man. Moderate levels of hypothermia, 29°–30°C. are easily obtained by simple irrigation techniques. When these are combined with moderate body surface hypothermia, profound levels, 17°–19° C., may be developed. This condition is rapidly introduced, easily maintained, and amenable to spontaneous rewarming. The systemic effects are inconsequential. Twenty-one patients have been subjected to topical cooling at craniotomy, using either the irrigation or gas chamber method. Two such patients underwent topical cooling and general hypothermia as a background for excision of neoplasm. During this they were given vincristine as a cytotoxic agent by vein with the hope that (based on experimental study) this agent would gain access to the tumor periphery in extraordinary amounts.

Ventricular fibrillation coincident to hypothermia has been related to cardiac excitability. It is found that regardless of the anesthestic agent, depth of anesthesia or sympathetic block offer significant protection against this increased cardiac excitability.

Moderate cerebral hypothermia is coincident with an increase in internal carotid vascular resistance which may be as great as 100 percent and rises to a peak somewhere between 30°–25° C. Paradoxically, it seems to return to normal values as the temperature falls to 20° C. This effect can be prevented by a total sympathetic block.

Anesthesia

The effects of halothane and total epidural anesthesia on internal carotid blood flow have been studied. Deep halothane anesthesia produced a 40-percent decrease in mean blood pressure and no change in blood flow, while total epidural anesthesia produced a 30-percent decrease in blood

pressure with a 15-percent decrease in blood flow. Halothane increased internal carotid blood flow when superficial anesthesia was maintained. A similar increase in intracranial pressure was then observed. As a corollary to this study of intracranial carotid blood flow in anesthesia, it was noted that simple craniotomy of itself undertaken with only local anesthesia produced a small decrease in internal carotid vascular resistance, with a corresponding increase in blood flow. These findings apply when the monkey's head is in a horizontal position at craniotomy. However, when the head is elevated to 45°, there is a considerable decrease in internal carotid vascular resistance, without a corresponding increase in blood flow.

Effects of Surgical Lesions

Retrograde transsynaptic degeneration has been noted at two levels in the visual system of the macaque monkey. In two animals examined 20 months after a surgical lesion of the optic chiasm, there was complete loss of ganglion cells in the medial retina and cystic degeneration in the inner nuclear layer. In a single animal studied 48 months after an occipital ablation there was severe loss of ganglion cells in the retinal half ipsilateral to the lesion.

At this time, seven chimpanzees have been subjected to mesial cerebral incision. This incision separates callosum, anterior and posterior commissures, floor of third ventricle, periaqueductal structures, pons, tegmentum and medulla as far caudally as the obex. All the animals subjected to this radical hemispheral separation recovered consciousness soon after anesthesia. Gross defects are minimal and include only monoparesis in the right upper extremity, and a metabolic defect in the form of elevated blood sugar in one instance. There has been no abnormal fluid intake or output, nor has there been any particular alteration in weight. The animals respond normally to social environment. In two instances in which two other chimpanzees were subjected to mesial cerebral incision, the animals died suddenly some months after operation. In each of these instances, the death was related to a stressful situation—one, light general anesthesia of short duration; the other, a physical conflict in which the animal was actually attacked by his peers in the run. One supposes that the anterior extent of this incision affects hypothalamic mechanisms and somehow prevents adequate reaction to stress.

Effects of Radiant Energy

Subsequent to the previously reported study on the effects of alpha particle radiation and glycogen in the brain, attempt was made to see whether gamma radiation produced similar changes. X-ray irradiation of the head of the rat resulted in the accumulation of histochemically demonstrable PAS-positive granules which were identified as glycogen. The first abnormal appearance of glycogen was observed in rats which received 1,200r. The amount of glycogen granules increased progressively with larger doses of irradiation. The peak of glycogen accumulation was reached at 24 hours. The quantitative analysis revealed 40 percent increase in glycogen in the brains of rats receiving 3,000r and sacrificed after 24 hours.

The thermal effects of ultra high-frequency radiation of the primate brain have been investigated with a device in which a digital wireless temperature telemetering system is incorporated. Thus, temperature can be read to .02° C. with a constant scale factor correction of approximately 1° C. as operational in a r.f. field. The device has been used to establish a r.f. exposure threshold so that the duration of exposure at a given power level can be related to the time required for the animal to recover its normal temperature. EEG signals have been monitored during ultrahigh-frequency radiation without significant artifactual interference. Similarly, blood pressure recordings have been made. With this array, the r.f. was pulsed at approximately 30/sec. with a 100-volt peak as the animal was exposed in a TEM node coaxial cavity occupying a volume of less than 1 cubic foot and suitable for 388 mc. Thus positioned, the animal became drowsy and failed to respond to pain during the period of the radiation, but recovered his normal sensory responses and became alert thereafter. The wireless temperature recording device did not reveal a significant rise in mean brain temperature. The average mean power in the field was calculated at something approximating 5 watts.

Technical Developments

During the past year, air sampling studies of the operating room environment have continued

and have reached a stage of significant conclusion. Less than five organisms per cubic liter for 4 hours have been found. None of the organisms deposed were pathogenic. This study, which will be formally reported in the Journal of the American Hospital Association, provides objective data for assay of traffic, ventilation, lighting, and other physiological factors which involve our operating room environment. The work on eye comfort previously reported continues and has now reached the fabrication stage so that useful color patterns for draping and for eye glasses are available. Likewise, a dural instrument has been developed.

Language and Memory

In depth stimulation of the human, arrest of speech, voluntary movement and confusion has been elicited from the vicinity of the head of the caudate nucleus. The responses described were elicited from the caudate head, the adjacent deep frontal white substance and the frontal limb of the internal capsule. All 10 patients were right handed and the responses were obtained from the left hemisphere in 7 and from the right hemisphere in 3. With 60/sec. 2.5 ma. unidirectional square pulses, the effective peak currents ranged between 5–15 ma. Stimulation in the lower current range would cause the patient to cease counting. In some instances they would stop performing a repetitive act, such as opening and closing the hand. When asked why they did so, usually some trivial excuse unrelated to the stimulus was given. With greater currents, the same response was seen with shorter latency and often the head and eyes were slowly turned toward the contralateral side. If the patient were given a series of words during such a stimulation, he might not recall them or would reproduce them in a garbled fashion. In three patients, an ictallike state was produced in which the patients became unresponsive and following turning to the opposite side, sat up, stared and fingered clothing or equipment or tried to remove it. There was no later recall of the episode. No stimuli were followed by generalized seizures. Repeated stimuli at a level only sufficient to induce speech arrest were often followed by confusion of mild degree.

In two patients in which cerebral dominance was determined by Wada test as left hemispheral, the fronto-temporo-parietal cortex was exposed at craniotomy and cooled to a temperature range of 20° C. by topical techniques. The patients, who were alert, cooperative, and under local anesthesia, did not notice any relevant subjective changes, and objective speech testing was unchanged throughout the period of this cooling, despite the fact that the electrocorticogram was flattened. These findings suggest that major speech representation may well be subcortical.

The carotid amytal test of Wada and Rasmussen has been used on about 100 patients. When the amytal is injected into the "dominant" hemisphere there is a brief period of speech disruption. A set of 20 common objects has been used on the last 49 cases to investigate the nature of the "induced dysnomia." The rather surprising result was that the percent error for the 20 objects was related to the type of initial and final phonemes of the names of the objects. Objects with names that have initial phonemes of low frequency and final phonemes of high frequency tended to be identified correctly more often than objects with names that have initial and final phonemes of opposite incidence. We suggest that the tendency for better performance with nouns ending with more frequent phonemes could be defined as an advantage afforded by greater familiarity for finishing correctly the pronunciation of a word. Poorer performance with nouns starting with more familiar phonemes may result from weakening, by the amytal, of some inhibitory mechanisms necessary for proper initial selection among practiced nouns.

A comparison of postoperative and preoperative scores on the Gorham's Proverbs Test indicates that the left temporal lobe removals in males in which the left side is dominant do not do as well as males with right temporal lobe removals, but in women there is no differential effect as regards the side of operation.

Recent memory has been studied in the monkey. These animals were trained in a digital-type automatic apparatus with a "matching from sample" problem using colors and involving delays varying up to 15 seconds. After satisfactory behavioral criteria was achieved, the animals were subjected to bilateral hippocampal and amygdaloid ablation by a surgical approach through the pyriform cortex. To date, nine animals have been trained in this performance and four have had surgical procedures as described above, three with complete lesions and one as control. As of this

date, observations reveal no loss of ability to perform the matching from sample with delay, nor is there any inability to learn a new discrimination task. These findings might indicate that, at least in the experimental model under consideration, the hippocampal and amygdaloid structures are irrelevant to recent memory.

Two hundred and seventy-four patients participated in the clinical investigations of this Branch, on an inpatient basis. Three hundred and thirty-nine outpatients were seen, in a total of 465 visits. There were 118 major operative procedures. There were 23 reports prepared for publication.

OPHTHALMOLOGY BRANCH

In the past year the research activities in laboratories and in the clinical unit showed continuity of investigative efforts in most of the individual areas. The overall results may be summed up as a widening of our knowledge in the basic fields of vision research and growth of information on problems of ocular physiology and disease. This report lists the accomplishments of laboratory and clinical research with some emphasis on the extent to which laboratory studies could be used to convey a message for a better understanding of diseases.

The new and important step in Dr. Fuortes' fundamental work on vision is the introduction of a well developed physical model which consists of a series of electrical units. For background information it should be said that according to Dr. Fuortes, the potential changes in the visual nerve cells evoked by brief flashes, or by long lasting steps of light of different intensities (accurately measured by means of intracellular microelectrodes) cannot be interpreted on the basis of a simplified hypothesis. The form of the responses requires a concept which encompasses complex processes which operate in the transformation of light into the potential changes. The good agreement between the performance of the eye of the horseshoe crab and that of the model suggests that visual processes are based on mechanisms analogous to those of the model. The form of the responses can be produced quantitatively by the model which consists of a series of identical units able to introduce delay and some attenuation of input and output signals. If the model is fitted with an additional system which operates by decreasing the value of the resistances of the components and increases the output signals it becomes possible to reproduce quantitatively the features of responses of the eye to long lasting steps of light such as the high initial phase, the decay to the later steady state and the logarithm relation between the input intensity and output amplitude. In brief, adjustment of the values of some of the model's components reproduces the changes of the responses of the eye obtained by various conditions of light and dark adaptation. This new approach of studying visual processes will be continued with the hope to correlate biological structures and processes to the performances of the electrical components of the model.

In the Electroretinography Laboratory of Dr. Fuortes' section, Dr. Gouras and Dr. Gunkel have successfully increased the sensitivity of electrophysiological techniques by using computer summation of responses and by adding Arden's method of electro-oculography (which measures the DC potential of the eye in responses to light) to routine electroretinography. By this development, the clinical aims of identifying, or grading retinopathies, have received a new incentive and the progress in electroretinography and psychophysical testing remains the backbone of our clinical project dealing with retinal degeneration. The combination of these methods with electroencephalography promises to provide further information of lesions in the visual pathway not located in the retina. Another important development for the analysis of the electroretinogram is the use of sinusoidal modulation of the light input. Thus, Dr. Gouras showed that the outer layers of the retina demonstrate resonance to light stimuli which depend solely on the frequency responses of the photoreceptors. When the same techniques are applied to the electroencephalograph and the study of brain waves they may pave the way for a better comprehension of visual mechanisms in the normal and disease states of the human.

In the same laboratory, Dr. Gunkel has continued his interesting study on age induced changes of scotopic visibility thresholds. Increase of such thresholds for short wave lengths was erroneously correlated, by workers in other laboratories, to loss of retinal sensitivity in old age. Dr. Gunkel determined relative spectral sensitivities in different age groups, in aphakics, albinos, and patients with cataracts and demonstrated that the thresholds were only slightly elevated in old age upon stimu-

lation with ordinary light. In contrast to this, the level for short wave light stimulation was raised about three times in the old age group. Interestingly, aphakics of old age proved to be nearly one log unit more sensitive to the short wave stimulus than young subjects. This is convincing evidence for the decisive role of the absorption of short wave lengths by the yellowing lens nucleus of the aged, although the lens of the young absorbed some light of such wave lengths.

As in preceding years, acknowledgment should be expressed to Dr. Gunkel for making his exceptional talent, for devising and constructing optical instruments, available to all who asked for help and particularly for designing a good deal of the equipment employed in the Laboratory of Electroretinography.

In the Section of Cell Biology, Dr. Bonting and his coworkers have, in great measure, widened the scope of the investigation on the Na-K ATPase which was initiated only last year. They could demonstrate the presence of the enzyme in all tissues known to possess a digitalis-sensitive cation transfer and showed that there were marked differences of the sensitivity of the tissues to the inhibitor ouabain depending on the studied species and tissues. This might explain some of the erratic clinical results of cardiac glycosides which will be mentioned in a subsequent report. Particularly impressive is the good correlation between the Na-K ATPase activity and the cation flux which was confirmed in various tissues of different species. It is concluded that the enzyme is actively involved in the maintenance of ionic gradients in single cells, in salt and water transport across epithelium membranes and is connected with the repolarization processes in nerves and muscles.

In last year's report, Dr. Bonting and coworkers described the relationship of the enzyme to the formation of aqueous humor and showed that ouabain inhibits inflow rate of aqueous humor of the cat by 70%. They also mentioned that in patients a decrease in aqueous inflow rate of 46% followed the inhibition of the enzyme by cardiac glycosides. Dr. Simon observed a significant lowering of the intraocular pressure in several patients with congenital glaucoma. Later observations showed that such an effect does not occur consistently or frequently, despite the marked drug-induced bradycardia. A warning was addressed to the ophthalmologic profession, therefore, to exert great caution in the use of cardiac glycosides in patients with glaucoma even when the intraocular pressure cannot be controlled by other means. The number of studied patients is too small at present to arrive at a clear indication for the use of this therapy.

Recently, Dr. Bonting extended his study of the enzyme to another tissue of the eye by exploring the part it plays in the active cation transfer in the lens. He determined the enzyme activity in various portions of the lens and found its highest concentration in the epithelium where it is closely linked to the active cation transport system of this tissue. Since this system appears to be necessary for maintaining ionic gradients between the lens and the aqueous humor and for regulating the water content of the lens, it is reasonable to assume that the absence or malfunction of the enzyme may lead to cataract formation. It should be stressed that Dr. Bonting's results of the studies on the enzyme in connection with the digitalis-sensitive cation transport have been confirmed fully by other investigators in their work on several tissues, including the lens.

Another offshoot of Dr. Bonting's exciting work with the Na-K activated ATPase is the study on the role it plays in the formation of cerebrospinal fluid of the cat. Dr. Thomas S. Vates of the Medical Neurology Branch conducted the experiments and from the data obtained so far, it is tentatively concluded that the enzyme has an important function in the formation of cerebrospinal fluid. The formation in the lateral ventricle was found to be decreased by 19% following the injection of 0.2 mg./kg. lanatoside C. Ouabain injected directly in the ventricle stopped flow completely and, under this condition, the Na-K activated ATPase enzyme of the choroid plexus was decreased to 31% of its normal value. As in the study of enzyme inhibition of aqueous humor formation, any conclusions as to the clinical applicability of enzyme inhibition in diseases of the human are premature; but, there is no doubt that the integration of biochemical studies, experimental work on the animal, and treatment of human diseases has to be considered a most fruitful approach.

It has been shown recently that adrenergic agents can influence the outflow mechanism in the angle of the anterior chamber of man. The distribution and nature of adrenergic receptors in certain parts of the anterior uvea is of consider-

able interest. Dr. van Alphen used his muscle strip technique on tissues of the cat ciliary body and iris and identified, by means of the adrenergic blocking agents, receptors of the alpha type in the dilator of the iris, whereas the sphincter muscle carries predominantly receptors of the beta type. Beta receptors, alone, appear to be present in the ciliary muscle. The implications of the distribution of adrenergic receptors of various nature in different parts of the iris-ciliary body system for regulating the intraocular pressure is not yet understood.

Dr. van Alphen devised an elegant technique to visualize, *in vivo*, the ciliary processes in cats and to follow the appearance of a highly diffusable dye, fluorescein, at this site where the formation of aqueous humor takes place. These observations on the living eye have been supplemented by experiments, *in vitro*, in which the preparations were placed in a pressure chamber and perfused via the long ciliary artery. Under these conditions an 8-minute delay in the appearance of the dye was noted, but the delay disappeared when acetylcholine was added to the perfusion fluid. This suggests that under the conditions of the experiment acetylcholine affected the rate of formation of aqueous, if one assumes that the diffusion of the fluorescein indicates a bulk flow from the blood to the aqueous humor across the cilary epithelium.

Dr. Macri's studies of the correlation between the state of the intraocular vasculature and the eye pressure proceeded successfully in two separate studies: (A) the relationship of venous pressure to intraocular pressure, and (B) the dependence of the intraocular pressure upon the degree of constriction of the iris artery in the cat. The previously reported concept of Dr. Macri on the connection between the venous pressure and the eye pressure has been further supported by additional experimentation.

The extensive work on the relationship between pressure or caliber of the iris artery and eye pressure has been conducted by Dr. Macri, in great part, by means of his refined bioassay on iris preparations, but was extended to studies on arterially perfused ciliary processes and intact eyes. Dr. Macri's attempts to identify the substance circulating in the serum which he had shown produces constriction of the iris artery are not all conclusive but strongly suggests that the agent is angiotensin. On the basis of the experimental data, some tests with angiotensin were conducted by Dr. van Alphen in patients admitted to the unit with glaucoma with generally negative results. For the present, Dr. Macri considers the available information as insufficient to definitely associate this pressure substance with aqueous humor dynamics.

In another phase of this comprehensive study Dr. Macri demonstrated, by means of bioassay, that constriction of the iris artery, which is followed by a fall of the intraocular pressure, can be produced by sympathomimetic agents as well as parasympathomimetic agents, by inhibitors of cholinesterase or Na-K dependent ATPase, and carbonic anhydrases. Other substances such as histamine and serotonin have a similar effect, although in the case of histamine vasodilation precedes the constrictive phase. It appears then, that drugs which are known to lower intraocular pressure have a constricting effect on the iris arteries in the cat preparation. This points to a vascular mechanism contributing to the pressure lowering action of these compounds.

In Dr. Macri's laboratory, Dr. Wong undertook preliminary studies of some pharmacological effects of serotonin on the eye of the cat. He used Dr. Macri's microcannulation technique to follow changes in the resistance of flow in the isolated posterior segment of the eye and observed an increase of this resistance when serotonin in dilutions of 1×10^{-6} and 1×10^{-8} was perfused. Subsequent dissection procedures on such preparations suggested that the serotonin sensitive part of the vascular tree is located in the annulus of vessels which surround the optic nerve near its entrance in the sclera. This work was initiated by Dr. Wong on the basis of his clinical observation in a patient with the carcinoid syndrome who showed peripheral retinopathy and a decrease of the pressure in the retinal arteries during the attack.

Clinical projects on lens pathology have several counterparts in basic work in different laboratories. One of these was described in the new studies of Dr. Bonting. In the Biochemistry Section of Dr. Resnik, the work on lens proteins has been continued. The many data he has published in the past on the physical and chemical properties of lens proteins, particularly alpha crystalline, are widely quoted in literature. Certain fractions of this protein contain leucine amino peptidase activity. The properties of this enzyme were studied extensively by Dr. Resnik and Dr. Wolff and emphasis was

placed on the interaction of the enzyme with metal ions. The activation of the enzyme by cobalt depends on the presence of magnesium.

Dr. Wanko, in the Laboratory of Electron Microscopy, continued cooperative efforts with several other units in the Institute despite limited space which caused considerable hardship. There are three general areas in which his studies contributed essentially to the understanding of individual problems. The lens and conjunctiva remain the main subject of his work on eye tissues. In the past, Dr. Wanko has provided a sound description of the structural characteristics of cells, fibers, and membranes of the lens in various species, and his electron micrographs and interpretation are sought after by all who write on the anatomy of the lens. The lens nucleus could not be explored in previous work because of insufficient penetration of the fixative into the central part of the organ. Modifications of the technique have now permitted more satisfactory preservation and preliminary data indicate that it will be possible to define structural details of the lens nucleus at high magnification. The controversial issue of the nature of the pigment in the aged lens nucleus was also investigated. Melanin granules were not definitely identified but some structures were observed which resembled developing pigment granules in the pigment epithelium of the retina. Recently Dr. Wanko added to his various studies of cataractous lenses observations on a new metabolic cataract produced in rats by triparanol feeding. It is expected that the high resolution of the electron micrographs will add to the understanding of the pathogenesis of this metabolic cataract which is under study in the Cytology and Histopathology Laboratory. Dr. Wanko's work on the normal and pathologic conjunctiva has progressed with the accumulation of new material. The sequence of morphological changes in the secretory cycle of goblet cells of the conjunctiva appears to depict stages of a physiological process in a convincing pattern.

Two studies of the Electron Microscopy Laboratory were carried out in cooperation with the Laboratory of Neurochemistry. One of these studies deserves special attention since attempts to correlate interpretation of fine morphological structure with the results of biochemical examination hold great promise. In the present study, brain slices were incubated both with and without the addition of ammonium chloride to the medium. The neuronal nonchloride and nonsucrose fluid spaces were found reduced in the slices treated with ammonia. The morphologic examination of material from this group showed considerable swelling of astrocytes, mitochondria, and endoplasmic reticulum. The investigators considered the possibility that the fixative osmium tetroxide could have influenced the fluid distribution and the morphology. If this were the case the question would arise whether or not the effects of fixation would alter the chemical measurements of fluids and electrolyte spaces and those of the fine structures of the tissues in a parallel manner.

In the Cytology and Histopathology Laboratory, my coworkers and I continued the studies on cell proliferation in various eye tissues with particular attention to corneal endothelium and lens epithelium. Autoradiographic techniques after intravenous injections of H^3-thymidine as a marker were combined with colchicine studies. Labeling and mitotic indices were determined in the corneal endothelium at varying intervals after the incorporation of the isotope. The collected data obtained with these techniques and the use of a new marker—the appearance of pairs of equally labeled nuclei—permitted estimation of the duration of the DNA synthesis period and the G_2 period. The mitotic duration in the endothelium of the rabbit's cornea determined with the colchicine technique was found to be 45 minutes and in the lens epithelium of the rat, 1 hour and 12 minutes. The mean lifespan of cells in the proliferating area of the endothelium is approximately 120 days and the G_1 period, which almost equals the intermitotic time, occupies about 99% of the entire cycle. Diurnal fluctuations of the mitotic activity of the endothelium seem to be associated with variations of the extent of DNA synthesis which, however, seems to follow an inverse pattern. In the lens epithelium of the rat the mitotic index and the turnover time could be estimated separately for the equatorial, preequatorial, and central area. The information gained by these studies is closely linked to problems of physiology and pathology of growth, regeneration, age-induced changes, renewal potentialities of tissues, and form a working basis for studies of the influence of various agents on cell proliferation.

In the same laboratory, a new form of metabolic cataract was examined by biomicroscopic, histo-

logic, histochemic, and cytologic techniques. Triparanol, which has been used to reduce serum cholesterol, produced cataract formation as a side effect, sometimes, in humans and also in rats. The biomicroscopic and histopathologic changes associated with this lens opacity are characteristic and can be distinguished from those of other forms of experimental cataract. The primary involvement of the lens fibers is accompanied by the appearance of peculiar layered structures which can be stained with Sudan black but can be demonstrated also in sections which have been passed through lipid solvents. This experimental cataract seems to be of special interest since it may give some insight in the pathogenesis of a cataract which, in most other incidences, remains obscure.

A new congenital form of cataract in the rat was studied in the same section by Dr. Howard Bernstein and coworkers. According to C. F. Cisar, the inheritance of this cataract is recessive. Irregular defects in the lens capsule and striking excrescences of the anterior capsule differentiate this cataract from other forms of congenital lens opacities, but may have some morphological features in common with the cataract of Mongolian idiocy as described by Cogan.

Again, in the same unit, Dr. Bernstein participated in a study of Dr. Stanton Segal of the Clinical Endocrinology Branch of NIAMD dealing with cataract formation in the newborn of rats which were on a high galactose diet during pregnancy. It is interesting to learn that cataract formation occurs in a high percentage when the mother rat was galactosemic only for a short period during the last week of pregnancy. Similar to experimental galactosemic cataract in young adults, the lens epithelium and the nuclei of the lens fibers are not affected despite extensive hydropic fiber changes. It may be concluded that galactosemia in pregnancy of humans might induce damage in utero to the lens of the fetus.

From the following synopsis of projects which in the main were carried out in the nursing unit, it is evident that all the clinical studies were connected with laboratory investigations. In some instances the integration of the investigational efforts in the two areas was intimate, in others broad gaps existed between the fundamental knowledge and that derived from observations of the human eye in health and disease.

The studies on chloroquine retinopathy belong to the first group. The multidisciplinary approach is illustrated by, the accumulation of clinical and histopathologic data in which Dr. Bernstein has the greatest share, the electrophysiological studies by Drs. Gouras and Gunkel, and the most valued cooperation of Drs. Rubin and Zvaifler from the Departments of Biochemistry and Medicine at Georgetown University, whose studies focus on pharmacology of chloroquine. The course of the disease was reconstructed on the basis of observations on nine patients who were visually incapacitated to varying extents by this most serious side effect of a widely and successfully used therapeutic agent. Visual field defects, fundus pathology, and the results of electrophysiology and psychophysical testing led to a characterization of the retinopathy. Histopathology was studied in sections of one eye with an obviously very advanced lesion. The prolonged storage of chloroquine in the tissues of patients treated with this drug was evidenced by measurable chloroquine levels in the blood and urine of patients years after the actual treatment with the drug. It has been shown previously that chloroquine excretion can be increased by various means, but it remains unclear whether such treatment can change the course of the retinopathy. An important, but still preliminary observation of Dr. Bernstein, deals with the unequal distrbiution of chloroquine in pigmented and nonpigmented tissues. The chemical analysis of the dissected eye showed much higher content of the drug in melanin-containing tissues.

In the project on toxoplasmic uveitis, the cooperation of Dr. Leon Jacobs of the Laboratory of Parasitic Diseases, NIAID is greatly appreciated. Dr. Conrad Giles is the principal investigator of an experimental and clinical study to evaluate a new antibiotic, Spiramycin, in the treatment of toxoplasmic uveitis. The data collected from several series of experiments in rabbits suggests that the course of the infection can be beneficially influenced by spiramycin therapy, but the best results were observed by combining this treatment with daraprim medication. On the other hand the antibiotic does not seem to have any effect on toxoplasma parasites in tissue cultures. The results of the therapy in patients with toxoplasmosis are inconclusive so far since side effects frequently necessitated the interruption of the treatment. At

present the daraprim-sulfa therapy, combined with steroid treatment, remains the preferred therapeutic approach. The simultaneous use of folinic acid has prevented bone marrow depression by daraprim which had repeatedly been noticed previously.

The mystery of the etiology of uveitis in the majority of patients is still unexplained and the lack of any progress in establishing the cause of uveitis is disconcerting if the great number of examinations and laboratory tests carried out in these patients is considered. New diagnostic procedures designed to discover autoimmune mechanisms related to the disease were unrevealing. The occasional reports of parasites in the stool deserve more critical interpretation than it has been possible to obtain so far.

As seen in the reports of Drs. van Alphen and Macri, and Drs. Bonting and Simon, studies on aqueous formation, aqueous dynamics in general, on intraocular pressure changes induced by pharmacologic agents and the relation to intravascular pressure within the eye are important projects under study in these laboratories. The clinical complement is the investigation of aqueous humor outflow mechanisms as studies in the tonography unit by Dr. John Nicholas. He makes use of the unique opportunity of examining the patient repeatedly over long periods of time. With the accumulation of data, the significance of various provocative tests could be evaluated and the reasons for aberrant results analyzed. Thus, Dr. Nicholas showed that the water drinking test has to be repeated several times before results can be considered reliable. A relatively good correlation between serum-sodium levels and the blood osmolar changes after water drinking on one side and the tonographic record on the other adds to the understanding of these important tests.

Recently Professor Goldmann reported observations of high intraocular pressures of a patient who had been treated for a long time with steroid drugs. Dr. Bernstein followed a patient treated systematically with steroids who exhibited increased intraocular pressure when on therapy, but became normal when the steroid medication was discontinued. Repeated tests carried out in the Branch and later at the Washington University School of Medicine in St. Louis suggest that in this case, and in other patients, the uncontrolled use of systemic or local treatment with steroids gives rise to a marked increase of the intraocular pressure in disposed individuals. It is planned to continue these studies both in the tonography unit and in the laboratory. The clinical trials of administering compounds which might influence intraocular pressure on the basis of laboratory observations have been mentioned in the works of Drs. Simon and Bonting and Drs. van Alphen and Macri. At present the practical applicability of treatment with digitalis and angiotensin must be questioned, but more observations are necessary to arrive at a more definite statement.

It has been demonstrated clinically and in certain experimental animals that injection of silicon in the anterior chamber or vitreous space of the eye is well tolerated. Although these findings are not confirmed by Dr. Bernstein in preliminary experiments on rabbits which have shown pronounced damage of the cornea after intracameral injection of silicon, such treatment was used in a patient with a painful bullous keratopathy upon the suggestion of Dr. A. Edward Maumenee, consultant to this Branch. When the silicon injection was combined with the external use of an ointment containing 5% sodium chloride the cornea cleared considerably and tearing improved satisfactorily. The course of events of this patient, who is followed by Dr. Bernstein, suggests that in some instances bullous keratopathy, an often hopeless corneal disease, might be treated by replacing the aqueous humor with silicon with apparently good results.

ELECTROENCEPHALOGRAPHY AND CLINICAL NEUROPHYSIOLOGY BRANCH

1. Diagnostic Service

This service continues to account for a large part of the activities of the Branch. A total of 1,873 EEG examinations have been performed—between the last report (prepared December 1, 1961) and the present one (prepared November 30, 1962)—on patients referred from the different Institutes with the following distribution:

NCI	322
NHI	104
NIAMD	186
NIMH	79
NINDB	1,182
Total	1,873

The number of monthly examinations continues to occupy more than one-half of the time of the professional staff. As in the past, the large majority of referrals have been from our Institute (about 63%), followed by the National Cancer Institute with an average of about 27 patients per month.

In addition to the routine EEG (scalp) examinations, there have been 17 records obtained directly from the exposed cortex during operations performed for surgical treatment of seizures.

The situation of the Branch in this diagnostic service activity has greatly improved since July 1, 1962, when Dr. Kristof Abraham joined our staff as Medical Officer.

2. Research Activity

Besides the above-mentioned diagnostic service, part of the time has been spent in analyzing and elaborating the data obtained from previously described research projects, and in organizing them for publication. Three papers are presently in press or have already appeared.

(A) A major project consists of three types of investigations carried out in experimental subjects with involuntary movements and in which numerous electrodes have been chronically implanted in various subcortical structures. Briefly, we are studying: (a) the spontaneous activity of these structures during the waking state and sleep; (b) the modification of cortical activity subsequent to selective subcortical (therapeutic) lesions; (c) by means of combined electrical stimulation and recording of evoked potentials, the interconnections between different nuclei in the depth and between these and the frontal lobe. The collection of data is presently completed but their analysis is still in process and the organization of the complex results should be achieved in the course of the coming year.

(B) Two projects deal with experimental epilepsy. One presently in progress is complementary to similar investigations carried out in the past by this Branch and continues the analysis, at the single neuron level, of the epileptogenic focus artificially elicited by different methods in the cerebral cortex of cats. Specifically, the project deals with the analysis of the mechanisms involved in the "triggering" of the epileptiform potentials by various types of afferent impulses and in facilitating or inhibiting the discharges themselves.

The other, now completed, deals with experimentally induced epileptogenic foci, but its emphasis is on the study of distant ("projected") electrical events. The problem is of direct interest to clinical epilepsy since, in the routine EEG diagnosis of many cases, abnormalities are often recorded not only in proximity to the probable epileptogenic lesion but also in other regions of the cerebral cortex. With this study it has been possible to analyze, at the unitary level, the different electrographic characteristics of the primary and secondary foci.

(C) Two other projects also deal with the problem of epilepsy but are essentially of a clinical nature. One draws attention to a little known association of two syndromes: precocious puberty and petit mal seizures. The latter may or may not be present clinically but their typical, characteristic electrographic manifestation is obvious. Four patients with this unusual association of signs have been studied and only three other comparable cases could be found reported separately in the literature. These findings are of interest for two reasons: (a) from a practical diagnostic viewpoint, since the presence of these EEG changes in case of precocious puberty strongly indicates that the latter is true precocious puberty of a cerebral type; (b) from a more theoretical, pathophysiological viewpoint, since the presence of organic lesions in proximity to the III ventricle in cases with 3/second wave-and-spike complexes in the EEG ("centrencephalic" pattern) would suggest a close relationship between the two events and thus provide further evidence for the subcortical "origin" of the latter.

The other study deals with temporal lobe epilepsy and with the effect of sleep upon its electrographic manifestations. This is a minor project which is mentioned only because of the recently revived interest in nocturnal sleep. Since it requires a very particular patient material, its completion might have to wait a long while and in fact might be eventually limited to a simple case report. The study, carried out with chronically im-

planted electrodes in direct contact with large areas of the cortex, consists of an analysis of the behavior of paroxysmal, epileptiform discharges during the different stages of nocturnal sleep. It is of interest that in the case so studied, the numerous discharges tend to disappear during certain periods of sleep which corresponded to the occurrence of dreaming.

(D) Another project is an analytical critical discussion of the pathophysiological substratum of status epilepticus. This has been prepared upon the specific invitation of the chairman of the International Colloqium on Status Epilepticus which was held in Marseille (France) in October 1962.

(E) This laboratory is also conducting a critical review of about 250 recent papers dealing with various aspects of epilepsy. Such review has been specifically requested by the Editor of "Progress in Neurology and Psychiatry" and will represent the chapter on Epilepsy for the 1962 edition of this book.

(F) Also under investigation are the mechanisms involved in sensory perception. An experimental study is being carried out on the dorsal column nuclei of cats by means of microelectrodes in an attempt to analyze feedback sensory mechanisms at that level. No data are as yet available but the project should be completed within the next 12 months.

3. Other Activities

Training facilities in clinical electroencephalography were again provided by the Branch for guest workers and for residents in neurology. The technical personnel, as in the past, has been of great help and has efficiently assisted the Branch Chief and Dr. Abraham in this activity.

The Branch Chief was officially invited to participate at the IV Symposium on Electroencephalography at Princeton, N.J., sponsored by the New Jersey State Department of Health. At this Symposium, he presented a paper on "New Electroencephalographic Methods: Extradural, Subdural and Depth Electrography With Chronically Implanted Electrodes," and took part in the Panel Discussion on "The Medico-Legal Aspects of Epilepsy."

Two papers were presented at the Meetings of the Eastern EEG Society (December 1961) and the American EEG Society (June 1962) and another was read at the XXII International Congress of Physiological Sciences (Leiden, September 1962). As already mentioned (Project 36C) a report was also given at the International Colloquim on Status Epilepticus (Marseille, October 1962).

The Branch Chief has been elected President of the American EEG Society for 1962–63, and is a member of the Council of the American Epilepsy Society. He will serve for the next 4 years as chairman of the Board of Qualification of the American EEG Society. He has continued in his duties as the chief editor for America and the Far East of the international journal "Electroencephalography and Clinical Neurophysiology.

LABORATORY OF NEUROANATOMICAL SCIENCES

One of the most established programs of the laboratory continues to define the specific neuronal interconnections which exist among the several levels of the auditory and vestibular systems. Dr. Grant Rasmussen's pioneer investigations have established the existence of efferent as well as afferent auditory nerve connections. Dr. Robert Boord and Dr. Rasmussen recently completed a comparative study of the reptilian and avian cochlear nerve. Placement of small diverse lesions in the cochlear and lagenar ganglia at different points along their length, followed by a study of axonal and myelin sheath degeneration preparations, has established the tonotopic connections of the cochlear nerve in the bird. An associated efferent cochlear bundle was previously demonstrated in the bird and reptile by Drs. Boord and Rasmussen. These findings open the way for physiological studies on the hearing mechanisms of lower forms that are particularly well suited to this type of experimentation. As part of another project Dr. Rasmussen seeks to establish, with the collaboration of Dr. C. Smith of St. Louis, the ultimate termination of the efferent fibers of the cochlea.

Initial experiments indicate that transection of the efferent cochlear bundle in the brain of the chinchilla is followed by the degeneration of some of the endings of the granular type in the cochlea. The major project in this study is to map the afferent and efferent interconnections between all the

auditory nuclear stations including the cerebral cortex. For these purposes especially modified histological and histochemical techniques will be supplemented by electronmicroscopic investigation.

Dr. D. K. Morest is devoting particular attention to the cellular composition and connections of the medial geniculate body in a study using Golgi methods as well as axon degeneration techniques. Both the analysis of the internal cellular structure of the medial geniculate body and the mapping of the fiber pathways which enter it suggest more and more strongly that the medial geniculate may contribute to central integration of more than one sensory modality.

In another study Dr. Rasmussen is employing experimental neuroanatomical methods to explore the interconnections between the medial geniculate body and the auditory cortex. Findings thus far in the cat demonstrate that the medial geniculate body is not strictly concerned with the auditory function as is commonly believed. Only the ventral part of this nucleus is clearly associated with auditory connections, while the rostrodorsal half receives connections related to the visual system.

In addition to increasing our understanding of how information from the environment is filtered and ordered by nervous circuitry the work of this section is providing detailed information concerning the manner in which this information is integrated with that coming through other sensory channels.

Just as the cytoarchitecture of the nervous system in large part determines the manner in which it marshals the body's responses to changes in the external and internal environment, so the type, number, and spatial distribution of organelles in the individual neurons and glial cells reflect the roles which each type of cell plays in the nervous system and the ways in which they relate functionally one to another. The neurocytologic techniques associated with electronmicroscopy now make possible all but exhaustive inventories of cell parts which lie just above the molecular level of organization. The Section on Neurocytology actively continues its development and improvement of techniques such as perfusion fixation which minimize distortion and artefact in studies of the fine structure of the nervous system. Drs. Keith C. Richardson, David E. Wolfe, and Ronald A. Naumann have developed an all-glass dropping chamber which permits the perfusion of physiological saline and fixative into anesthetized animals under carefully controlled conditions of temperature, pressure and rate of flow. Developments such as this, coupled with the successful use of hypothermia during perfusion, have brought much closer the goal of obtaining fixation of nervous structures with consistently high quality. Drs. Naumann and Wolfe have utilized these techniques to study the development and fine structure of the subcommissural organ, a structure which is present in the diencephalon of a number of vertebrates including man, and is believed to play a role in the control of electrolyte levels in body fluids. The cytogenesis of this neurosecretory organ is under study in the rat. In addition, this investigation has led to the discovery of a heretofore undescribed material related to the endothelial and parenchymal basement membrane of the vessels of the organ. This material has a macroperiod of about 1070 A, and may be some unique form of collagen. This possibility is currently being tested because of its possible significance in connection with the absence of a blood-brain barrier in the subcommissural organ.

While much remains to be learned from the use of the electronmicroscopes for descriptive studies of normal cells, increasing emphasis is being placed on its use to follow the changes which are produced by experimental manipulation of the nervous system. This trend is reflected in the program of the Section on Neurocytology. Dr. Milton W. Brightman has continued his studies of the cytomorphology of ependyma in the rate brain and is analyzing its role in the exchange of substances between cerebrospinal fluid in the ventribular compartment and the brain. Ferritin, which was introduced into the lateral ventricles of conscious rats through indwelling catheters, appeared to move across the ependyma largely by a transcellular route. Fusions at the tight junctions between the cells of this epithelium appear to shunt the bulk of the protein through the ependymal and glial cytoplasm which may thereby be given an opportunity to modify the substance. Dr. Brightman has further focused attention on the biological significance of attachments between cells in a collaborative study with Drs. R. Kirstein and P. Gerber. A virus (SV_{40}) was used to induce ependymomas in new born Syrian hamsters. Electron-

microscopic comparison of these neoplastic cells with cells of normal ependyma revealed an absence of haptoneres (tight cell-to-cell junctions) among the tumor cells. The hypothesis that the absence of such intercellular fusions may be correlated with the invasiveness of the neoplasm is being tested.

A remarkable series of investigations into the fine structure, composition, and functional changes of the autonomic nervous system have provided us with a new endpoint for both pharmacologic and pathologic studies and have opened the way for following changes in the levels of the effector substances of the individual nerve cell. Early in the year Dr. Richardson completed and published an electronmicroscopic description which clearly characterizes autonomic nerve terminations on smooth muscle fibers (in the longitudinal muscle coat of the vas deferens). These terminals are believed to provide for transmission between the autonomic fibers and smooth muscle cells. Granular and nongranular vesicles in the autonomic axons and their terminals were described as possible sites of formation and storage of catechol amines, or other transmitter substances. In addition, specialized areas of intimate contact between adjacent smooth muscle fibers may represent regions at which excitation can be transmitted from one smooth muscle cell to the next. Following this, Drs. D. E. Wolfe, J. Axelrod, L. E. Potter, and K. C. Richardson utilized a combination of electronmicroscopy and autoradiography to demonstrate the localization of exogenous tritiated norepinephrine in the granular vesicles of sympathetic axons in the pineal gland of the rat. These results, coupled with previous findings, permit the use of granulated vesicles as a criterion for the identification of adrenergic sympathetic axons in electronmicrographs.

Drs. Richardson and Potter compared biochemical assays of norepinephrine with counts of granular vesicles in the cytoplasm of autonomic nerve endings of reserpinized rat vas deferens. They found remarkably good agreement between the chemical assays on the one hand and the counts of granular vesicles on the other during the time following the administration of reserpine.

This series of investigations has uncovered a morphological endpoint just above the molecular level which appears to make it possible to assess, at the level of the individual cell, the effects of pharmacologic and pathogenic agents on the pool of transmitter substances.

Another dominant theme in the program of the laboratory is represented by studies of nerve regeneration which were carried out in the Section on Experimental Neurology. To assess regenerative capacity in the central nervous system of lower vertebrates, Dr. Feringa injected tritiated thymidine into adult newts at intervals following transection of their spinal cords. Maximal mitotic activity in the ependymal epithelium was noted 13 days after cord transection. Autoradiographic analysis of the spinal cords of these animals in later months met with technical difficulties. The approach should, however, enable us to determine whether or not lower vertebrates are capable of replacing lost neurons. Dr. Jerald J. Bernstein is currently giving attention to central nervous system regeneration in the fish. Electrophysiological, behavioral, and anatomical criteria are being used to determine the ability of the fish and other inframammalian vertebrates to regenerate extirpated portions of the central nervous system. This project has been underway for a short time and only preliminary and tentative results are available. The approach is a promising one for determining where and when, phylogenetically and ontogenetically, central nervous system regeneration occurs. It will make possible an identification of correlative factors which permit or inhibit regeneration.

Dr. Lloyd Guth has under investigation the parameters of regeneration in the peripheral nervous system. This program seeks to identify the factors that are involved in the establishment of connections between regenerating nerves and their end-organs in rats. Crushing of spinal nerve L4 was followed by collateral sprouting of L5 nerve fibers which grew to adjacent denervated muscle fibers within the plantaris and soleus muscles. When fibers from L4 regenerated they established connections with the muscle fibers that had already been innervated by the L5 collaterals, thus hyperneurotizing these muscle fibers. This result corroborates his previous observation that there is no selectivity in the relationship between muscle and nerve fiber. In a separate experiment the circumvallate papilla, which is innervated by both glossopharyngeal nerves, was denervated unilaterally. One week postoperatively the number of taste buds was diminished 10%, and the number of taste cells per taste bud was diminished at least 25%. There was no further change 6 weeks postoperatively, indicating that this organ, unlike muscle, does not

undergo collateral sprouting in response to partial denervation. In yet another experiment hypoglossal nerve was implanted directly into normally-innervated or into denervated sternomastoid muscle to determine whether the implaned nerves would induce muscle to form cholinesterase at the site of implantation. Cholinesterase was formed at the site of implantation only in the denervated muscle. This experiment demonstrates that normally-innervated skeletal muscle fibers cannot be hyperneurotized. This barrier to additional innervation breaks down when the muscle is denervated. In addition it appears that the neuromuscular junction does not serve merely to facilitate the transmission of the nerve impulse, but also mediates trophic nervous effects which exert some measure of control over the muscle metabolism (inasmuch as formation of cholinesterase involves protein synthesis).

Investigations of the anatomical basis of visual function constitute another program of the laboratory. Dr. Bernstein has continued to use conditioning in the fish coupled with forebrain ablation to evaluate the role of the telencephalon in color vision in this lower vertebrate. The ability to make hue discrimination is lost immediately following forebrain ablation but returns after a suitable postoperative period. It is planned to continue these studies to ascertain precise roles played by the forebrain and by the pretectal area in the integration of color visual information.

The eye is a precision optical instrument which, when it has completed its morphogenesis, must be appropriately shaped in all its parts so that a focused image will be formed at the level of the rods and cones. A program in the Section on Experimental Embryology directed by Dr. A. J. Coulombre is revealing the sequence of steps during development which shape the vertebrate eye, and is disclosing the factors which operate at each step to shape, or misshape, this sense organ. The chick embryo has been used for this work. Attention has been given to a ring of imbricated membrane bones (the scleral ossicles) which surround the corneal limbus, and which play an important role in the shaping of the eye. During development the scleral ossicles are foreshadowed by a ring of papilliform thickenings in the conjunctival epithelium. These transient thickenings have been demonstrated to be responsible for the development of the bones in the underlying mesenchyme. The number of papillae which form is a function of the rate growth of the eye. The number of ossicles which form correspond to the number of papillae. The shape of the individual ossicles is determined, among other things, by the mutual inhibition which they exert one on another. Thus, the factors which shape this bony ring, which, in turn, plays so important a role in shaping the submammalian eye, are being identified progressively. In a related project descriptive and experimental work has revealed the following sequencies of events in the development of the chick eye. Following closure of the choroid fissure vitreous substances accumulates in a closed compartment (the vitreal chamber), and thus generates tangential forces at the level of the eye wall, which are a necessary condition for the growth of the eye. The pigmented epithelium of the retina, unlike the neural portion of the retina, increases in area only in direct response to the forces generated by the growing vitreous body. Its increase in area is brought about principally by the enlargement of individual pigmented epithelial cells. Since this cellular enlargement depends upon the growth of the vitreous body a mechanism is provided for tailoring the area of the pigmented epithelium to the size of the retina and the eye wall at all stages during development. A third project in this program focuses attention on the developing lens whose size and shape help to determine the optical properties of the eye. Microsurgical procedures applied to the embryo, and other techniques, are being used to determine to what extent the lens increases in volume during development independently of the remainder of the eye, and to what extent its shape is dependent upon tensions generated by a burgeoning vitreous body and transmitted by way of the zonular fibers.

A relatively new program in the laboratory deals with the effects of neuropharmacological agents on the developing embryo. Drs. Daniel B. Drachman and A. J. Coulombre have perfected a method which permits prolonged or programmed infusion of substances into the vascular compartment of the developing chick embryo throughout most of the embryonic period. One of the objectives of this program is to ascertain to what extent drugs, which may have only transient effects when given at the adult stage, may produce long-lasting or permanent changes when applied to the more plastic embryonic stages. To date extensive stud-

ies have been made of curare, succinylcholine and decamethonium. When these substances are administered for periods of 24 hours or more to chick embryos, congenital ankylosis occurs in many joints. This experimental model supports the hypothesis that paralysis or immobilization of the embryo leads to joint deformities which are experimental counterparts of congenital clubfoot and arthrogryposis multiplex congenita. In addition, the study has revealed that the minimum paralytic dose (corrected for body weight) of these three substances remains constant before, during, and after the development of the myoneuronal junction. This finding raises interesting questions concerning the site of action of "neuromuscular blocking agents" in the developing embryo, and provides a unique tool for determining the site and mode of action of a wide variety of agents far better than any provided by the study of adult organisms alone.

LABORATORY OF NEUROPATHOLOGY

In the Laboratory of Neuropathology, the Section on Experimental Neuropathology has as part of a long-term plan continued to attack problems of fundamental nature, such as the reliability and adequacy of procedures of fixation, the normal pattern of organization in a perfectly fixed organ, the structural characteristics of neurons in different animals and age groups, and the early manifestation of experimentally induced cell damage.

Fixation by a two-step perfusion procedure followed by the delayed autopsy, which originated in this Laboratory, has proved to give dependable results for studies of brains and spinal cords from normal young and old animals and from experimental animals with unaltered vascular permeability. Some inherent defects of the original procedure are currently being checked in experiments designed both to improve the reliability of the histologic techniques and to avoid artifacts other than cytoplasmic hyperchromatosis and nuclear pyknosis, already eliminated. The specificity of reactions obtained with histochemical techniques is being tested in order to determine their usefulness in neuropathology.

Studies on cytoarchitectonic characteristics of the central nervous system have been extended to cerebellar cortex and the facial nucleus; new concepts about the spatial relationship between the vasculature and both neurons and oligodendrocytes have been confirmed. Two Purkinje cell types in the cerebellar cortex and two motor neuron types in the facial nucleus were identified, each in contact with separate blood vessels. The observation that the granule cell nuclei and oligodendrocyte nuclei have a dozen microscopical features in common led to the hypothesis that they serve similar functions; these may be associated with intrinsic blood flow control as previously proposed here for oligodendrocytes. It may be relevant that the facial nucleus of a small animal like the mouse has fewer oligodendrocytes than that of a large animal like the rabbit.

The distribution, staining intensity and particle size of Nissl substance vary with region and species in the two recently identified cell types. The perikaryon of each can be identified by the differential staining of its cytoplasm with periodic acid-Schiff reagent, whereby "pink" and "blue" neurons are discerned (in rabbits and others), or by the different particle sizes of its Nissl substance, whereby "small" and "large" neurons are distinguished (in mouse). With due regard to these differences, it will be required that in future neuropathological investigations, only such techniques be used which permit the selection of comparable cell forms.

The appearance of neurons was found to vary with aging in animals; the severity of such a change depends on region and species. The aging process affects both the small sized basophil granules, which become less numerous, and the large ones, which exhibit more intense staining. Therefore, comparable cell forms can be studied only in animals of same age and the effect of an experimental agent determined in animals of known age.

Acute pathologic neuronal changes after axotomy apparently result in complex disturbances of intracellular protein metabolism. There is an initial loss of basophil material along the cell periphery, which has not been previously described, and a subsequent deposition of basophil material subjacent to the neuronal membrane. The intensity of changes is not uniform in the two types of neurons described in mouse and rabbit; in general those in the mouse react more severely than those in the rabbit, and the neurons are more sensitive in older adult rabbits than in younger ones. In conclusion, the pathologic reaction of neurons depends on age, species and cell type.

These studies are continued on various animal species of different ages in order to determine the consistency of these observations.

LABORATORY OF NEUROPHYSIOLOGY, INTRAMURAL RESEARCH

General Neurophysiology

The long controversy concerning cortical projection to the caudate nucleus in the cat has been settled by Dr. Rocha-Miranda a Visiting Scientist from Brazil. Unitary analysis of orthodromically evoked synaptic reactions by means of microelectrode recording has established a direct projection from the sensory motor cortex and possibly the cingulate gyrus. This has been demonstrated with cat preparations anesthetized with nembutal, chlorolose and with decerebrate preparations. Under chlorolose long latency reactions are found which are not seen in decerebrate preparations. Most of the observations consisted of extracellular records of single caudate cells with enough intracellular observations to substantiate adequately the former. Detailed studies of the striate system are very important for a better understanding of the elaborate mechanism of posture and locomotion.

The elaborate mechanisms which make possible the many nearly automatic processes of posture and locomotion are under study by Dr. Barbara Renkin. These control mechanisms extend from the cerebral cortex to the sensitive stretch receptors in the skeletal muscles. Multiple feedback circuits are found at all levels. Work has been done with an aim of analyzing the functions of the large and small muscle spindle afferents and evidence has been obtained that the large fibers carry information chiefly dealing with velocity of stretch and the small ones chiefly for measuring length. Further work is in view on analysis of the control of final motor neuron by the peripheral signals from muscle spindles and the control information from the brain.

Section on the Spinal Cord

This Section has continued its research in the area of its primary interest—basic mechanisms operating in the central nervous system at the single cell level. Work can be grouped into five projects. The purpose of four of these projects has been to extend the fundamental knowledge about nerve cells, built up largely through studies of motor horn cells of the adult mammalian spinal cord, to nerve cells of other structures and other species. But none of these extensions has been intended merely to determine the generality of mechanisms which have already been worked out. In each case there has been a specific purpose in applying the now widely accepted electrophysiological techniques of intracellular recording to new nerve cells.

Through techniques developed by Dr. Naka for studying the spinal nerve cells of fetal kittens it has been learned that inhibitory synaptic connections are more powerful but develop later than excitatory connections, but both types have been clearly demonstrated in fetuses 2 to 3 weeks prenatal. The latency of excitatory synapses shortens markedly during the late prenatal period when the fetus is developing the first signs of reflex coordination, suggesting that this type of "learning" may be the result of an increased effectiveness of specific synaptic connections. No evidence was found to suggest that developing cells can produce "A" or axon type action potentials before they show "AB" type spikes, indicating activity of the cell soma. However, there was some evidence for a "patchy" nature of the soma membrane.

Dr. Erulkar of the University of Pennsylvania has been collaborating with Dr. Nelson of this Section on a project for studying synaptic mechanisms in mammalian auditory pathways. They find that clicks, tone pips and steady tones produce both excitatory and inhibitory synaptic potentials in nerve cells of the inferior colliculus and medial geniculate body of the cat. The latencies for generation of these membrane potential changes are so critical as to suggest that their interaction provides a basis for explaining binaural directional discrimination. Both excitation and inhibition appear to be significant in the process of discrimination of steady tones. Up to 200 cycles per second, cells in these areas may follow the stimulus frequency. Higher frequencies produce firing patterns with varying degrees of periodicity which may contain information about the stimulus frequency. Models have been constructed in an attempt to account for the observed behavior of single cells.

Dr. Frank, working at the Institut Marey in Paris with Dr. Tauc, applied voltage clamp and space clamp techniques to ganglion cells of the

snail and aplysai. Some of these cell somata fire and some are passive, and those that are active may have passive patches. Whether the membrane shows a large or a small "sodium" conductance change, this is always followed by a large "potassium" or late conductance change. If this phenomenon also occurs in mammalian nerve cells it would explain the observed behavior of spinal motor horn cells under voltage clamp conditions. Acetylcholine applied to a clamped spot on the cell soma may increase the membrane conductance by many fold. In some cells (the "H" cell of Tauc) the equilibrium potential for this change is five to ten millivolts hyperpolarizing, while in others (the "D" cells of Tauc) it is in a depolarizing direction. Chloride ions introduced into the cell reverse the equilibrium potential for the conductance change in "H" cells but have no effect on "D" cells. The possibility that it is the presence of this ion (Cl^-) inside "D" cells which distinguishes them from "H" cells has been raised but is not resolved.

Dr. Nelson is working with Dr. Hunt and Dr. Katz at University College, London, on a project to study the effects of denervation on single nerve cells in the frog's sympathetic ganglion. Miniature excitatory synaptic potentials occur with amplitudes and at rates like those observed at the neuromuscular junction. Stimulation of the single presynaptic fiber synapsing on a cell produces a large, unitary synaptic potential. The latter result makes this preparation an important one for studying fundamental synaptic mechanisms.

Dr. Smith, working on cats' spinal motor horn cells, is also studying basic synaptic mechanisms through a statistical analysis of the nature of the miniature synaptic potential. Attempts are currently under way to determine the time course of membrane conductance changes associated with synaptic activity and the dependence of these changes upon membrane potential.

Dr. Van Buren is studying the nature of the electrical fields produced in a uniform volume conductor by various distributions of electrical sources. His objective is to compare these with the fields observed in the central nervous system when a localized pool of nerve cells fires synchronously. Success in this project could lead to an increased usefulness of electrode techniques in diagnosing clinical lesions of the brain. But the fundamental importance of such studies is their contribution to a better understanding of the origin of the electrical potentials generated by normal nervous activity.

LABORATORY OF BIOPHYSICS, INTRAMURAL RESEARCH

The year has seen the Laboratory of Biophysics progressing steadily along numerous lines—all part of the revoution initiated by the now familiar voltage clamp of the squid giant axon membrane. This continues to be part of the period, after the new, important and fundamental facts have become established, of the opening of new avenues and examination of detail in the search for the next major step. In it the Laboratory has been investigating the ion permeabilities of more membranes, more carefully, in more ways and under more conditions than ever before.

It is still not possible to predict how or when there may emerge a plausible descripiton of the process by which an ion moves through a living membrane. Profoundly impressive however is the first demonstration that the negative conductance —which is the basis of nerve activity—can be a steady state for one ion crossing one membrane. As a climax to the discovery a few years ago that a frog axon was excitable in iso-osmotic potassium chloride has come the direct measurement under similar conditions of a negative conductance for lobster axon membrane which is stable for at least 100 milliseconds. In contrast to far more usual transient behavior, this result greatly encourages more immediate and powerful attempts to understand the physics and chemistry of the process.

This finding was part of a collaborative project with the Naval Medical Research Institute by Julian, Moore, and Goldman in which a lobster axon was 'myelinated" with surcrose to achieve an adequate voltage clamp. The results are generally similar to those of squid, frog, and toad axons but with other and provocative differences. The power of the experiment has been increased by Taylor and Chandler with a shortened response time and their data on potassium currents are in the process of analysis. Work with this axon is being reactivated at the NIH by Taylor and Ehrenstein and some of the sucrose effects are being investigated.

The analyses of squid axon data from preceding summers are moving towards publication. Now both the outward potassium and the inward sodium clamp currents with sucrose diluted sodium are satisfactorily explained by Adelman and Taylor in terms of independent movements of the sodium and potassium ions. However they find a specific sucrose effect of the retification current from hyperpolarization which may affect the resting potential. Taylor and Chandler continue the interpretation of the precision membrane impedance measurements as functions of temperature and deterioration. An unconventional distribution of currents and potentials within the membrane is being explored and in part with the assistance of the NIH computer.

Digital computer investigations that were only possible a decade ago in collaboration with the National Bureau of Standards have been expanded but can now be largely handled at the NIH. The scope and speed of the solutions depend upon an understanding of the problem and FitzHugh has the programing of established routines well underway. The Hodgkin-Huxley equations have been inadequate expressions for the properties of the squid axon membrane as they appear in the phenomena of repetitive firing. His investigation of modifications of the potassium parameters with the analog computer have been partially successful and should help to understand adaptation. These equations have however been found by FitzHugh to contain some recent observations for the increase of threshold with temperature for long stimuli and the decrease for short stimuli.

It has been possible to expand the experimental work on the squid axon. The previous electronic equipment has been made more reliable and an independent and simplified clamp equipment was put in routine operation by Binstock. A novel wire and sucrose clamp technique has been developed, tested, and used by Adelman. And a belated venture into deep water animal collection at Woods Hole has also contributed.

By flushing out the axoplasm, Adelman and Gilbert obtained successful internal perfusions of the axon and these were particularly encouraging after the frustration of the previous season. Although much of the work by others was confined, clamp experiments with about 25 percent of the axoplasm remaining indicate considerable and unexplained roles for the normal interior of the axon. Much needed and more conventional clamp work was carried out by Armstrong and Binstock on the effect of a series of alcohols. Most interestingly the shorter chain alcohols reversibly eliminated the inward current without affecting the later outward current at the concentrations which block normal activity. At the same time the axon was found to conform to the generalizations that have been evolved for the effects of these agents on the more usual phenomena in other preparations.

LABORATORY OF NEUROCHEMISTRY

The research activities of the NINDB Laboratory of Neurochemistry during 1962 are most appropriately considered under a single heading. In retrospect, it is clear that most of the research efforts of the Laboratory Sections have imperceptibly and spontaneously gravitated toward a broad but concerted attack on the nature and mechanisms of function of neural conducting membranes. The approach comprises essentially a six-pronged attack on the problem.

(1) Under Drs. Trams and Kanfer in the Section of Lipid Chemistry, much effort is being devoted to the elucidation of the structure and macromolecular organization of complex neural glycolipids, which are integral components of neural membranes; to the mechanisms of biosynthesis and catabolism of these glycolipids; and to the questions of how these lipids may function either in myelinated axons or in membranes directly concerned with processes of cation transport. Collaboration with Dr. Rudin and associates (at Eastern Pennsylvania Psychiatric Institute) has made available their technique for reconstituting such lipids in isolation as functioning biomolecular layered membranes, with which the functional attributes of such lipids may be explored in a simple two-compartment model system.

(2) Studies by Dr. Trams in collaboration with Dr. Irwin continue on the identification of potential receptor substances. A new glycoprotein, sialectrin, has been isolated and shown to be composed of at least 10 different amino acids linked in peptide chains, plus carbohydrate (hexose) moieties and neuraminic acid. This glycoprotein is particularly prevalent in brain and in electric eel electroplaxes.

In addition to its structural novelty, it appears to be a promising candidate for the elusive receptor

protein for acetylcholine, the cholinergic nerve transmitter agent.

(3) Under Dr. Albers in the Section on Enzymes, a detailed study of the mechanism of action of the enzyme, sodium-potassium activated adenosine triphosphatase (Na-K-ATPase), is underway. A number of previous studies on a variety of tissues have implicated this enzyme as a necessary and direct participant in the transmembrane, energy-linked transport of cations (Na^+, K^+), a process directly at the basis of maintenance of nerve conduction. More or less simultaneously with Dr. Bonting, Ophthalmology Branch, Dr. Albers recognized that the electroplax of the electric eel (*Electrophorus electricus*) provided the richest and purest source of the Na-K-ATPase enzyme. Utilizing this source, a study of the detailed mechanism of action of the enzyme has been undertaken. Currently the mechanism can be separated into two stages: (a) a reversible reaction involving phosphorylation of the enzyme by energy-rich ATP [ATP+Enzyme$\rightleftarrows$P$\sim$Enzyme+ADP]; and (b) an irreversible reaction, activated by Na^+ and K^+ and inhibited by oubain (which also inhibits cation transport), involving dephosphorylation of the enzyme [P$\sim$Enzyme$\rightarrow$Enzyme+P_i] with release of energy. If the enzyme is directly concerned with cation transport, this second step would be the relevant one. An immediate goal of these studies is the isolation and characterization of the P$\sim$Enzyme intermediate, so that it can be utilized to study the second stage of the reaction in full detail. The approach here is clearly pertinent to an understanding of the true nature of the role of this enzyme in cation transport.

(4) In the course of other studies in the Section on Proteins and Amino Acids, cerebral cortex proteins from subcellular fractions have been isolated and characterized for their content of glutamic acid, aspartic acid and amide residues. The microsomal fraction was found to exhibit a high content of these two amino acid residues and on subfractionation into ribosomal and endoplasmic reticulum membrane proteins, the latter have been found to contain at least twice the usual or "average" content of glutamic and aspartic acid residues together with a low amide content. Thus, a protein subfraction, isolated from a predominantly neuronal, membrance structure which is strongly suspected of being involved in cation transport, possessed an unusually high proportion of anionic (negatively charged) groups. A protein with such properties is presumed to be one of the potentially essential features of membranes concerned with cation transport. A more detailed study of this protein subfraction is now in progress in order to evaluate this possibility.

(5) As a consequence of the foregoing studies, the long-term studies by the Section on Proteins and Amino Acids of cellular electrolyte metabolism, utilizing whole cell, incubated slices of cerebral cortex *in vitro*, take on additional importance. This type of preparation represents the final, most complex test system for studies on cation transport, since it embodies the complete cellular organization of which the aspects cited in the above studies represent detailed portions only. With greater complexity of the system, the analytical and interpretive difficulties are greatly magnified. It is toward the solution of such problems that these particular studies are currently being directed. Extensive use of morphological (electron microscope) correlations are essential here to interpretation of fluid distribution and shifts among the various cell types and subcellular compartments. Techniques have been sufficiently developed so that it has now been possible for studies of cation fluxes in these whole cell slice preparations under controlled *in vitro* conditions to get under way.

(6) Finally, attention is being devoted to the pathological aspects of these same problems. The relevance of seizure phenomena to derangements of cation transport across excitable membranes is obvious, and is an integral part of the program of studying electrolyte metabolism just cited. In a different context, studies under Dr. Brady in the Section on Lipid Chemistry are under way to evaluate the relevance of immunochemical factors to demyelinating diseases. These studies represent a very sophisticated approach to an old and well-worn, but still unsettled problem. Two developments have permitted this reexamination: (a) The development in the Section of techniques for producing and studying antigens and antibodies for the various lipid constituents of myelin sheaths and neural cell membranes; and (b) the availability through collaboration with Dr. Bornstein (Mt. Sinai Hospital) of neuronal tissue culture preparations where myelination and demyelination under controlled, reversible conditions can be studied. It is too early to discern or predict the

course and outcomes of these studies, but they give promise of clarifying many of the uncertainties about immunochemical factors in the demyelinating states.

Thus, a major portion of the research activities of the NINDB Laboratory of Neurochemistry is being devoted to the study of the mechanisms subserving the functioning of neural membranes in reception, conduction and transmission of impulses. Included are detailed studies of molecular structure and organization; examination of the mechanisms of relevant enzymes; utilization of model systems, tissue culture, electron microscopy, and immunochemistry; and studies at all levels of cellular organization and complexity, including resort to appropriate clinical material. Since the nervous system functions primarily in terms of membranes and their surfaces and interfaces, the rationale and significance of these studies individually and collectively are obvious. It is worth reemphasizing here that this development of the Laboratory program was not preconceived or organized as a team approach, but represents independent, parallel convergence on a fundamental problem from a variety of subdisciplines utilizing a host of techniques and principles. There is a place for preplanned, directed team approaches, but one has the feeling that spontaneous convergence on a problem indicates a ripening and maturation of the problem and a greater possibility of a rich harvest of knowledge and understanding.

If neurochemistry really comprises a special field and is not just an organ-oriented facet of general biochemistry, it is because, as Professor Oliver Lowry has put it, "The brain presents an interesting challenge to the chemist. It would be hard to imagine a more complex structure than the brain with its axons, dendrites and cell bodies, from nerve cells of every size and shape, all mixed together with several different kinds of glial cells." To which could be added the further complexity of topographical and functional organization. In its inception as a special field, neurochemistry dealt with the nervous system almost as if it were an homogeneous organ and concentrated on the fueling and energy production aspects of its metabolism. Subsequently neurochemistry turned more to the structural aspects, the biosynthetic and maintenance mechanism and the subcellular distributions involved. But now the emphasis is again shifting to considerations of the organizational integration of structural and metabolic components, an integration which focusses on membrane and surface chemistry, as a prelude to delineating the functional attributes of such chemical systems in initiating, maintaining and controlling the physiological activity of neural tissues. Although much of the neurochemical research in this Laboratory and elsewhere may seem far removed from the pressing clinical problems which, it is hoped, neurochemistry can solve, this report suggests that the gap between the two is in reality very small. Knowledge about the workings of neural membranes is surely one of the keys to neurological problems, and once this key is available, the solutions to a number of these problems will be much simpler, if not at once obvious.

LABORATORY OF MOLECULAR BIOLOGY, INTRAMURAL RESEARCH

The laboratory intends to investigate mainly those phenomena and problems which occur in all biological systems, and it aims to explain and solve them in molecular terms. In these terms the major problems can be stated quite simply: First, we want to know the complete molecular structure of the hereditary material, as it occurs in chromosomes, episomes, and viruses, and to determine how this structure can be altered by recombination and mutation, the latter being inducible by chemicals, viruses, and other agents. Second, we have to discover which properties of DNA and of other cellular components which govern the production of specific messenger RNA. This control of messenger RNA is responsible for the control of enzyme synthesis observed in enzyme repression or induction. Third, we have to unravel the details of protein synthesis from messenger RNA and understand the properties of functional and nonfunctional proteins. Again, some control mechanisms work at the protein level, involving the liberation of finished enzymes from ribosomes, the conversion of enzyme precursors into active enzymes by mechanisms other than protein synthesis, and the function of enzymes by feedback inhibition. Eventually, when the genetic and molecular elements of enzymatic control are better understood, we may approach the problem of differentiation.

It is clear that such a program requires a variety of talents. Biologists who discover new biological phenomena and outline biological assays, biochem-

ists who isolate macromolecules and analyse their reactions in vitro, physical chemists who determine the properties of these macromolecules, and chemists who analyse the composition of the molecules and find new chemical ways of isolating and altering them. Common to all these scientists must be the desire to obtain an integrated picture of cellular processes and not to get lost in the overdetailed examination of processes that are specific to one particular type of organism. This laboratory, not restrained by tradition, is in the fortunate position of extablishing such an integrated program of different scientific disciplines which, in most other places of research, belong to separate departments that are often located far apart from from each other. However, the desired integration will be possible, only after the new building of the NINDB will have been erected.

Since we wish to obtain decisive results as rapidly as possible it will be necessary to restrict our studies to the simplest biological systems in which a given phenomenon has been observed. At present we have to limit ourselves to unicellular organisms, in particular to bacteria and their phages, since here the genetic mechanisms are simple, the generation times are small, and statistical work with large populations is feasible. These advantages are so enormous that certain phenomena have been elucidated only in micro-organisms, while in higher organisms where, similar phenomena have been observed and by analogy attributed to similar causes, a scientifically unquestionable proof for their causes seems nearly impossible. We are convinced therefore, that our work, although restricted to micro-organisms, will be indispensible for the discovery and understanding of certain medical problems and, by analogy, may suggest means of a cure. Although the very principle of our approach assures that our results are of general significance to all organisms, it may be occasionally worthwhile for us as well as the reader, to be aware of the consequences of our work to medicine. This time the significance of work with mutations shall therefore be sketched.

Mutations first have been artificially induced by X-radiation and still today research on radiation induced mutations exceeds by far that on chemically induced mutations, partially because radiation can be easily quantitated and partially because the AEC greatly encourages this work. Nevertheless, all biologists agree that the frequency of spontaneous mutations is normally much higher than that of mutations induced by natural or artificial radiation. However, work with bacteria and bacteriophages has shown that the so-called "spontaneous" mutations are often not due to an inherent property of DNA replication but are *induced* by chemicals that are either made by the cell or fed to it with the nutrients. The ability of cells to produce certain mutagens is inherited, i.e., there are bacterial strains which do and others which do not produce the mutagen; this reminds one of the inheritance of cancer production in certain animals. In addition to chemical mutagens certain viruses have recently been found to induce a very high rate of mutations (up to 50%) in bacteria that are infected and survive. Many of these phenomena could hardly have been discovered and analysed in animals, for which mutation work is rather slow; but they suggest that certain human nutrients or pharmacological agents, viruses, or genes, may also cause mutations and thus be responsible for abnormal growth of certain tissues. Our studies indicate that certain compounds should be screened for their mutagenicity in humans and suggest which antimutagenic or antiviral agents might reduce abnormally high mutations rates which are inherited or induced by viruses and which may be responsible for many kinds of abnormal developments of body and brain.

Major Results of Research

1. Mutations in Transforming DNA

It has been difficult to observe induced mutations in transforming DNA since no selective system was available. We have developed a new method of "linked mutation induction" in which we select for a DNA piece containing a given genetic information and then look for mutations in this piece. Since one usually works at transformation levels of about 0.1% this implies a selection by a factor 1,000. Using this method we have found that the mutation induction by hydroxylamine greatly increases with the temperature, with the unusually high Q_{10} value of 10, and that transforming DNA is about 1,000 times less sensitive to mutation induction than DNA of bacteriophage T4. One factor, responsible for this large difference, is the fact that isolated DNA is less reactive than DNA coiled up in the head of a

phage. The coiling must cause some DNA bases to loop out of the DNA strand, since DNA is otherwise a rather stiff molecule; our work has shown that DNA reacts much more when its strands are partially or completely opened. Another factor, causing the different mutagenicity, may be the difference in the base composition of DNA, phage T4 containing hydroxymethylcytosine instead of the normal DNA base cytosine. This possibility is under investigation. But it is already clear that the detailed chemical composition and structural arrangement of DNA in a chromosome can greatly influence the mutagenicity of various chromosomal areas.

2. The Temperature and pH Dependence of DNA Melting and the Rate of Uncoiling

DNA consists of two strands which repel each other because of their many negatively charged phosphate groups and which are kept together by hydrogen bonding between complementary bases. When one exposes DNA to increasing temperatures, the chemical equilibrium between the state in which hydrogen bonds are formed and the state in which they are separated shifts more and more towards the separated state. Since many hydrogen bonds collaborate to keep DNA strands together their separation occurs rather abruptly within a small temperature range. This melting temperature is about the same in the pH range between 5 and 9 but decreases rapidly within the range of the pK values of the DNA bases. Above the melting temperature the biological activity of transforming DNA decreases rapidly and drops to about 1/50 to 1/100 where it remains for long times, provided that the reaction occurs at high pH and low temperature, conditions under which secondary chemical reactions of DNA are negligible. Since the melting phenomenon results from a shift in the equilibrium between hydrogen bonding and strand repulsion towards the latter, the rate of strand separation increases with pH and temperature. At very high pH (13.5), however, all DNA bases are negatively charged and the speed of DNA strand separation is merely determined by the Brownian motion of the DNA strands, this being nearly independent of temperature. It has been possible to measure this fast speed (2 seconds) by transformation. The result makes it unlikely that all DNA of a chromosome exists in the form of one long double stranded molecule which duplicates as such; rather the DNA should have some swivel points at which one of the two strands is interrupted and therefore rotation can occur.

3. Organization and Function of the Head Protein of T-bacteriophages.

The heads of T2 phages can assume two different forms whose relative frequency in solution depends on temperature, pH, and certain ions. This behavior can be attributed to the properties of the protein sub-unit of which the phage head is composed. A histidine residue, near the C-terminal end of this protein, seems to play the major role in the head changes, since methylene blue, which can rupture the histidine ring by photodynamic action, irreversibly eliminates the short head form, suggesting that histidine maintains the long head form. A comparison of different T phages has shown that also T4, T5, and T6 can change their sedimentation constants, i.e., head forms, while T1 and T3 seem to exist in only one form. The head form seems to be related to infectivity, since in those cases in which head changes occur, phages seem to be infective only if they can assume the short head form; after infection the phage ghosts that are attached to the bacteria are all found in the short form. To assist these studies a monochromatic UV system for the Beckman Utracentrifuge has been developed.

4. Mechanisms controlling enzyme synthesis

The synthesis of the biosynthetic enzymes that are necessary to make a particular amino acid is usually repressed by the presence of this amino acid in the medium. We have found that alanine dehydrogenase (ADH), which makes alanine from pyruvic acid, is an exception to this rule, because its synthesis is induced by the presence of alanine in the culture medium of B. subtilis. This exceptional behavior is understandable, since alanine serves also as a carbon source in germinating spores and induces germination. We have shown that chloramphenicol, an inhibitor of protein synthesis, immediately stops ADH formataion while actinomycin, which blocks messenger RNA synthesis, permits ADH to increase for another 3 minutes. This shows that, after in-

duction by alanine, ADH is made denovo using an induced specific messenger RNA as template. Experiments are now underway to determine whether and how the decay of messenger RNA can be controlled both by the cell and from outside.

The molecular nature of compounds that cause repression or induction of enzymes is not known We are presently combining genetic and biochemical methods to attempt their determination. To aid this work chromatographic columns are being developed which permit a better separation of various RNA components of the cell.

NATIONAL INSTITUTE OF DENTAL RESEARCH

Introduction

With the recent expansion of the Dental Institute's direct reserarch operations, it is important that assessments of progress be made in the broad context of interpretation of mission and scope of responsibility.

Viewed in retrospect, the intramural program of the Institute has provided a significant element of leadership to the total national dental research effort. Currently and prospectively, this influence on research development in our universities and other nonfederal institutions gives evidence of further extension into the sphere of dental education. While earlier contributions to outside programs accrued as fringe benefits, there is today an increasing awareness by the Institute's scientists of a new role related to the broadening perimeters of their laboratories. In ever expanding degree, dental research is reaching into and complementing the allied biomedical, physical, and behavioral science fields.

Most responsible perhaps for this change has been the rapid and productive expansion of collaborative research ventures by Institute scientists and an increasing participation in the research and clinical associateship programs available at the National Institutes of Health for in-service training. In the conduct of these activities, the Dental Institute is well aware of its catalytic role in spurring the need for and the ultimate establishment of additional categorical dental research centers in university environments; each with the opportunity to influence further the shape of dental research, dental education, and dental practice.

More than a century ago, dentistry in this country ventured forth as an autonomous health profession, committed to a course of independence from medicine. With early educational emphasis on technical, material, and mechanical aspects of dental practice, it followed that the predominating research efforts in dental schools were channeled in parallel directions. It is not surprising, therefore, that the late-coming incorporation of the biological and medical sciences into dental educational curricula and allied research programs was more a grafting procedure than a naturally conceived part. The contrasting origin and development of the Dental Institute's research activity within a broadly conceived and multidisciplinary biomedical complex is perhaps the principal factor for its assumption of a pioneering role in the future of dental research and education in this country.

The diversity of activity represented in several recently initiated collaborative research programs is perhaps indicative of some trends, advances, and opportunities that lie ahead. While the details of these programs will be described in later sections of this report, the following listing will give evidence of their scope.

1. The Human Genetics Section, in collaboration with the University of Michigan, and under the sponsorship of the U.S. Atomic Energy Commission, the Rockefeller Foundation, and the Association for Crippled Children, is currently in its fourth year of study to evaluate the effects of inbreeding in a population where a large number of consanguineous matings had occurred and where detailed records of parents and offspring exist. Using facilities of the Atomic Bomb Casualty Commission in Hiroshima and Nagasaki, Japan, and with the cooperation of the Japanese National Institute of Health, the team of investigators sought to determine the average numbers of deleterious genes in the selected populations, and assembled considerable data pertinent to stature, weight, and dental eruption. At present, the collaborating team member from the Dental Institute is on assignment to the Department of Genetics, University of Michigan, where he is completing data analysis.

2. In another collaborative study, the Human Genetics Section is participating with the National Institute of Neurological Diseases and

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Blindness in a contracted research program at the Clarke School for the Deaf in Northampton, Mass., designed to analyze physical, biochemical, audiometric and clinical signs and symptoms incident to specific genetic types of hearing loss. The principal statistical method applicable to the analysis of assembled records and pedigree data will be by an electronic computer program designed by one of our staff for the study of discrimination and segregation patterns.

3. For the past 5 years, the Gnotobiotics Section, NIDR, has been engaged in a series of collaborative projects with Dr. Bengt Gustafsson of the Karolinska Institute in Stockholm, Sweden. The results of this association have been particularly fruitful in that it accomplished a partnership of competency in oral microbiology with a competency in nutrition and the development of germfree technics. For a period of 6 months during the current calendar year, the Chief of the Dental Institute's Gnotobiotic program was on assignment to Dr. Gustafsson's laboratory for the conduct of studies on the microbial aspects of dental and renal calculus formation in the rat. A report on this collaborative project will be forthcoming at an early date.

4. Following the demonstration by Dental Institute scientists that certain streptococci could serve as transmissible cariogenic agents in rodents previously free of caries, it became of importance to seek analogous situations in man. A group of hospitalized children at the National Children's Cardiac Hospital, Miami, Fla., and at Heart Haven Hospital, Lancaster, Pa., seemed to offer particularly suitable study material insofar as they had been maintained on an antibiotic regimen sufficient to depress any caries producing oral microflora. Accordingly, collaborative studies were negotiated which may provide the essential clinical followup information to the experimental lead that caries is a specific and transmissible bacterial disease. In parallel with these projects, a further collaborative study is being initiated with the Bureau of Indian Affairs to evaluate the cariostatic effect of a broad spectrum antibiotic of minimal toxicity (bacitracin) on selected groups of schoolchildren.

5. In the course of recent studies in the Dental Institute's Oral and Pharyngeal Development Section, it became apparent that currently available technics and devices for analysis of palatal impairments fail to distinguish normal valving from that found in disturbances of speech. Parallel observations by investigators at the State Institute for Speech Defectives in Copenhagen, Denmark, provided the basis for a contracted collaborative program to develop a device which would portray and measure air flow separately from nose and mouth during speech. Progress, to date, indicates that the new apparatus will provide accurate discrimination of speech valving at the palate in normal and abnormal subjects. It is anticipated that this contribution will make it possible to evaluate more effectively the benefits of prosthetic obturation and various forms of surgical repair in patients with partial and complete clefts.

6. During the past year, final field examinations were made in the 17-year study of the effects of fluoridation of community waters in Grand Rapids. This program, conducted by the Institute's Epidemiology and Biometry Branch in collaboration with the University of Michigan, the Michigan State Department of Health, and the City-County Health Department of Kent County, Mich., not only established the practicability and efficacy of water fluoridation as a caries control measure, but also provided important evidence that optimal doses of fluoride, in the range of 1 ppm, caused minimal degrees of fluorosis (mottled enamel).

7. During the current calendar year, investigators from the Institute's Epidemiology and Biometry Branch collaborated with the Interdepartmental Committee on Nutrition for National Defense in surveys in Trinidad, Burma, Jordan, Bolivia, and the Blackfeet and Fort Belknap Indian Reservations of Montana. These studies continue to provide important information on patterns of oral disease prevalence and severity in various ethnic groups, and presented leads and challenges for future studies in depth. For example, it was found in Trinidad that unusually high prevalances of caries and periodontal disease coexisted in the same individuals. This is contrary to the usual finding in population groups of a significant dominance of one disease or the other and raises some question regarding the validity of the long held view that dental caries and periodontal disease are mutually inhibiting.

The cited collaborative programs represent a rather limited number of intramural extensions made possible, in part, by contractual mechanisms.

Proportionately, support for these activities is considerably less than the expenditures of other Institutes for their contracted collaborative programs which are, in large part, biometric, field and service oriented, and range from eleven percent of their total direct operating budgets to more than four times the apportionment provided for intramural laboratory and clinical research at the National Institutes of Health. However, a more representative portrait of the Dental Institute's breadth of activity and contribution to the broad field of biological and physical sciences may be provided by the extent of its less formal participation in joint research enterprises with individual scientists and investigator teams of other categorical Institutes and outside institutions and aboratories.

Based on information recorded in the annual project reports of the Dental Institute for the previous calendar year, it is estimated that 56 percent of the professional staff participated in active collaborative studies with one or more scientists from each of the other six categorical Institutes, as well as with a large number of other Federal and non-Federal institutions. This compares most favorably with the estimated extent of similar, "between-scientists," collaborations engaged in by the other six categorical research divisions of the NIH which, as recorded in last year's individual project reports, ranged from 32 to 48 percent of their respective complements of principal investigators. To a substantial degree, therefore, early concern has been dispelled on the matter of whether geographical consolidation of the Dental Institute's program under a single roof would contribute to an undesirable degree of segmentation and isolation of personnel and programs.

While the mechanisms for initiating collaborative research are often unpredictable, and the resulting program is the true measure of accomplishment, it is of some interest to note that, by a conservative estimate, at least 50 percent of the Dental Institute's collaborations were consequent to other investigators seeking particular competencies among Dental Institute scientists. This fact alone provided our staff with a sense of prideful participation in and contribution to the total mission of NIH as a unit structure as well as parent organization.

As we take note of today's major accomplishments in dental research, and speculate on the advances they might promise for tomorrow, the clarity with which we see the future is, of course, dependent on the distance in time that we try to bridge. It is evident, however, that unlike the frequent solo performances of the past, our activities of tomorrow will increasingly demand multidisciplinary partnerships of personnel competent to deal with the complexities of modern research. While speculation on matters of research can be a most hazardous undertaking, the more evident events on the horizon would seem to be related to the impact of dental research itself on the teaching and practice of dentistry.

Following the discovery some 10 years ago, that dental caries cannot occur in the absence of bacteria, further successes were gained by the isolation of decay producing organisms, and the establishment of a direct cause and effect relationship between the disease and a particular oral flora pecular to the animal species under study. While the evidence of such highly specific host-parasite relationship served to emphasize the magnitude of the problem still faced in attempting to identify a similar causative microbial factor in man, an already considered objective is to develop a method of caries prevention by specifically acting antibacterial agents or related means. Leaving the dental practitioner manpower problem of the future to others more qualified to anticipate and estimate needs, one can be sufficiently objective to forecast the possibility of prophylactic procedures for prevention of dental caries becoming a matter so routine as to be handled by pediatrician and dentist alike.

In parallel with this expected achievement in the prevention and control of dental caries, there emerges the interesting speculation that as more teeth are saved from caries, more susceptible tissue sites may be preserved for attack by periodontal disease. How realistic a forecast is this for the dental practitioner of 1970 or 1980? Since formation of dental calculus in the gingival area is recognized as perhaps the most immediate exciting factor in periodontal disease, systematic studies of calculus are essential. One such investigation has shown that various species of streptococci outnumber all other types of micro-organisms at all stages of calculus formation. This, we recall, is contrary to the long held view that filamentous forms predominate, and may present to some a possibility that adequate measures can be found to prevent

ectopic calcifications by the elimination of suspected causative bacteria. Other studies are seemingly pointing to comparable relationships between specific microorganisms and certain types of periodontal disease. Thus, the prospect is for the development of prophylactic measures against periodontal disease similar to those envisioned for the prevention of dental caries.

With parallel advances expected in other categories of oral disease, the bridge that has long separated the basic from the clinical fields of dentistry promises to become a well travelled road of personnel exchange and communications. Similarly, there is every assurance of a further narrowing of the still existing gap between dentistry and its allied health professions. Thus, in the definition of and approach to long-range program goals, principally through the effective utilization of scientific manpower, the Dental Institute has continued to follow a practice of formally established collaborative and integrated planning among its program leaders. Quite naturally, this undertaking requires an appraisal of current programs in order to establish a baseline upon which to make reasonable assumptions of future trends and to construct and reconstruct along lines of promising productivity. In this responsibility, the Institute's scientific leaders have received an important measure of support in the expressions of confidence by the Board of Scientific Counselors.

The following account of activities, contributed by the Institute's program leaders for collation, details in more specific terms the accomplishments required to translate goals into tangible assets.

LABORATORY OF HISTOLOGY AND PATHOLOGY

In prior years the Summary Statement of the activities of the Laboratory of Histology and Pathology has taken the form of a descriptive catalogue of research accomplishments. During the current year certain marked changes in the attitudes and ambitions of key members of the Laboratory staff have become evident which make it advisable to include additionally within the narrative report an assessment of program activities in terms of present scope, long-range potential, and practical steps that might be taken to meet immediate needs. The above-mentioned reactions are not to be interpreted as reflecting dissatisfaction on the part of the staff, but on the contrary bespeak a realization that the research problems that have hitherto been considered as independent investigations have now reached proportions that merit extension into broader programs. As background information, the original nucleus of this Laboratory was a small group working with physical methods, primarily electron microscopy and diffraction, on the structure and properties of the calcified tissues. Activities were subsequently added in histochemistry, mainly directed toward the study of connective tissues, experimental pathology, dealing primarily with experimental dental caries, and crystal chemistry, concerned with atomic structure of tooth and bone mineral. The total professional complement has always been small, and interestingly enough, almost every individual has been inclined toward conservatism regarding expansion, preferring to wait for the time when justification seemed adequate. It is not surprising that the desire for expansion in all four areas should be expressed simultaneously, for the responsible investigators are all senior scientists, whose careers have run more or less in parallel. These individuals have by now gained international reputations, and their maturity in research is well established. Perhaps the most influential factor in the present situation, aside from the previously stated sharpening of research focus, has been the markedly improved working conditions and facilities over the past year.

The fact that the research of the past year has been most fruitful is attested to by the fact that the professional Laboratory staff, consisting of 7 permanent members and 6 visiting scientists (all of whom were not on duty for the full year), contributed to the production of 37 publications. On 26 of these they assumed first authorship. This statistic and a recognition of the varied subject material covered, bear out the facts that there is no dearth of ideas among these scientists, and that to expect productivity beyond this without some provision for expansion would be unreasonable. A start in this direction has been made within the last month by the establishment of a Section on Crystal Chehmistry, the implications of which will be discussed below.

At this point note should be made that recruiting, even on the small scale possible over the past several years, has presented serious problems, es-

pecially in the area of biophysical scientists. Competition from industry and universities has proved overwhelming. This is demonstrated by the fact that for one reason or another of 22 individuals contacted in the past 2 years, none were recruited. Admittedly, some of these did not prove to be desirable candidates. The fact that this unavailability of permanent personnel cannot be accounted for by scientific or physical unattractiveness of NIH is evident, because it has been easy for this Laboratory to recruit Visiting Scientists of the highest order of competence, as well as trainees (Special Fellows, Guest Workers, and Research Associates). Fortuitous as this latter circumstance may be for the perpetuation of the Laboratory program, it will be essential to improve our recruiting system. The problems may not be immediately soluble, however, inasmuch as the primary considerations appear to be either salary or general lack of individuals qualified in the research areas in question. It may be necessary to resort further to recruiting individuals who have biological or dental backgrounds and are amenable to specialized on the job training. This has worked very successfully in two cases and might provide a partial answer to the personnel problem.

With the preceding discussion in mind, the following summary of research accomplishments, together with projected needs is presented according to the subdivisions into which the activities of this Laboratory may be classified:

Biophysical Research

In this area the projects have primarily consisted of continued basic studies of the ultrastructure of various calcified tissues, dental embryology, and mineralization mechanisms, as well as new investigations in cytology and microbiology. As would be expected in research involving physical methods such as specialized light microscopy, electron microscopy and diffraction, contact and projection microradiography, densitometry, etc., considerable time and effort must be spent in technical and instrumental development. This year, for example, the studies on crystal development became so exacting that improvement of embedding methods and precise orientation of specimens for thin sectioning was essential. In addition, a rather extensive survey of the properties and usefulness of numerous newly suggested embedding media had to be made. A major effort concerned the development of adequate techniques for contact and projection microradiography, as well as modification of commercially manufactured equipment so that it could meet the experimental demands. As matters now stand, many of the difficulties in specimen preparation have been solved, cameras of various types have been constructed and are in regular service, and the projection X-ray microscope has been modified so that specimen accommodation, focusing, camera inadequacies, magnification calibration and several other inherent limitations no longer present problems. Microradiography has by now reached the dimensions of a specialized field, the coming aspect of which is microanalysis.

Work on the structure and properties of mature human tooth enamel has continued, and a good start has been made in explaining through electron microscopic studies various of the hitherto unclarified morphological configurations long observed under the light microscope. Perhaps the most important findings concern the striation patterns in the enamel prisms, which have previously had several different interpretations, and which have structural implications in the progression of dental caries. Apparently these rhythmic striations do not reflect differences in the ratio between organic and inorganic components, but may be accounted for in terms of regular variations in microcrystalline orientation which produce summatively the observed optical effects. In other studies on mature enamel, the surface alterations and changes in solubility effected by stannous fluoride, especially as compounded in dentifrices, have been explored further. A principal finding has been the surprising degree of *in vitro* protection against acid action provided by very low levels of available stannous fluoride.

Several observations of major importance have come from the studies of enamel development. In microradiographic examinations aimed at defining the calcification sequence through density changes, a disturbing physical effect was found which indicates the need for reassessing the validity of essentially all of the recently reported X-ray data. It was discovered that X-ray absorption is much higher when the radiation passes through the enamel crystals parallel to their c-axes than when it passes through perpendicularly. The explanation for this phenomenon is still being sought, but its

implications insofar as quantitative estimations of the degree of mineralization in enamel, where crystal orientation varies considerably from area to area, are self-evident. On the positive side, some excellent data have been obtained regarding crystal growth in enamel, and on the structural interrelationships between the organic and inorganic components. The work on crystal growth has required high resolution electron microscopy of precisely prepared sections containing cross or longitudinally cut crystals. In combination with electron and X-ray diffraction studies, the data show clearly that there is very rapid growth in length (c-axis) following nucleation in the newly formed organic matrix at the immediate ends of the formative cells, and then a slow and steady growth in width and thickness until the final hexagonal cross sectional contours are developed. Observation of parallel lines within the tiny crystals, which are in fact reflections from atomic intracrystalline planes, indicate a degree of structural perfection that essentially rules out the presence of organic matter within crystals. Further progress, on the other hand, has been made in defining more precisely the distribution of organic matrix between and immediately around the apatite crystals. The importance of exact characterization of the inorganic-organic relationships in enamel is in the fact that herein may lie one of the most important keys to an understanding of how the enamel disintegrates in the caries process.

Inasmuch as the origins of all the calcified tissues are basically cellular, work has been continued on the ultrastructure of the cells involved in enamel, dentin and cementum formation. Aside from the purely anatomical connotation of such studies, the major points to be settled concern exactly what any of these cells have to do with the elaboration of the organic matrices, and the possible role they lay in the calcificataion process. Other aspects in cytology which will be given attention are the devopment of adequate methods of tissue handling and staining procedures, so that histochemical investigations can be made at the electron microscopic level, and observations on the structure, properties and embryonic sequences of cementum.

Through the training of a microbiologist over the past year, it has been possible to extend collaborative studies on bacteria mineralization, as well as initiate ultrastructural investigations of certain oral micro-organisms. The latter project, concerned mainly with genus Bacteroides, has led to the observation of what may be conjugatory bridges. Although the phenomenon of conjugation, a process by which genetic characteristics are transferred between bacteria, has been known to occur for a long time, it seems to be a very rare event. If the present interpretation of the electron microscopic data should prove correct, it is the first time visual evidence of actual interconnections between micro-organisms has been recorded.

Other collaborative projects with workers in laboratories within and outside NIH have dealt with aspects of the mineralization process, and cytological, microbial, and viral problems.

Crystal Chemistry

Concentrated work in this area was begun a year and a half ago with the establishment of a program for the conduct of crystallographic studies. Here the major approaches are made through X-ray diffraction in all its forms, and through infrared spectrophotometry. By these techniques investigations are being made of the crystal texture of hard tissue apatites and synthetic calcium phosphates of similar composition, in which crystal size and strain are under study first from the standpoint of pure basic definition, and second from the standpoint of structural improvement that may be achieved by various induced chemical alterations. Single X-ray analyses are also being conducted for the purpose of elucidating the exact atomic arrangements within crystals of bone and tooth mineral, as well as the substitution sites of various ions and groups in both natural and synthetic apatites. Coupled with these studies, hydrogen bonding in the calcium phosphates in general is being investigated by infrared absorption spectrophotometry. The basic aim here is to gain an understanding of the manner in which electrical neutrality is maintained in structures which prove to have less than the stoichiometrically required number of calcium atoms. It is thought that this is achieved through the presence of hydrogen bonds between orthophosphate oxygens. The significance of this phenomenon lies in the effect it has on the stability and solubility of bone and tooth mineral.

During the past year, it was found that fluoride and hydroxyl ion occupy identical structural positions in hydroxyapatite when substituted for each other, while chloride ion assumes a different position. A most important observation was made with respect to the effect of fluoride ingestion in humans and laboratory rats on the crystalline character of bone mineral. The X-ray studies have shown that with increased fluoride content the bone apatite crystals tend to be larger and more perfect structurally, while the infrared observations have demonstrated further a crystal perfection through reduced calcium deficiency. Thus, for the first time some concrete evidence has been obtained as to the exact way in which fluoride acts upon biological mineral to make it more stable, by increasing crystallinity and by the production of a less soluble fluoride-substituted apatite. The continuation of these initial studies will be a refined investigation of the role of fluoride in synthetic apatite crystal nucleation and growth, followed by a reapplication of the findings to other biological tissues, especially tooth enamel. In addition, the changes in stoichiometry of biological apatites with age, disease, and dietary changes will be important to determine as background for experiments with not only fluoride compounds, but also other possible media for effecting improved mineralogical status.

Emphasis in the immediate future will be placed on crystal texture in organized tissues and on single crystal structure. This work will be extended to include crystal nucleation and growth, and important studies will be undertaken on the organic components of the calcified tissues. One of the most immediate needs in this latter area is to characterize the chemical nature of the organic matrix of enamel. This cannot be done by chemical analysis, and so far there remains considerable dispute as to the real nature of the fibrous keratin-like protein in enamel. To extend this program, it is planned to recruit at least two more physical chemists or physicists, and to set up facilities for synthesis of the larger amounts of experimental compounds required. As might be expected, much of the work will continue to be performed in collaboration with other workers outside the section, who have specialized knowledge either in allied instrumentation or in the handling of biological specimens.

Histochemistry and Experimental Pathology

Studies in this area have continued to concentrate on the chemical composition and reactive groups in normal and diseased connective tissues. The significance of the oxytalan fibers discovered here in 1958 has become more clear, and it is now thought that they belong to the elastic family of connective tissue fibers. There is an implication in this year's result that they may actually be a precursor of elastic fibers.

In another histochemical study of periodontal disease in human autopsy material, it has been found that degradation of connective tissue and bone may occur at sites distant from the inflammatory focus. This suggests a weakening of periodontal structures in advance of any actual inflammatory process. Data such as this provide another link in the search for sequential processes involved in periodontal disease, and it is hoped that through the histochemical approach some of the mechanisms will be clarified.

Stemming from the observation several years ago of transmissibility of dental caries in animals, it became obvious that serious attention should be given to the microbial factor. The extension of this important work has been in collaboration with the Laboratory of Microbiology and is discussed in a later section of this report. In a like demonstration of productivity accruing from such cooperative enterprises, a significant advance has been made in the development of a laboratory technique for inducing and studying periodontal disease. This finding is described in the following section.

LABORATORY OF MICROBIOLOGY

During 1962, the research programs of the Laboratory of Microbiology continued to fall quite naturally into a long range pattern encompassing the categories of periodontal disease, dental caries, microbial taxonomy, microbial physiology, immunology, and virology as they pertain to a wide variety of the oral microbiota. The work of each individual investigator, however, extends into several areas, as it should in a healthy research program.

Periodontal Disease

From the viewpoint of direct relevance to oral disease, the most notable achievement of the year

was the first fruition of a collaborative investigation with the Laboratory of Histology and Pathology, which demonstrated for the first time the transmissible infectious nature of a form of periodontal disease in hamsters and identified a specific type of filamentous microorganism as an essential etiologic factor. The availablity of this model system opens the way for controlled study of the undoubtedly multiple factors, nonmicrobial as well as microbial, that determine the course of periodontal disease in general. Since in many ways this experimental syndrome resembles chronic periodontitis in man, further study of its pathogenesis should help elucidate the nature of this major human disease. Already, the ecologic subtlety of the experimental disease has been revealed by the fact that the essential etiologic microorganism can be established readily in germ free mice but that it produces no periodontal pathosis in them. These observations may indicate the necessity for species-specific host factors; on the other hand, they may indicate the necessary participation of additional microbial species.

The accumulation of dental calculus, with its accompanying microbial components, is recognized as the most important immediate provocant of periodontal disease. Previous observations in this Laboratory indicated that, contrary to the general impression, filamentous forms did not bear a specific etiologic relation to calculus formation; a wide variety of other oral micro-organisms predominated in all stages of developing human calculus, and calcified equally well in a model system *in vivo*; namely, within cellophane sacs implanted intraabdominally in rats. The latter system has now been extended to include calculus deposition on sterilized teeth within the sacs. Under these conditions, present results indicate that filamentous bacteria may play a rather specific role, e.g., deposition occurred in the presence of *Actinomyces iraelii*, but not on oral diphtheroid. On the other hand, our gnotobiotic studies have shown that calculus accumulates about the teeth of germfree animals and has a microstructure very similar to that of ordinary calculus, except for the substitution of an amorphous organic matrix for bacteria. Evidently the tendency to form calculus is inherent in the host; the critical determinant is a suitable organic matrix upon which mineralization may occur, and bacteria constitute a particularly effective one. This conclusion is substantiated by other experiments showing that calculus deposition in ordinary germ-bearing animals is invariably more extensive than in comparably treated germ-free animals; that it is not much affected by a variety of chemical, nutritive, and physical alterations of the diet; but that it is strikingly reduced by inclusion of a minute amount of an antibiotic, erythromycin, in the diet.

Dental Caries

Collaborative studies established previously the transmissible infectious nature of experimental dental caries in hamsters and rats and identified a particular kind of streptococcus as an essential microbial factor. These studies have been recognized repeatedly by awards, culminating in 1962 with the Joachim International Research Prize of the International Dental Federation, which has been awarded only once every several years and is the subject of keen international competition. Continuation of these studies emphasizes the importance of quite sophisticated correlated investigations in microbial physiology; that is, to date no characteristics have been found to explain why certain strains of streptococci are cariogenic in hamsters and rats, whereas other strains, seemingly the same by a variety of criteria, induce no caries. Such investigation should be greatly facilitated by development during this year of a simplified, chemically defined culture medium for these streptococci, which makes it possible to study them under closely controlled conditions.

Intimately influencing the action of microbial agents of caries are genetic and nutritional determinants; however, we know now that in past studies the significance of the latter was obscured by doubt whether the experimental animals harbored the essential cariogenic flora. Experiments in this Laboratory have now confirmed the inherent genetic resistance of certain strains of rats to experimental caries, even when they are adequately exposed to a cariogenic oral flora and are maintained on a diet conducive to caries. As expected, the progeny from mating such rats with caries-susceptible strains showed intermediate degrees of susceptibility. Past reports have stated that maintenance of pregnant rats on a high-sugar diet resulted in increased susceptibility to dental caries in the offspring; however, it has now been shown in this Laboratory that when measures were taken to equalize the oral flora, the progeny of mothers

maintained on a low-sugar diet developed just as much caries as the others. The apparently greater susceptibility of the "high-sugar" group evidently resulted from transmission of an intensified cariogenic flora from the mothers to the offspring.

Microbial Taxonomy

Our attempts to characterize specific cariogenic streptococci from rats and hamsters, and the filamentous form essential for periodontal pathosis in hamsters, have emphasized anew the continuing great importance of classic systematic study of the oral flora, which has lagged behind that of most other parts of the animal and human body. Without a large reservoir of such fundamental information, we are seriously limited in our understanding of the ecological and pathological potentialities of these organisms, and different investigators cannot be certain whether they are examining the same organism. Thus, the streptococci and the filamentous organism just mentioned seem to belong to previously undescribed groups, and will require further analysis. During this year, it has become possible to delineate a much more accurate cultural, biochemical, and serological characterization of the genus *Veillonella*, the second most numerous bacterial in the human mouth. Particularly significant is serological analysis of the toxic lipopolysaccharide antigens of the cell surface, because it reveals hitherto unsuspected sharp distinctions between the strains of *Veillonella* from different individuals. Similar analysis has revealed equally numerous and specific serogroups in the fusobacterial, which have long been implicated in periodontal disease. Also during this year, criteria for the genus *Actinomyces* were reviewed. It was concluded that the characteristics of these organisms had been stable on prolonged cultivation; and that the species of this genus must be differentiated by morphological criteria and comparative rates of growth, for the usual physiological and biochemical tests were of no value. Finally, an important contribution was the development of a simplified improved culture medium for oral and other spirochetes, which should greatly facilitate their isolation and mass cultivation.

Microbial Physiology

In the field of microbial physiology, unusual metabolic functions of the vitamin, lipoic acid in *Butyribacterium rettgeri* were elucidated further. Though essential for growth on lactate media, lipoic acid does not participate in pyruvate fermentation by this organism, and is not required for its growth on glucose media; however, lactate is a major product on the latter media. Extracts of *B. rettgeri* grown on glucose contain a DPN-linked lactic dehydrogenase, lacking in cells grown on lactate. Extracts of the latter, however, contain an enzyme system capable of coupling anaerobic dissimilation of lactate to the reduction of ferricyanide. It is believed that the DPN-linked lactic dehydrogenase functions primarily in formation of lactate from pyruvate, whereas the ferricyanide reaction reflects an enzyme system primarily involved in dissimilation of lactate. Since end products are the same, whether from glucose without lipoic acid or from lactate with lipoic acid, the latter seems to function primarily in the electron transport system involved in disposition of lactate hydrogens. In another study, continued investigation of the biochemical factors initiating and controlling morphogenesis of *Dictyostelium discoideum* showed that the amino acid, histidine, stimulated morphogenesis at pH 5 but not at pH 7. Since equal amounts of histidine were taken into the cells at these pH values, its action seemed not to depend on intracullular accumulation but to be external. Histidine decreased the uptake of exogenously supplied amino acids into the internal free pool; it decreased the leakage of ultraviolet-absorbing material from the cells but not the leakage of protein. A direct correlation was found between the ability of materials to stimulate morphogenesis and their ability to prevent leakage of ultraviolet-absorbing material from the cells. Another project continued to study enzymatic synthesis of the vitamin, folic acid, by lactobacilli: from data obtained by electrometric titration, acid hydrolysis, enzymatic hydrolysis, and spectrophotometry, it was concluded that enzymatic coupling brings together 2-amino-4-hydroxy-6-pteridylmethyl-pyrophosphate and *para*-aminobenzoylglutamate to form dihydrofolic acid and free pyrophosphate.

Immunology

Immunologic investigations emphasized the immunochemical analysis of endotoxic lipopolysaccharide antigens of oral bacteria and the alteration of nonspecific host resistance by these sub-

stances. Lipopolysaccharides from species of *Veillonella*, *Fusobacterium*, *Leptotrichia*, *Selenomonas*, and *Neisseria* were characterized as to molecular weight (greater than 50,000 and polydisperse), saccharide units (glucosamine, galactosamine, methylglucose-like, sialic-acid-like), and lipid components (cephalin, plasmalogen, fatty acids). Although the isolated lipopolysaccharides did not engender antibodies when injected parenterally into rabbits, they sensitized erythrocytes to agglutination by homologous antibody and formed specific precipitates with antibodies. By a combination of agglutination and antibody-absorption techniques, the lipopolysaccharides were shown to be part of the mosaic of surface antigens of their parent bacteria. By indirect hemagglutination and its inhibition by solutions of the lipopolysaccharides it was possible to delineate clear-cut serogroups of strains of *Veillonella*, *Fusobacterium*, and *Leptotrichia*, which should assist studies of the distribution of these organisms. In other experiments, it was shown that prior treatment of mice with vaccines of oral gram-negative bacteria, or with minute doses of their lipopolysaccharide endotoxins, effects a biphasic nonspecific change of their susceptibility to experimental bacteremia with oral viridans streptococci, to which they are ordinarily quite resistant. During the first 12 hours, susceptibility is increased tenfold; after 24 hours, resistance is greater than normal. The latter effect depends not only on an increased blood clearance by the reticuloendothelial system but also on increased phagocytic capacity of mononuclear and polymorphonuclear leucocytes in the treated animals. This state seems to be attributable at least in part to the nontoxic lipid moiety of the endotoxin, which is released by enzymes of the liver; that is, markedly increased nonspecific resistance to infection can be induced without a lag period by injection of minute amounts of the partially purified lipid, released *in vitro* by either acid hydrolysis or digestion with liver homogenate. Another aspect of the biology of endotoxins has been the question whether animals become more reactive to them following exposure to an endotoxin or to the bacteria producing it. We have now answered this question affirmatively by showing that mice bearing the usual coliform flora of their species are much more susceptible to an *E. coli* endotoxin than are their germ free or coliform-free counterparts.

Virology

In virology, our continued serological analysis of herpes simplex virus has shown in a number of cases that a different strain is present in each successive recurrence of fever blister in the same individual. This observation throws a new light on the natural history of this common infection, which usually has been attributed to exacerbations of the identical virus. Other experiments have now succeeded in producing primary herpetic ulcerations in the oral mucosa of rabbits, thus providing a useful model system for study of immunity, exacerbation, and therapy of such infections. In another direction, investigation was started on the mechanisms and consequences of animal cell transformation, with chromosomal aberrations, induced by herpes simplex virus. In collaboration with the National Cancer Institute, investigation continued on the virus designated as lactic dehydrogenase (LDH) agent, which was discovered as a contaminant of a number of transmissible tumors of mice, in which it resulted in a five- to tenfold elevation of the plasma LDH. Now it has been shown to produce elevations also of the plasma isocitric dehydrogenase, malic dehydrogenase, phosphohexoxe, isomerase, and glutamate-oxalacetate transaminase, but not of alkaline phosphatase or aldolase. The well known elevation of plasma enzymes that accompanies development of transmissible tumors has been shown to be truly attributable in part to the tumors, but infection with the LDH agent superimposes an additional increase. This virus is characterized by several unusual properties, including rapid initiation of multiplication within a few hours after infection, associated with extraordinarily high titers of virus in plasma within 24 hours; viremia persisting for at least a year, suggesting a poor antibody response; similarly prolonged elevation of a number of plasma enzymes, evidencing some sort of cellular alteration; and absence of discernible illness and gross or histological evidence of pathologic change. Spontaneous transmission of the LDH agent from infected to uninfected mice in the same cage is infrequent; the virus has been recovered from saliva, feces, and urine only early in the course of infection. The properties of this agent make it an uncommonly promising subject for investigating virus-host cell interactions.

LABORATORY OF BIOCHEMISTRY

Protein Chemistry

The activities of the Section on Protein Chemistry have centered principally on the structure and function of connective tissue proteins.

In a recently completed study, it was demonstrated that collagens from several species (dogfish, carp, rat, chick embryo, and guinea pig) and several tissues (skin, tendon, and bone) have the same general structure, i.e., in each case the molecule contains three subunits, two of one type and one of another type. Designated $\alpha 1$ and $\alpha 2$, these subunits, have different, but related, amino acid compositions. The structure is stabilized by intramolecular crosslinks between chains which form at a rate and to a degree characteristic of the tissue.

Lathyrism

Toxic agents such as β-aminopropionitrile injected into experimental animals develop symptoms which are related to a connective tissue disease called lathyrism. Following an earlier demonstration, that collagen from lathyritic animals contains a much reduced content of crosslinked subunits, it was established by isotope and tissue culture technics that the primary effect of lathyritic agents is to break or prevent the formation of the crosslinks. The gross symptoms develop later as a result of the incomplete maturation of the collagen fibrils and the consequent loss of integrity of the tissue.

The results obtained from the study of lathyrism indicate that intramolecular (and perhaps intermolecular) crosslinking is an important aspect of connective tissue maturation. Further, the observation that the degree of crosslinking varies from one tissue to another indicates that this may have an important relation to function. Presumably, either hypo- or hyper-crosslinking might produce a connective tissue disorder.

Collagen, Molecular Weight, Structure and Function

The molecular structure of collagen is established, in part, by precise molecular weight data on both the native molecule and its subunits. Owing to aggregation phenomena, it has been extremely difficult to obtain accurate molecular weights of these complex compounds. Recent work has shown, however, that sedimentation equilibrium technics offer high precision and give the best estimate of homogeneity of a sample. Whereas previously the best collagen samples had been shown to have molecular weights near 360,000, the new, more accurate, value now stands at 300,000. In confirmation of our proposed subunit structure, $\alpha 1$ and $\alpha 2$ had molecular weights of about 100,000. The covalently linked dimers formed from $\alpha 1$ and $\alpha 2$ have about twice this weight, as expected.

The fact that collagen is found throughout the animal kingdom and may have diverse functions in a single species (for example, tendon, skin, bone, and dentin have somewhat different properties and functions), indicates that relatively minor variations within its basic molecular structure could affect its properties. In addition to the possible role of crosslinking already mentioned, amino acid content and amino acid sequence all may have major importance. Indeed, previous studies of collagens from different tissues of a single species have demonstrated varying contents of lysine and hydroxylysine and recent studies in this laboratory have shown that the amount of 3-hydroxyproline also is not constant. 3-hydroxyproline is a recently discovered imino acid related to the more common 4-hydroxyproline. These two imino acids may occupy interchangeable positions in the amino acid sequence.

The mechanism by which collagen may function to initiate and control calcification is not known. One approach to the problem is to study proteins which calcify as part of an abnormal process. One of these is elastin as found in aorta. In a recent study, an *in vitro* system, employing rat aorta and serum, was developed. Since it is known that elastin in aorta is calcified in the atheromatous lesions found in atherosclerosis, the *in vitro* system may closely parallel the disease state. The independent study of experimental variables such as inhibitors and activators, levels of calcium and phosphorus, and chemical modification of the elastin can be evaluated. For example, the observation that the deposition of mineral (apatite) is confined to elastin even though collagen fibrils are present, demonstrates that the protein matrix is only one part of a calcifying system. Other regulatory factors are present. In this respect, the presence of a lag phase indicates that inhibitors are present in serum. A further observation is that the presence

of certain amino acids affects the rate of mineralization.

Calcification

The fundamentals of skeletal and dental tissue calcification are obscure in many details. During the past year experiments were conducted to evaluate the relation of fluoride to calcification and particularly its role in bone resorption. There has been increasing interest also in the possible role of fluoride and bone metabolism have been motivated by this particular problem in bone disease. Using the white rat as the laboratory animal, studies relative to calcification are attempting to evaluate fluoride effects in association with cortisone. Whereas hydrocortisone depressed urinary excretion of citrate, an important minor constituent of bone, fluoride had little or no such effect. With reference to bone-citrate per se large differences were found in the dental tissues of different species. The possibility exists that a variable citrate content affects bone and dental tissue crystallinity.

In parallel experiments, further evaluation was made of the effects of fluoride-induced changes on bone metabolism and bone-mineral crystallinity. Using X-ray diffraction analysis it was shown that fluoride incorporation improved bone apatite crystallinity. Bone and tooth mineral metabolism was investigated further in rats receiving 25 and 50 ppm of fluoride in their drinking water. In this case it was shown that the uptake of Ca^{45} and Sr^{89} in the tibia ends of the bones was decreased by these quantities of fluoride. Incisor tooth apatite, however, was not affected but there was a discrimination against strontium vs. calcium in the base of the incisor teeth.

In connection with the problem of osteoporosis, the influence of the level of calcium ingestion is of major importance. Thus additional studies were conducted with white rats receiving low and high calcium diets. The effect of fluoride was studied with these calcium-adequate and calcium-deficient diets and labeled calcium as well as strontium were administered during the course of experiments. The relative absorption of calcium vs. strontium gives basic information on the exchange mechanisms of bone mineral. The results of this study suggest that there was a high skeletal uptake of Ca^{45} by rats receiving the calcium deficient diets and this increased uptake was due principally to an increased rate of calcium exchange rather that to an accretion of calcium. Somewhat contrary to expectations, fluoride decreased slightly the uptake of radiocalcium. However, there was an increase in total bone ash, due to fluoride. The facility for absorption of calcium in the intestinal tract is an additional important facet of adequate skeletal mineral metabolism. In further experiments it was shown that the intestinal absorption of Ca^{45} was greater than Sr^{89} as the level of calcium ingestion was increased. On a low calcium diet the absorption of both these isotopes was increased. Thus under normal conditions the organism adapted to an efficient utilization of a calcium deficient diet.

Dental Caries—Relation to Phosphates

Interest in the cariostatic role of phosphates continues with experimental studies being directed toward the role of organic phosphorus compounds. With remarkable consistency and significance, certain organic phosphorus compounds, i.e., phytin, sodium phytate, calcium phytate and β-glycerol phosphate were caries-preventive in the white rat. These results follow previous studies and similar results using inorganic phosphates. However, the mechanism of an anticaries effect of a soluble inorganic phosphate may indeed involve a less complicated action than that of an organic phosphorus compound. This significant anticaries action of both of these types of phosphates is highly provocative and may have practical applications in human caries control.

Congenital Malformation and Teratogenic Agents

Using pregnant Sprague-Dawley rats, ACTH, cortisone, 17 hydroxycorticosterone, glutithionide, and thalidomide were investigated as teratogenic agents during specific periods of gestation. The treated animals were killed on the 20th day of pregnancy and the young examined for malformations. This particular strain of rats proved particularly resistant to these treatments, thalidomide being the only agent which produced congenital malformations. When the latter drug was administered, 6 to 9% grossly malformed fetuses were found. Among the more common defects were malrotations of the hind limbs, hamartoma of the palate, accessory incisors and microsomia. Further studies revealed the presence of a subcutaneous cartilagenous type mass of tissue from middorsal region to the tail of the fetus.

In a parallel study, a special effort was made to evaluate the role of the hydrostatic pressure of the amniotic fluid, as well as amniocentesis (puncture of wall membrane) on congenital malformations. The principal defects following amniocentesis were cleft palate and limb deformities. It is of interest that cleft palate occurred only following amniocentesis on the 14th, 15th and 16th gestation day whereas limb deformities developed if amniocentesis was performed on any day except the 13th. A bacteriological study showed that infection was not a factor in teratogenesis by this procedure.

Saliva and Salivary Glands

Since salivary glands are reportedly enlarged and salivation is increased by isoproterenol hydrochloride, it was in our interest to investigate this observation with respect to susceptibility to experimental dental caries. Recently, investigators in this laboratory observed that this drug caused marked enlargement of the submaxillary and parotid glands in the rat, but did not affect the sublingual gland. Excessive salivation was also evident. When these animals were placed on a cariogenic diet, a 50% higher caries score resulted than in control rats. Interesting histological changes were observed in both the thyroid and salivary glands.

In other studies, biochemical data have revealed the presence of four times as much inorganic phosphate in human saliva as in rat saliva. However, human saliva was markedly lower in tyrosine, tryptophan and protein. Additionally, acid phosphatase activity of human saliva was five to six times as high as that of rat saliva whereas alkaline phosphatase was only ⅛ that of rate saliva. Finally, uric acid was found in the human but not in the rat saliva. These differences may foretell important differences in the dental caries susceptibility of these two species.

Enzyme Chemistry

At the present time approximately 1,000 enzymes have been described, and many of these have been studied intensively from physical, chemical, physiological and kinetic points of view. Despite years of effort by many teams of investigators in many institutions the precise structure of no single enzyme has been determined, and the mechanism of action of no enzyme has yet been established. It is the program of the Section on Enzyme Chemistry to participate in the elucidation of enzyme structures and the nature of the catalytic process. Enzymes differ from each other in many essential respects; therefore, the work of the Enzyme Chemistry Section has included studies on different types of enzymes that permit various techniques to be applied, providing insight into the differences that exist as well as similarities.

Many enzymes contain metal ions built into the protein structure. Kinetic studies of carboxypeptidase B have described the complicated roles of different metals, zinc, cobalt and cadmium, in affecting the binding of various substrates to the enzyme and in determining the rates at which these substrates are hydrolyzed. The formation of carboxypeptidase B from its precursor has also been studied. Similar studies with procarboxypeptidase A of pig pancreas (instead of the conventional beef pancreas) have yielded an enzyme that differs in certain characteristics from the beef enzyme. In the activation process several intermediates and products have been detected by electrophoresis and ion exchange chromatography; one fraction that has enzyme activity has been obtained in crystalline form. Related studies in collaboration with NIAMD have resulted in the demonstration of an enzyme in pig kidney that hydrolyzes specifically glutamic and aspartic acid groups from proteins and peptides.

The enzyme aldolase is an essential catalyst in glycolysis, and is the subject of active investigation in many laboratories. In other laboratories the reversible dissociation of aldolase into thirds in acidic and urea solutions has been demonstrated recently. Parallel studies in this laboratory have shown similar dissociations to occur in alkaline solutions, and further dissociation into sixths has been indicated by ultracentrifuge studies at high pH values. The relationship between the fragments has been studied by chromatography and electrophoresis of the components separated by enzymatic digestion; these methods indicate that the thirds are identical to each other, but that the smaller subunits of each third are not identical. The kinetics of aldolase have been studied intensively with native and enzymatically modified preparations. In collaborative studies with Yale University, a partial reaction catalyzed by aldolase, in which there is no net chemical reaction, but merely exchange of one specific hydrogen atom

with the hydrogen of the medium, has been shown to be greatly affected by modification of aldolase. The modified enzyme shows a primary isotope effect when deuterium or tritium are substituted for hydrogen in the substrate, whereas the native enzyme attacks all forms of hydrogen at the same rate. This change in relative rates of steps in the catalytic process is paralleled by changes in the rate of exchange of products of the net reaction into the substrate. The kinetic and structural studies can be combined to permit the construction of a theoretical model of the enzyme that is being examined by further experiments.

Vitamin B_{12} has recently been found to occur in a modified form in which it participates as a coenzyme for several enzymes. In collaboration with NHI, the mechanism of the conversion of the vitamin to the coenzyme has been described. A single enzyme has been shown to be responsible for the replacement of a cyanide group by deoxyadenosine derived from ATP. A unique feature of this reaction is the liberation of inorganic tripolyphosphate from ATP. Vitamin B_{12} also participates in the formation of methionine from homocysteine. The role of vitamin B_{12} in transferring methyl groups from tetrahydrofolic acid to homocysteine with a bacterial enzyme has been established, and the influence of vitamin B_{12} on the analogous animal liver enzyme has been described.

A special low molecular weight form of nucleic acid, S-RNA, is known to participate in protein synthesis by accepting amino acids from specific amino acid activating enzymes and transferring them to the polypeptide chain being synthesized by ribosomes. The establishment of the structures of individual types of S-RNA is a major biochemical problem, since the specificity for reaction with the individual activating enzyme and also the specificity for transfer to the appropriate part of the protein (i.e., reading the genetic code written in messenger RNA) are built into the S-RNA molecule. Until now studies on the structure of this material have been hampered by the lack of an adequate method for separating the more than 20 types of S-RNA from each other.

In contrast to the empirical methods of separating nucleic acids, a specific method is being developed based on the reaction of amino groups of amino acids with N-carboxyanhydrides. This method has been shown to result in the formation of an amino acid polymer that precipitates with any S-RNA molecules that are carrying amino acids, but not with "uncharged" S-RNA.

Work is in progress to establish optimal conditions for quantitative separation of charged from uncharged S-RNA, and methods are being developed for the analysis of the component nucleotides of the precipitated nucleic acid.

Control of Metabolic Processes

An objective of this phase of our enzyme research is the study of levels of coenzymes in their relation to control of metabolic pathways. In addition, abnormal products of metabolism were studied in relation to alterations in metabolism controls as may be caused by chemicals and disease products. Thus the levels of the nicotinamide nucleotide coenzymes were found to be present at high levels in several species of bacteria with high metabolic rates. These coenzymes also occur at high levels in mammalian liver and spleen where the metabolic rate is high. They are present in lower concentrations in soft and hard structural tissues where the metabolic rate is corresponding low. Apparently the nicotinamide nucleotide coenzymes thus have a significant relation to metabolic activity in the body organs and tissues.

The glycolytic action of a rat-cariogenic streptococcus may possibly serve to explain a relation of this organism to dental caries etiology. Since it has been shown that sodium bisulfite inhibits experimental dental caries, it is of interest to study the effect of this chemical on the glycolytic activity of this particular streptococcus. Sodium bisulfite decreases the amount of lactic acid produced by this organism from glucose with a subsequent increase in some of the more neutral products. This effect has not been found to follow the known alternate pathways of glycolysis, where products other than lactate are formed by micro-organisms.

EPIDEMIOLOGY AND BIOMETRY BRANCH

Research activities of the Epidemiology and Biometry Branch continued in 1962 to be concerned with obtaining and analyzing data to elucidate the descriptive and determinative epidemiology of oral diseases. Because specific etiologies of most oral diseases are unknown and appear to be complicated and dynamic interactions between numerous factors within the agent-host-environment relationship, investigations have been necessarily broad in scope. Objectives of studies

undertaken by the Branch have varied widely within a broad program which included: (a) Nutrition surveys in cooperation with the Interdepartmental Committee on Nutrition for National Defense; (b) studies of the fluoride-dental caries relationship, including a survey of dental fluorosis; (c) clinical trials of other caries inhibitory agents; (d) investigation of the Keyes-Fitzgerald hypothesis of the communicability of dental caries in animals, to determine whether it is applicable to human caries; (e) a survey of occlusal anomalies in a population; and (f) studies of the epidemiological characteristics of periodontal diseases.

Some of these projects, described in the following sections, were initiated during the year; other were continuations of studies begun in previous years.

Nutrition Surveys

Investigators from this Branch continued to collaborate with the Interdepartmental Committee on Nutrition for National Defense in surveys designed to evaluate the nutritional status of selected populations of various foreign countries. Findings from studies in Vietnam, Lebanon, and Burma were under analysis. To date, field examinations have been completed in 14 countries or geographical units in such diverse areas as Africa, North and South America, Southeast Asia, and the Middle East. These surveys employed a team approach and within this framework dental epidemiologists worked closely with specialists in nutrition, medicine, biochemistry, food technology and agriculture. During the course of these studies, oral examinations were done on thousands of persons. Examinees represented diverse ethnic, social and cultural backgrounds, lived under widely different environmental conditions and subsisted on traditional diets of varying quality and composition.

Vast quantities of information to describe the prevalence and severity of oral diseases in groups with divergent characteristics were obtained through participation in these surveys. Findings have been analyzed for individual studies and collectively as components of an overall program. Attention has been directed toward contrasting findings within and between groups in an effort to better understand the relative significance of contributory factors in the complex etiology of oral diseases—particularly dental caries and periodontal diseases.

Results of work during 1962 on this continuing program provided additional information on the occurrence of oral diseases and corroborated and expanded previous concepts. To date, results indicate that prevalence of dental caries varies widely. High prevalence levels, similar to those reported for the continental United States, were seen in the larger villages of Alaska, throughout Trinidad and in most areas surveyed in South America. Conversely, a very low attack rate from dental caries was observed in Ethiopia; an average of less than one tooth per person being affected at all ages up through 40 years. Dental caries was almost as rare in Eskimos of remote Alaskan villages, in South Vietnam, in Palestinian refugees in Lebanon, and in Burma. Preliminary analyses have elicited no consistent relations between dietary or nutritional findings and dental caries findings beyond general tendencies for low caries experience to be found associated with marginal caloric intake and limited use of simple sugars. Inhibition of dental caries was seen in a series of populations with adequate intakes of fluoride. There was evidence that fluoride was fully effective at levels below those considered optimum for domestic water supplies in the United States, in populations where children receive little water from fluid milk. Such children, drinking relatively larger quantities, seem to receive an adequate amount of fluoride from water containing relatively less of the fluoride ion.

Generally, periodontal diseases constituted a much greater problem than dental caries in most of these populations. Disease levels lower than those commonly observed in groups within the United States were seen only in remote areas of Alaska and in the Jivaros of Ecuador. Gingival disease with relatively little tooth loss from this cause was reported from Ethiopia. Elsewhere the onset of periodontal diseases was early and advanced destruction was common even in young individuals. In adults, prevalence approached 100 percent and severity was extreme. Despite a favorable dental caries experience, as many Lebanese as United States citizens were edentulous in middle life due to tooth loss from destruction of supportive tissues. Equally high levels of disease were seen in Trinidad and the three countries

of Southeast Asia—Vietnam, Thailand and Burma. As yet, no consistent patterns of association between nutrition and periodontal diseases have been developed from these data.

Fluorine and Dental Caries

The pioneer study of the practicality of domestic water fluoridation was initiated in Grand Rapids, Michigan, beginning in January, 1945. This study was intended to determine if controlled fluoridation of water would result in the same inhibition of dental caries as seen in persons using water containing natural fluoride. Efficacy of fluoridation was obvious after a few years of observation. The final field examination required by design of this study (a survey of dental fluorosis in children aged 12–14 years) was reported this year. Less fluorosis was found than had been predicted on the basis of observations on children using water with comparable amounts of natural fluoride. Findings from this study support the original estimates of the safety of controlled fluoridation at the recommended level.

After the efficacy of fluoridation became apparent in the Grand Rapids study, a corollary investigation of the fluoride-dental caries relationship was begun to determine in what manner the inhibition of caries was affected. This was designed on a longitudinal basis. Examinations were to be conducted for a period of ten years following fluoridation of water in Prince Georges and Montgomery Counties, Md., in January, 1952. The 10th year examinations were completed this past March. Repeated observations of the same children have indicated that most of the assumptions accepted from conventional cross-sectional field study were valid. Teeth in eruption at the time of fluoridation continued to decay at essentially the same rate as before. Attack rates were less on smooth surfaces of teeth fully calcified, but not in eruption, at the time of fluoridation. However, there was little effect on development of pit-and-fissure lesions in these teeth. Caries of all tooth surfaces was inhibited in teeth still undergoing calcification at the time of fluoridation. Detailed analyses of data from this study are continuing. Results are expected to yield a family of findings ranging from actuarial-type tables which will permit estimation of the impact of fluoridation upon public health dental programs for children, to inferences concerning the sequence and mechanics of development and calcification of teeth.

Another study of the effects of fluoride on dental and oral health is currently in progress. This was designed primarily to investigate the epidemiological characteristics of periodontal diseases and the relation of these diseases to deposits of oral debris and calculus in children using fluoride free and fluoridated water. Additionally, information will be obtained on the action of fluoride during the formative periods of the teeth.

Other Studies

The hypothesis, described previously in this report, that dental caries is a specific, infectious and transmissible disease in laboratory animals raised the obvious question of a parallel process in humans. Inasmuch as micro-organisms associated with the carious process in animals were shown to be inhibited by adequate concentrations of penicillin, a pilot study was begun on young rheumatic fever patients to determine the effect of a daily regimen of penicillin instituted prior to eruption of the permanent teeth. Findings, to date, show that dental caries experience in these children is importantly less than in public school children of the same community who had not received antibiotics. However, some lesions of caries were observed in the study children. Possibly, drug concentrations were inadequate to completely suppress the contributory effects of the oral flora to the carious process. Plans for further field study presently are being formulated to better evaluate these findings.

As reported by the Laboratory of Biochemistry, animal studies have suggested that, under certain conditions, phosphate may be an effective inhibitor of dental caries. A clinical trial of the effects of dental caries of a phosphate containing dentifrice is in progress in Pennsylvania. The study, not yet yielding definitive data, was designed for a term of 2 years, and involves a minimum of three clinical and X-ray examinations.

An epidemiological investigation of possible relations between variations in anatomical characteristics and orthodontic problems was also initiated during the past year. On this project, efforts have been directed toward development of criteria to describe prevalence and severity of occlusal anomalies in populations and to use these criteria to elucidate relationships between the factors un-

der study. Clinical examinations have now been completed on a selected group of young adult males and a report of findings is in preparation.

In addition to direct research activities and biometric services to our professional staff, a considerable amount of time was spent in consultation on the design and conduct of field studies being undertaken by others, particularly research grantees of the Dental Institute.

CLINICAL INVESTIGATIONS BRANCH

Seven years ago, when the Clinical Investigations Branch became a functional unit of the National Institute of Dental Research, the major attention was given to investigating the common oral and dental diseases which were considered of immediate importance. The studies involved, for the most part, the purely categorical dental diseases, such as caries, periodontitis, and certain prosthetic conditions. Soon thereafter, studies in oral biology, systemic physiology, human genetics and, finally, basic studies in oral and pharyngeal development and function were added. These studies involve basic anatomy, neurology, general pathology, and many other of the medical disciplines which have as their ultimate objectives the understanding and control of oral and dental disease.

For the purpose of this report, the activities of the Branch can best be described in four general categories: (1) studies involving oral surgery and oral medicine; (2) studies in human genetics; (3) studies in oral and pharyngeal development and function; and (4) dental services provided to the total National Institutes of Health's program.

Oral Surgery and Oral Medicine

Research projects on the oral diseases include those concerned with the teeth, such as caries and erosion, pulpitis and periodontal disease, and those concerned with pathological conditions of mucous membranes and underlying oral structures, such as aphthous and herpetic stomatitis, leukoplakia, lichen planus, and desquamative gingivitis.

It is recognized that one cannot dissociate the health of the oral structures from the health of the entire body, and that one cannot dissociate the health of the individual patient from that of the family and the social and genetic community from which he has come. It is also recognized that clinical investigations should include related laboratory studies on physiological and biochemical mechanisms, as well as studies of similar disease conditions in model animal systems.

A brief review of the activities in the broad program of oral surgery and oral medicine follows:

(*a*) *Dental Caries*

There is under way a comprehensive clinical and laboratory study of the multiple factors involved in the etiology of rampant dental caries. This study has included the use of advanced instrumentation for making intraoral observations and measurements of the caries process and the evaluation of predisposing systemic and familial factors in children with this type of caries. Concurrently, multifactorial experiments have been carried out in laboratory animals to determine the effects of the strain of the animal, the cariogenic properties of various diets, and the cariogenic and infectious potentialities of specific types of oral bacteria derived from patients with rampant caries. The fluorescent antibody technique has been used to trace specific microorganisms. Although the study deals with the etiology of dental caries in all of its complexities, it is hoped that a clarification will evolve of the relative importance of different basic factors concerned in the caries process, with particular reference to the host-parasite relationship as affected by diet.

As more knowledge is gained on the parasitic and cariogenic properties of different microorganisms, particularly in tracing specific organisms using the fluorescent antibody method, a more spefic control of potentially cariogenic microorganisms may be developed.

(*b*) *Studies on the Human Dental Pulp*

This project is continuing to evaluate the response of the human dental pulp to changes induced by dental drilling procedures 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 amalgam.

Because of the delayed and reduced inflammatory response of the pulp following high speed cutting technics, the time lag period for the production of reparative dentin has been greatly pro-

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longed. 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 slow response of the pulp to repair itself 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 and to more effectively seal the dentinal tubules are being sought, as well as drugs and technics to reduce the lag period in which reparative dentin is formed.

(*c*) *Periodontal Disease Investigations*

Animal experiments to assess the effect of skim milk and cornstarch on the production of calculus have demonstrated that it is the carbohydrate per se and not the caloric factor which is responsible for the calculus producing effect. Further studies will evaluate the effects of salivary gland and pituitary gland extirpation. Although the exact etiology of calculus formation is not known, these studies are significant in that it is accepted that once calculus has formed it becomes a very important factor in intensifying periodontal disease.

In a more practical approach to certain periodontal problems, findings to date indicate a significant effectiveness of positive pressure appliances for postoperative control of dilantin gingival hyperplasia and recurring gingival hemangiomas.

Other studies on the occurrence of periodontal disease in subhuman primates (the gorilla) have shown a prevalence as high as 76.4%, in a series of 300 skull specimens examined at the Cleveland Museum of Natural History. The most characteristic change was a peculiar and severe vertical type of bone loss, presumably resulting from a confluence of increasing horizontal resorption and a fenestrating type of alveolar bone resorption.

In order to establish a system for correctly evaluating the three dimensional aspects of the periodontal tissues microscopically, another investigation is seeking to describe a group of so-called "periodontal surface profiles" in the hope of increasing the knowledge and understanding of the periodontium in both normal and diseased conditions. Efforts are also being made to document histologically the essential criteria of periodontosis.

(*d*) *Anesthesia Studies*

A collaborative study with the Clinical Center's Anesthesiology Department of 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 almost 5 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 with the exception of Fluothane, which drug is next on the schedule for study.

Among the more significant findings from a practical standpoint are:

(1) Consistent hypertension in all ambulatory anesthesias, which directly parallels the intensity of the surgical stimulation;

(2) Preoperative and operative tachycardias in almost 100% 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).

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 and the dental profession in general.

(e) *Studies of Soft Tissue Lesions*

1. Recurrent Aphthous Stomatitis: Study of this fairly common and painful disease of unknown etiology is continuing in an attempt to determine possible relationships with bacterial in-

fection, abnormal body metabolism, iron deficiency anemia or hormonal imbalance. A recent finding of significance is the presence of PPLO (pleuropneumonia like organisms) not only in the oral tissues of several patients suffering with the disease but in the blood stream as well. This promising direction of study, in collaborataion with the Division of Biologics Standards, may provide additional leads as to the role of bacteria in such other conditions as desquamative stomatitis and erosive lichen planus. Therapeutic investigation is also under way on all these stubborn and resistant chronic debilitating diseases.

2. Mucus Membrane Changes Associated with Age and Certain Diseases: 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 to eliminate errors in diagnosis due to the age factor. Related studies are providing a description of histopathological changes associated with systemic disease including amyloidosis and multiple myeloma, and an evaluation of mucosal changes following systemic chemotherapy.

3. Postsurgical Tissue Healing: There is some recent evidence in the literature to suggest that, under certain circumstances, removal of impacted and third molars is followed by irreparable damage to the periodontal and soft tissues of adjacent teeth. This damage relates specifically to such factors as type of impaction, presence of preexisting disease around the second molar, and age and physical condition of the patient. Findings to date promise to provide important information to assist the oral surgeon in making a judgment of postoperative risk whenever he advises the removal of an impaction.

Human Genetics

The major new project of the Human Genetics Section undertaken in 1962 was a study of the genetic causes of deafness (in collaboration with the National Institute of Neurological Diseases and Blindness, the Clarke School for the Deaf in Northampton, Mass., and the Bionetics Research Laboratories). Material for analysis consists of genetic and audiometric data on the pupils and alumni of the Clarke School accumulated over a 40-year span. To date, physical, dental and laboratory examinations have been completed on 530 subjects. While representing only a part of the total to be examined, significant physical differences associated with certain forms of deafness are already apparent. Clinical thyroid abnormalities occur in about 10% of the adult alumni and in 6% of the pupils, ages 5 to 17 years. There also is a significant increase in the frequency of nontasters for PTC among the hereditary deaf, but not the sporadic deaf. Lack of response to the cold caloric vestibular function test is most frequently associated with a history of meningitis.

Data from this and other studies are being processed by an automatic data processing program, SEGRAN, designed and programmed by the statistical activity of the Human Genetics Section. This program represents a major advance in handling genetic data and has widespread application to some of the more complex segregational problems in human genetics.

Studies on the genetically determined constituents of saliva and their relation to normative traits and disease states have overcome some of the major technical difficulties inherent in this area of research. Reproducible electrophoretic patterns of the electrophoresable salivary components have been obtained. One major finding indicates that secretor factor is produced differentially by the various salivary glands, which probably explains failures of previous genetic studies on whole saliva to yield information relative to secretor titer behaving according to genetic theory. A second major advance is the production of an antiserum system in rabbits immunized to human parotid saliva that reacts with some but not all human parotid saliva. This newly discovered genetically determined system appears to be a common polymorphism affecting about 40% of the population. Its significance relative to disease states is being investigated.

Study of hereditary biochemical defects associated with disorders of speech, hearing and mastication have opened up a new concept of speech problems.

These studies were conducted with the cooperation of investigators of the National Institute of Arthritis and Metabolic Diseases, who described a specific biochemical defect, histidinemia, that is associated with a speech defect but with no other apparent clinical abnormality. The finding by this Section of a similar situation in Sjogren-Larsson syndrome, where in addition to speech defects

there is an associated disorder of mastication, is being intensively investigated because these disorders are theoretically treatable. Hereditary renal dysfunction and deafness appear associated with abnormal serum lipoproteins, but the basic biochemical defect has not been defined.

Cytological methods have now been worked out for the rapid smear technique to assist in the diagnosis of a number of nonneoplastic oral lesions including pemphigus, hereditary benign intraepithelial dyskeratosis, white sponge nevus, Darier's disease, herpes, PPLO infections of the periadenitis mucosa necrotica recurrens type, and a cytological method of following the course of cancer therapy with methotrexate, 5-fluorouricil and 5-fluorodeoxyuridine.

By utilizing the two-dimensional peptide technique (fingerprinting), no difference could be demonstrated in the peptide patterns of fetal and adult myoglobin similar to that seen in these hemoglobins.

Studies in population genetics and the epidemiology of genetic disease have contributed descriptions of new populations and practical knowledge about blood group distributions in Chileans useful in disaster work. A knowledge of blood group distribution is imperative for the efficient collection and supplying of blood for emergency transfusions.

The first nationwide survey of a representative sample of Chilean civilians and military personnel has determined the frequency distribution for some 26 red cell antigens. Utilizing these data, comparisons were made with nutritional findings, dental caries severity and periodontal disease. No significant correlations could be found between blood phenotype and these states. However, a significant excess of persons with abnormal oral mucosa (scrotal tongue) were blood group O; and in addition a significant excess of persons with abnormal oral mucosa (scrotal tongue) were low or deficient in serum vitamin A. By utilizing a number of genetic markers, including haptoglobins, a new method of calculating genetic distances between population was devised.

A new isoantigenic system in baboons that cross-reacts with some but not all other baboon sera and with some but not all human sera has been described. This finding and the finding that two human sera that contained high precipitating serum isoantigens indicate that these systems may be equally important as the cell antigen systems in determining cross-reactions in blood transfusion problems.

A significant excess of persons with enamel hypoplasia has been found in sibships from ABO and Rh incompatible matings who did not present the overt signs of severe neurological damage (kernicterus). This important finding, coupled with the observation that there is a 10% fetal loss of type A children from A father x O mother matings, forms the basis of a part of the Montgomery County study to ascertain if the surviving 90% of children from such incompatible matings may show a lesser degree of damage such as mental retardation, dyslexia, and dysarthria.

Studies of histidine metabolism have definitely identified urocanic acid as a urinary metabolite in man. Methods for the quantification of urocanic acid and α-formiminoglutamic acid in human urine were described. Both urocanic acid and α-formiminoglutamic acid excretion are increased after oral histidine loading in normal control subjects and in patients with hepatocellular disease. The response to loading in patients with hepatocellular disease is exaggerated.

Experiments with loading albinos with tyrosine, studies of *in vitro* melanin production, and genetic studies indicate that there are two forms of recessive albinism in man. In the more common form, albinos have tyrosinase activity. In the rarer form, tyrosinase may be lacking or inactive. Pigmentation can be induced in the skin of the former type, but not on a therapeutic level.

The tooth defect in a rare form of vitamin D-resistant rickets was found to resemble that seen in hypophosphatemia, consisting of marked interglobular dentin formation and microfissures traversing the full thickness of dentine from enamel to pulp. This results essentially in a pulp exposure and abscess formation around clinically normal-appearing teeth. The presence of multiple abscesses around clinically-normal appearing teeth may be one of the first signs of this disease.

Mutation rates have been estimated in man for the gonadal dose from a full mouth series of X-rays. Based on the findings of three independent studies by others that the gonadal dose from a full mouth X-ray series using modern techniques is about 0.0016r, germinal mutation rates were calculated using the best estimates available for the number of mutable loci in man and the mutation

rate per r. If both parents of 1 million children received a full mouth X-ray series, we could expect that from 0 to 400 of the children would carry an induced mutation of any type, if these estimates are correct. As some of these mutations would be lethal *in utero*, even fewer children would show induced defects. This additional genetic load (0 to 400 mutations) was then compared to the additional load expected as the result of treatment of a single genetic disease (dentinogenesis imperfecta) before marriage. In 1 million U.S. children, about 125 would have dentinogenesis imperfecta. By treating all cases prior to marriageable age, 64 additional cases would be added to the next generation as the result of treatment of the parents. It appears that early medical treatment contributes a substantial number of deleterious mutations to subsequent generations and that the potential number of deleterious mutations contributed as the price for X-ray diagnosis is comparatively small.

Chromosomal satellites which have been thought to be associated with certain abnormal body constitutions have been studied in 40 normal subjects.

Approximately one-third of these have shown detectable asymmetry of satellite bodies. A family with an abnormal number one chromosome is being followed for linkage and other studies.

Studies to determine the nature of the mitotic stimulatory effect known to occur when extracts of kidney bean are added to human lymphocyte cultures have made possible a new technique for obtaining a relatively pure suspension of lymphocytes. In addition, a method to assay the mitotic stimulatory properties of phytohemagglutinin has been devised. Morphological variation of the Y chromosome in various strains of hamsters has been described, thereby ending a taxonomic dispute.

In collaboration with the National Institute of Allergy and Infectious Diseases, a particularly important study has been initiated. Simian vacuolating virus (SV–40), when introduced into hamsters directly will, after a period of time, induce tumors. If introduced into hamster cells in culture, these are transformed into malignant cells which, when reimplanted in the hamster, will cause a rapidly developing fatal tumor. Studies on virally transformed cells have shown chromosomal changes induced by the SV–40 virus to be indistinguishable from chromosome changes found in spontaneously occurring malignancies.

It is to be noted in the foregoing account that studies on gene, virus and chromosome lead from a fundamental scientific basis into all reaches of human biology, and that discoveries and techniques in genetics are useful in virology and infectious disease, metabolic disease and congenital malformations. Accordingly, these several areas are inseparable.

Studies on inbreeding effects of dental characteristics among Japanese children indicate that although there appears to be an increase in periodontal disease with increased inbreeding, the results are inconclusive. Inbred children appear to have more malocclusion than outbred, but no consanguinity effect was noted for tooth eruption status, caries, congenitally missing teeth or supernumerary teeth. Abnormalities of tooth number, both supernumerary and congenitally missing, were more prevalent in Japanese than in other reported populations. No clear-cut simple mode of inheritance can be established for supernumerary teeth. Positive associations were found to exist between congenitally missing permanent teeth. An association was found between tooth size and a predisposition to "missing" or "extra" teeth. No association in these data could be found between febrile disease in early childhood and enamel defects.

During the past year the Human Genetics Section had 30 scientific articles accepted for publication, including a book on dental genetics, 4 book chapters, and 25 publications in periodical literature. Sixteen of these have been published to date.

Oral Pharyngeal Development

During the past year, the Section on Oral Pharyngeal Development and Function has undergone progressive stabilization and accumulation of personnel, equipment, methods and clientele. Its orientation to the professional variety of its personnel has progressed well and with significant profit in development of concepts. To date, the complement of professionals in this activity includes competency in orthodontics, anthropology, speech, etc. The investigations of each of these persons is concerned with form and function of the mouth, pharynx and larynx.

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Activities

The implementation of studies related to feeding, vocalization and speech is now nearly completed, with the installation of instruments in our working areas and the construction of a sound-controlled room for speech research. In cooperation with the Clinical Center Diagnostic Radiology Department and the Photography Department, sound-correlated cineradiographic and cinephotographic studies of the pharynx and mouth performances are under way.

The patterns of skeletal development in the face and adjacent cranium are under study in a variety of mammals. By methods of vital staining with a succession of dyes, the successive sites of bone formation can be distinguished. These sequence patterns of skeletal form development are demonstrated in normal rats and rabbits, and methods of imposing skeletal distortions upon these immature animals are now under exploration. More detailed studies have been initiated to investigate the mechanisms that determine skeletal form. These include observations on the growth of limb bones of chick embryos in organ cultures, and the study of histological changes of bone undergoing mechanical deformation. Clinical abnormalities of facial and cranial skeleton associated with cleft palate are also under study. During the past year we have undertaken an investigation of skeletal deformation in infants and children having marked degrees of facial deformity. Anatomical studies of infant cadavers demonstrating cyclops, bifid face, cleft palate and Robin's syndrome are now under way. Infants or children having severe cleft of palate, cebocephaly, glossoptosis or laryngospasm have been admitted for inhospital study.

Other related activities include the study of progressive distortions of the skeleton associated with disorders of motor function or resulting from multiple dental extractions.

In a collaborative study with the Karolinska Institute, infant respiratory adaptations at birth, and cry and suckle feeding are being analyzed by extensive cinefluorographic and acoustical methods. Upper respiratory participations in natal transition have been described on the basis of cineradiographic and phonetic recording. The physiological and phonetic performance of cry has also been described in normal infants and the directions of abnormality of cry in neurologically impaired infants have been defined. In comparison with these observations in normal infants and previous studies of normal mature subjects, this investigator group has undertaken physiological studies of speech and feeding actions in a selected clientele of cleft palate infants and children, as well as in the other more severely impaired subjects mentioned above, and in a small number of referred subjects having more common oral or pharyngeal disabilities. The basic methods of these clinical studies are those of cineradiography and acoustical recording. The cineradiographic film is analyzed in a standard manner, with tracing of selected frames. The acoustical record is initially evaluated by ear, and selected portions are portrayed by sonagrams which display frequency and amplitude. Cinephotography, pressure recording and electromyography are employed optionally.

It has become progressively evident to us that the demonstration of motor performance alone is a very incomplete and inaccurate indication of performance. In the oral and pharyngeal region, the motor performance is most intimately related to local sensation since the motions themselves occasion most of the local sensation, modulate these sensations and are modulated by them. Accordingly, basic studies in cats have been undertaken to determine the pharyngeal sensations which modify respiration. This investigation has resulted in a preliminary mapping of respiratory-relevant sensations upon the pharynx; the first recognition that differential sensory representation was present in this area. Initial trials are under way also on the stimulation of epipharynx in anomalous infants and children. Preliminary observations were also made of sensation in the hard palate of the dog.

In parallel with these studies of local sensation and motor function, are studies of the central representation of pharyngeal area functions in the brain stem. To implement this project a neurophysiology laboratory has been equipped for observations in experimental animals of neuronal activity in the central nuclei which govern swallow and related functions.

Other studies in the oral pharyngeal field include an extensive exploration of oral sensation in the human, including introduction of histological techniques for the demonstration of neuroreceptors

in the oral mucosae and development of instruments for mucosal surface sensory testing. In this connection, a variety of simple plastic forms have been devised which are placed in the mouth and recognized by oral manipulation. These "oral stereognosis forms" have been standardized in a preliminary set of ten for detection of gross deficiencies in the sensory-motor function of neurologically mature, anatomically normal subjects. More subtle and discriminate variations of shape and size of the frames are now in design, manufacture or trial. Findings, to date, have shown a remarkable variety of motion patterns in subjects having anatomical differences, and a discrepancy between functional adequacy and anatomical form and proportion in the mouth and pharynx area. As a result, we are coming progressively to the inference and conclusion that the motor mechanisms of tongue and pharynx are currently or potentially capable of adequate speech and other upper respiratory feeding functions in most of the common variety of discrepancies of form. The critical deficit in many persons having minor or moderate disorders of "portal area" performance is that of local sensory elicitation and modulation of these motor functions, along with such central coordinative disorders as may be developmentally related to these elicitation deficits and distortions.

Activities of the Dental Services Section

In the course of the past calendar year, authorization was given to establish a Section on Dental Services in the Clinical Investigations Branch. This organizational change in no way altered or modified the Dental Department's responsibility for providing the research beneficiaries of the categorical Institutes with an optimum level of dental care. Additionally, however, the Section was given a clearly defined function to assure fulfillment of the research potential of the Dental Clinic, Clinical Center, through an effective utilization of National Institute of Dental Research and other National Institutes of Health patients.

That the above objectives are being met is attested to by the Section's contributions to important programs of research in periodontology, orthodontics, prosthodontics and operative dentistry.

In the discharge of its responsibilities to the total NIH research effort, the Section has active collaborations in progress with all the categorical Institutes. The following examples may be cited.

With the National Cancer Institute

Prosthetic devices, although very often complicated and time consuming to produce, frequently are necessary in the postsurgical management of intra- and extra-oral head and neck cancer. Intraoral splints for patients who have had large areas of their oral and masticatory apparatus removed, make a dramatic difference in the recovery of these subjects because of emotional, nutritional, hygienic, and other vital factors which lead to earlier physical and mental rehabilitation. In addition, artificial noses, ears and eyes, of such quality as to defy detection by even the critical observer, are produced for National Cancer Institute subjects in an almost routine fashion.

The Section renders further important contributions to the Leukemia Service of the National Cancer Institute in the handling of the myriad of oral problems associated with the disease, such as bleeding, infection, and necrosis.

With the 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 evaluation of antihypertensive drugs.

All patients with rheumatic heart disease present particular problems. 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.

With the National Institute of Arthritis and Metabolic Diseases

Surgery of any kind on a hemophiliac poses a most serious threat to life. All varieties of drugs, surgical splints and technics have been utilized in an attempt to overcome the hemorrhagic difficulties indigeneous to oral surgical procedures on patients with hemophilia. Plasma in large quantities and immeasurable amounts of professional time have to be expended, even after single extractions. Recently, Fraction I (fibrinogen fraction of Cohn), which has a concentration of AHG

(antihemophilic globulin), has been employed in the surgical management of three hemophiliacs. The first 2 cases involved the extraction of 8 and 11 teeth respectively in single operating sessions while in the third case, 25 teeth, 2 impactions and a generalized alveolectomy were performed in a single operation. In no instances were complications noted other than incidental periodic oozing and the infusion of Fraction I. Results, to date, have been most encouraging for a practical approach to oral surgical problems, trauma and elective general surgery in the hemophiliac patient.

DIVISION OF BIOLOGICS STANDARDS

INTRODUCTION

The Division of Biologics Standards, which has the responsibility for administering the provisions of the Public Health Service Act with respect to the control of biological products, discharges this task as a blend of research and administrative activities which are about equally divided between control and research. The research programs are concerned largely with the basic function of the Division—the control of biological products—although by their very nature, some of these activities could well be classified as basic or fundamental research. However, no programs are undertaken initially unless they have direct bearing on the responsibilities of the Division. The activities of the Division—both control and research—are therefore product-oriented, and their scope, direction, and intensity are dictated by the need to provide essential information for developing requirements and regulations for the licensing and release of biological products. The Division research program is vital in this respect.

It is characteristic of the type of responsibility which the Division has, with its ultimate objective the protection of the public against unsafe and uneffective biological products, that the Division is confronted with a series of problems which are often never completely solvable. The successful accomplishments of the Division reside in those instances where difficulties are anticipated, headed off, or abated. It is necessary to maintain an active scientific interest in products which have long since been routinely accepted by the medical and health professions, and in some instances even forgotten by the majority of scientists working within the field. The Division can never move its area of interest completely or abandon a concern for any of the products which remain licensed. Small pox vaccine is an excellent example.

During 1962, the Division faced problems similar in pattern to those encountered in 1961. Live poliovirus vaccine, types 1 and 2, were licensed in 1961, but the problems encountered with type 3 and which delayed its licensing were carried over into 1962. The type 3 strain is genetically the most unstable, and the major difficulties concerned the consistent production of consecutive lots of vaccine which would meet the requirements for a low degree of neurovirulence in monkeys as well as the specific marker characterizations required in tissue culture. The availabiliy of only two of the required three types of live poliovirus vaccine severely limited the large scale immunization programs which had been visualized by health authorities and local medical groups. However, many of these programs were started in anticipation of the eventual licensing of the type 3 vaccine which took place on March 27, 1962. During the latter part of 1962 the safety of the type 3 vaccine was called into question by the occurrence of a few cases of poliomyelitis in association with the administration of type 3 vaccine. This situation which was examined thoroughly over a period of some months by the Public Health Service and its advisors was finally resolved with the report by the Surgeon General in December 1962, which in effect stated that there was a minute hazard but that this appeared to be confined to persons over 30 years of age. This period of time was a difficult one for the Division. Even though the main type 3 problem has now been solved, the control of this vaccine calls for special vigilance.

The monkey neurovirulence tests required for all three types of live poliovirus vaccines involved extensive studies. More than 150 neurovirulence tests have been made in more than 4,000 monkeys since the first vaccine samples were submitted to the Division for testing by the manufacturers. This testing has provided a wealth of information and experience that is invaluable in judging the validity of individual tests.

Research, testing, and the development of standards in connection with measles vaccine was a second item of major concern during 1962, and by the end of the year, most of the necessary work

had been completed to the point that the general availability of measles vaccine as a licensed product appeared to be only weeks or months off.

In the development of requirements for measles vaccine, the Division has drawn on its experience with other vaccines grown in tissue culture, the most comprehensive of which was with the poliomyelitis vaccines. However, since each virus has its own characteristics, and the properties of live vaccines depend on such factors as how the vaccine virus enters the body, where it proliferates, and what elements—mechanical, physiological, immunological, and virological—favor or inhibit its ability to "take," one vaccine is not truly comparable to another.

The requirements cover the characteristics of the measles virus strains to be used in the vaccine; the exclusion of adventitious agents from the virus fluid used in manufacture; the employment of a variety of animal and tissue culture test methods; the demonstration of satisfactory clinical use of the vaccine, including clinical effectiveness; and the stability of the live virus vaccine strains in terms of capacity to revert to significant virulence for man.

As with all new biological products, the information most difficult to obtain concerns the stability of the product under conditions of actual use, yet without this information a realistic dating period cannot be set. In this regard field studies such as those conducted in Upper Volta are of great value.

An important need in the testing of measles vaccine is a supply of monkeys that are susceptible to measles virus. While monkeys are susceptible to measles infection, they are not exposed to it under natural conditions. However, during procurement and shipping, they readily develop an imperceptible measles infection on contact with infected monkeys or human beings. As a result, almost all of the animals have developed resistance to measles infection by the time they reach the laboratory. This renders them unsuitable for many of the tests required in the evaluation of the vaccine. Therefore, special procurement, handling, and transportation of these animals are essential, as well as special quarantine and conditioning requirements prior to and during their use in measles vaccine studies.

A difficult problem encountered in working with tissue culture produced vaccines is the occurrence of adventitious viruses, many of them previously unknown. Their presence in the tissue cultures used for production of viruses represents a complicating factor in production and testing procedures. Since their role in the cause of human disease is rarely demonstrable, the only course to pursue is to exclude them from the final product insofar as is possible with available testing methods.

Experience with the simian virus, SV–40, sharply emphasized the problem of viruses that are unidentifiable by tests normally employed. Although no relationship of SV–40 to the development of human disease has been demonstrated, this virus is associated with the production of tumors when inoculated into baby hamsters and, under certain conditions, tissue cultures infected with the virus show changes that suggest a tumor-producing potential. In order to insure that this adventitious agent would be excluded from commercial vaccines, new methodology was developed and adequate test procedures were designed as a result of extensive research conducted by members of the Division staff.

The production of measles virus in chick embryo tissue culture also presents a problem since a number of adventitious agents are known to be present in fowls. Although vaccine produced in the developing embryo of the hen's egg is well tolerated by human beings as demonstrated by the large-scale use of yellow fever, typhus, and influenza vaccines for more than 20 years, analogous experience with chick embryo tissue culture is limited.

One approach towards the resolution of some of the testing problems encountered with adventitious viruses is the development of continuous cell line tissue culture preparations which would obviate the use of primary cell cultures. Such a cell line has been initiated by the Division using kidney tissue of the African green monkey. The cell line is now at a high passage level and its continuous growth is considered to be established. These cells have not changed their sensitivity to SV–40 virus, and are sensitive to attenuated measles virus as well as a variety of other viruses. Continuous cell lines while useful for test purposes are not used at the present time for the production of virus harvests to be used in the actual manufacture of virus vaccines. The recent report of an NIH Committee on Tissue

Culture Virus and Vaccines on this subject should clarify the direction of future research toward this end. (Science 1963, 139, 15–20)

During the past year a number of variants of the influenza virus strains have appeared in this country. In cooperation with other interested laboratories, the Division has paid close attention to these variants with a view to the possible selection of an additional virus strain for inclusion in the vaccine now in use. The last change was in 1957 when the Asian strain of influenza virus spread throughout the world. By December 1962, it appeared that the incorporation of new strains in the vaccine would again be called for and appropriate studies were initiated by the Division in cooperation with manufacturers and specialists in this field.

Industry continues to attempt to meet the demand by the medical profession for multiple antigens that will immunize against several diseases simultaneously, although the preparation of these products is a complex process. This is especially true of the so called quadruple antigen vaccine which is used in pediatric practice. This product is composed of diphtheria and tetanus toxoids combined with pertussis and poliomyelitis vaccines. Studies conducted by the Division on the stability of this product revealed that there was a significant decrease in the potency of the pertussis component during the prescribed dating period. The reasons for this are obscure. However, as a result of the investigations, the dating period for this product was shortened and the potency requirement of the pertussis component increased in order to ensure that the potency of the pertussis component does not fall below the required minimum level even when the product is exposed to variable market conditions. Manufacturers are attempting to meet this problem by bypassing it. Double chambered syringes and dual packaging of the components are being offered.

The shortage of adequate laboratory and animal space continues to be acute for all activities within the Division. It is not anticipated that this will improve until the new facilities now in the planning stage are occupied, although the remodeling of animal quarters which have become available in Building 14 should help somewhat when this job is completed during the latter part of 1963.

A great deal of time and effort has been expended during 1962 in the investigation connected with alleged violations of the Biologic Law in the sale of plasma and whole blood. These investigations, carried out under direction of the U.S. Department of Justice continued to consume the time and resources of the Division's Laboratory of Blood and Blood Products. It is anticipated that these cases will come to trial early in 1963.

The recruitment of suitable personnel, particularly senior scientists willing to engage in the type of scientific work presented by the Division and willing to accept the kind of responsibilities involved, continues. The anticipated retirement of a number of senior staff members in 1963 will present additional difficulties in this respect.

The following summaries of the programs of each of the laboratories present more details of the activities of the Division.

LABORATORY OF CONTROL ACTIVITIES

This Laboratory is responsible for activities dealing directly with licensed establishments in relation to the licensing and control of biological products. It is supported by sections on control tests, pyrogens, and reference standards.

Its activities include:

(a) Determination of eligibility of establishments and of individual biological products for license. This determination is made on the basis of the integrity of management and technical personnel, the physical facilities for manufacturing and testing of products, the scientific and professional qualifications of personnel and the evidence developed by manufacturers and the Division of continued safety, purity, and potency of products, for which an application for license is being evaluated. License applications are reviewed individually when required by an *ad hoc* committee consisting of appropriate members of the staff of the Division.

(b) Supervision of annual and special inspections of licensed establishments and of those for which an application for license has been made.

(c) Releasing of individual lots of biological products for distribution by manufacturers on the basis of review of manufacturers' and of DBS tests and of any other available information relating to the safety, purity, and potency of the lot of the product.

(d) The establishment and distribution of physical biological standards, reference preparations, and control materials. A small culture collection

is also maintained mainly for the Division and for licensed manufacturers.

(e) Review of requirements and regulations now in effect for such constructive revision as needed and the development of requirements and regulations for new products.

(f) Maintenance of close working relations with other laboratories of the Division and other agencies to insure continuous knowledge of information needed for the licensing of establishments and new products and for the testing, release, and control of products already licensed.

The scope of activities carried out by this Laboratory is indicated by the fact that during the 12-month period, December 1, 1961–November 30, 1962, a total of 7,007 control tests were carried out to insure the sterility, safety, potency, and purity of licensed biological products as follows:

	Tests
Productions for release	6,103
Inspection samples	886
Complaint investigations	18
Total	7,007

These results served as a basis for the release or rejection of individual lots of products. In addition, 1,193 cooperative service tests were done on biological products not licensed.

During the same period 5,041 lots of biological products were submitted for release by licensed manufacturers. Of these 4,915 lots were released, 56 lots rejected, and 70 lots withdrawn from consideration for release by manufacturers.

To maintain an adequate supply of physical reference standards for use by the licensed manufacturers in their official control testing, it is necessary to prepare and standardize new liquid lots from the primary dried stocks. The number of lots prepared and standardized during the year were: antitoxins—19, serums—5, vaccines—1, toxoids—3, and toxins—3. A total of 701 tests was required to complete a satisfactory standardization of these lots. These include flocculation reactions, animal protection tests, animal potency tests, and a number of specialized tests for specific products.

Standards, reference preparations, and cultures are freeze-dried for greater stability during storage. The following were dried between November 15, 1961, and November 15, 1962:

	Ampules
Cultures	1,340
Serums	1,407
Vaccines	1,837
Viruses	2,630
Toxins	2,146
Total	9,360

Official standards, reference, and control preparations currently maintained include 75 items.

Standards, reference preparations, and cultures were distributed to research or control laboratories of licensed and other manufacturers, health departments, and universities in this country and abroad as follows:

Antitoxins	500
Serums	1,861
Vaccines	883
Toxins	356
Bacterial and virual cultures	2,271

LABORATORY OF BACTERIAL PRODUCTS

During 1962, the Laboratory was able to increase its research activities with the addition of scientific personnel. With the available space being used to capacity with 2 to 4 persons per room, an expansion of the program to cover bacterial products which need investigation must be curtailed until more space is available.

Allergenic Products

Dr. Harold Baer and his associates have made considerable progress in their studies on tuberculin and poison ivy extracts. Both carbohydrate and protein fractions which have been separated from tuberculin elicit skin reactions in sensitized guinea pigs. The carbohydrate fraction shows a greater specificity in homologously sensitized guinea pigs than those sensitized with mycobacteria of other species.

The skin reactivity of poison ivy extract and pentadecylcatechol, a reactive component, on sensitized guinea pigs has been shown to be directly related to the amount of the catechols as measured by gas chromatography. If these results are successfully correlated with human reactivity, tests of which are anticipated in the immediate future, sufficient information will be available for the establishment of a U.S. Standard of Potency for poison ivy extracts and the designation of a standard reference preparation. Fundamental infor-

mation on sensitization and blocking of sensitization is being obtained.

The liaison activities with the Committee on Standardization of Allergens (NIAID) of which Dr. Baer is a member continue. It is anticipated that current work of investigators on ragweed pollin will furnish basic information that will be applicable to the establishment of standards for ragweed products.

Bacterial Toxins

The addition of Dr. M. Carolyn Hardegree to the staff has strengthened the work on tetanus toxoids. The experimental work on potency assay of toxoids and the correlation on laboratory measured potency with field response in pregnant native women in the Territory of Papua and New Guinea for the prevention of tetanus neonatorum is well underway. A project has been initiated on the study of the toxins of *Clostridium tetani.*

The work on the Schick test toxin (diphtheria) and the revisions of the requirements for this product should be completed early in 1963.

Much of Dr. Michael Barile's work is devoted to the study of pleuro-pneumonialike organisms. He has formulated the test to be used for the detection of the presence of PPLO in measles virus vaccine and is currently engaged in screening lots of the vaccine submitted in support of license applications. He cooperated in a study with NIAID in which the Eaton agent was shown to be a PPLO.

Bacterial Vaccines

The cooperative study with the World Health Organization on typhoid vaccine is proving to be very profitable. It has been found that the route of vaccination of the mouse significantly affects the measured relative potency between the field trial vaccines which are prepared by different methods. The acetone-killed-dried vaccine in the field has given excellent protection which is three or more times better than that obtained with the heat-killed-phenolized vaccine. Only by the intraperitoneal route of vaccination has this relation been shown by us in animal tests. The final evaluation of the WHO project will be very valuable in prescribing methods for the preparation and control of typhoid vaccine.

An investigation on the cause of instability of pertussis vaccine in the presence of poliomyelitis vaccine has been initiated. The temporary removal of the quadruple antigen product from the market curtailed the investigation on the adequacy of the revised specifications for the product.

The revision in the toxicity test for pertussis vaccine has resulted in products of lower mouse reactivity without an apparent decrease in potency. The preliminary study with Dr. J. A. Bell indicates that there is a possible relationship between mouse toxicity and reactivity in children. More information is greatly needed.

Cholera vaccine in the mouse is as protective against the El Tor strains as against classic cholera strains. A revision of the requirements for cholera vaccine in which the newly developed potency test will be included is in preparation. The conditions whereby the hemolytic activity of El Tor vibrios can be uniformly determined have been defined by Dr. J. C. Feeley. The test should be helpful in epidemiological studies of cholera.

Non-Research Activities

During the year, 571 tests were performed relating to the control of pertussis vaccine, antipertussis serum and *Haemophilus influenzae* therapeutic and diagnostic sera.

Other regulatory activities included (1) formulation of revised standards for pertussis vaccine and (2) participation in the review of license applications to determine eligibility for establishments and of individual products for licenses, inspection of establishments and liaison activities between the Division and one manufacturer of pertussis vaccine.

A reevaluation of the potency of the U.S. standard pertussis vaccine Lot No. 6 was made and a number of courtesy pertussis-vaccine potency tests were made for reference preparations of national control laboratories and for WHO to determine if the international standard for pertussis vaccine was stable.

LABORATORY OF VIRAL IMMUNOLOGY

Live Polio Virus Vaccine

Work with the oral, live poliovirus vaccine has continued at an accelerated pace during the past year. Not only did new production laboratories make their appearance, but work with the type 3 virus was expanding. When the data had been received and analyzed, new licenses were issued to Pfizer, Ltd. of Sandwich, England, for type 3

Poliovirus Vaccine, to Lederle Laboratories, Pearl River, N.Y., for type 1, type 2, and type 3 Poliovirus Vaccines and to Wyeth Laboratories, Marietta, Pa., for type 1 and type 2 Poliovirus Vaccines on March 27, 1962. This was followed by the issuance of a license for type 3 Poliovirus Vaccine to Wyeth Laboratories on May 17, 1962. The pressures for vaccine to implement springtime immunization programs meant that the control facilities were strained to the limit. The need for serums with high antibody levels to permit the detection of SV–40 in preparations containing at least $10^{7.0}TCID_{50}$ of poliovirus per milliliter was met by concerted efforts of the staff of this laboratory. By early September when most of the demands had been met, analyses of the surveillance data showed that disease had occurred in some persons, mostly adults, who received the type 3 vaccine. This situation required action by the Surgeon General, who advised that the type 3 vaccine henceforth be given only to children. This action halted the release of all type 3 vaccines after September 6, 1962, and served to stimulate increased production of the Salk Vaccine by certain manufacturers.

Adenovirus Vaccine

The situation with respect to adenovirus vaccine remained quiescent during the period of this report. All of the manufacturers were attempting to prepare seed viruses free of extraneous viral agents. This work proceeded very slowly and only by the end of this period were reports received indicating that some success was being attained.

Immune Serum Globulin

Methods for the standardization of immune serum globulin preparations were worked out and surveys of antibody content of a number of preparations permitted the draft of regulations for their products. This draft is now under discussion by scientists of various interested laboratories.

Measles Vaccines

The appearance of measles vaccine, both the living, attenuated and the killed virus preparations, on the horizon of licensed biological products, brought rearrangement in testing responsibilities. The killed virus vaccine was added to the program of both the tissue culture section and the pathology section. The live, attenuated virus vaccine was made an additional responsibility of the pathology section. Increases in personnel in both these sections are now being implemented in order to meet these new duties.

The control program for poliomyelitis and adenovirus vaccine involved the following examination and release actions:

	Poliomyelitis vaccines	Adenovirus vaccines
Samples received	76	11
Vaccines released	58	11
Combined with other antigens	26	0
Vaccines rejected	0	1
Vaccines withdrawn by manufacturer	7	0

The control program for Poliovirus Vaccines, Live, Oral, involved the following examination and release actions:

	Type I	Type II	Type III
Samples received	39	38	42
Vaccine released	31	42	24
Vaccines rejected	1	0	1
Vacines withdrawn by manufacturer	4	1	6
Vaccines submitted for consistency purposes	0	0	2

SV–40

The research program of the laboratory has included work by Dr. Kirschstein and her coworkers who showed that ependymomas were produced in newborn hamsters after intracerebral inoculation of SV–40. In mastomys, however, it was found that ependymomas occurred following subcutaneous inoculation. Additional work with SV–40 revealed that some transformation of normal adult human thyroid cell cultures was produced by the virus. Carrier cultures resulted which had the capacity for limited growth when inoculated into the brains of monkeys. Virological studies by Dr. Gerber indicated that at least $10^{6.5}$ $TCID_{50}$ of SV–40 were needed to produce ependymomas in newborn hamsters. The virus could be recovered from

intact tumor cells but not from extracts of the tumors.

In a study of samples of Salk poliomyelitis vaccine which had been stored at 4° C. for 5 to 7 years, Dr. Gerber found that SV–40 was detected in tissue cultures which were inoculated with 16 of the 32 samples studied. Thus, another demonstration of the remarkable stability of this virus has been made.

LABORATORY OF VIROLOGY AND RICKETTSIOLOGY

Again this year the Laboratory of Virology and Rickettsiology undertook a wide variety of studies ranging from field work centered around the first attempt to eradicate measles from a nation to exploratory studies on natural defense mechanisms which assist the infected cell in eliminating intracellular pathogens.

Measles

Although licensed live attenuated measles virus vaccine has not yet become available, extensive work has been done in the United States and elsewhere with experimental vaccines produced in commercial facilities. The experience in the field and in the research and biologics control laboratories has accumulated to the point where a licensed commercial product can be anticipated shortly.

The Section on General Virology has made substantial contributions to the development of reference standards and procedures applicable to the biologic control of both live attenuated measles virus vaccine and inactivated measles virus vaccine and of human gamma globulin which may be used along with the attenuated measles virus vaccine in the immunization of children.

The SGV has added materially to knowledge regarding the use of live attenuated measles virus vaccine through its studies in West Africa. These, undertaken as a collaborative venture by the DBS, the Ministry of Health in Upper Volta, and the AID have demonstrated that Voltan children respond in the same manner as American children to the live vaccine, whether given alone or in combination with gamma globulin. Because measles is one of the serious diseases of Upper Volta, with mortality rates running as high as 50% at times, and because the Volta health officers and the people do not object to the mild illness which is common when attenuated virus is given without gamma globulin, plans were made for a large-scale measles vaccination program in Upper Volta using attenuated virus alone in all children under the age of three years. This represents the first attempt to eradicate measles from a nation. The original plan called for the inoculation of 100,000 children. Before the plan became operative in early November 1962, this was raised to 300,000 * on the basis of a new census of the population. At the moment it looks as if the actual number to be vaccinated will be appreciably more than a half million. Vaccine is being administered parenterally by jet injection by teams which comb the country, district by district. Each team consists of three indigenous Voltans employed by the Ministry of Health and is equipped with a jeep, two jet guns, an electric generator for operating the guns, and a portable refrigerator for transportation of the vaccine. Each of the eight teams, which cover a limited area before moving on, is visited every 2–3 days by the DBS investigators (Drs. Meyer and Hostetler and Mrs. Bernheim) who advise and assist in the work. During the first 3 weeks of the mass immunization program over 200,000 children were inoculated. The plan is to complete the work by the first of February 1963, which is the beginning of the annual severe measles epidemic in this region.

The effort to date has been most encouraging. The acceptance of the immunization procedure by the mothers is little short of amazing. When the word goes out that measles vaccination is to be done at a certain time and place, mothers walk in for miles, carrying their children. The populace does not view all immunizations with such enthusiasm. Indeed, sometimes the health officers who arrive for other purposes are chased out of the villages. In the first 200,000 Voltan children inoculated, serious complications have been recorded in only one instance; this was a spreading cellulitis which began at the site of jet injection and, in the absence of medical care, eventually resulted in death. While the ultimate success of the mass immunization program will be judged by the reduction in measles during the forthcoming annual epidemic, it is already apparent that the experience has provided worthwhile informa-

* The number actually given measles vaccine by the time the program was completed in early 1963 was 730,000.

tion on the means by which measles immunization can be accomplished in a developing nation when accompanied by the enthusiasm of the people and of the government of the country.

Before launching the mass-vaccination program mentioned above, the DBS team undertook a second series of pilot studies in which live attenuated measles virus vaccine was used in combination with smallpox vaccine and yellow fever vaccine administered by jet injection. Besides their scientific value, the pilot studies provided an opportunity for the Americans to train the Voltan teams who were to carry out the mass program. The studies indicated that a bivalent vaccine (dried measles and smallpox vaccines rehydrated separately and mixed just prior to connecting to the jet injection apparatus) and a trivalent vaccine (measles, smallpox and yellow fever mixed in the same manner) can be used without untoward effects in Voltan children. Serological tests to determine the response of the children to the mixtures of attenuated viruses remain to be completed. However, it is worth noting that previous experience had indicated that American children responded satisfactorily to a combination of measles and vaccinia viruses and American adults to a combination of vaccinia and yellow fever. If such polyvalent living vaccines prove to be immunogenic and safe, their use would be welcomed in developing countries.

Avian Leukosis Virus Complex and the RIF Test

Studies on the avian leukosis virus complex and Rubin's Rous interference factor (RIF) test were initiated early in the calendar year. In developing the regulations for live attenuated measles virus vaccine, which is prepared from virus grown in chick embryo tissue cultures, a safety test was included to ensure that viruses of the leukosis complex were not present in the vaccine. The only feasible control test for this purpose in the present state of knowledge is that described by Rubin—the RIF test. In principle it is simple. Wild viruses of the complex, including wild strains of Rous sarcoma virus, grow in chick embryo tissue culture monolayers without producing obvious signs of their presence. However, when present and growing in the monolayer, they interfere with the production of proliferative and degenerative foci which are elicited by an appropriate laboratory strain of Rous virus.

The complexities of the test are multiple, but two are outstanding. Tissue cultures prepared from embryonated eggs from most chickens are contaminated with avian leukosis virus, hence are unsuitable for the RIF test. Another difficulty with the RIF test is that it must be carried through three serial passages beyond the primary chick culture which was inoculated with the unknown, and then challenged with Rous virus before results can be obtained indicating that the test material is free of leukosis agents. The LVR was interested in the RIF test only as a control procedure in the safety testing of measles vaccine, nevertheless it was forced into investigative and developmental work on the RIF test because no other laboratory in the NIH had successfully employed the procedure.

During the year, a task force was drawn from various units in the LVR to establish Rubin's test on a working basis. After this was accomplished a permanent unit was established, under Mr. Jahnes, in a newly converted laboratory in the Section on Basic Virology and Rickettsiology to do the necessary biologics control testing and to investigate the role of this group of viruses in vaccines.

Hepatitis

Dr. O'Malley continued studies on the properties of the A–1 virus, an agent presumed to be causally related to some cases of homologous serum jaundice. Work reported previously had shown that jaundiced volunteers developed A–1 antibodies. Serologic studies on volunteers who failed to develop jaundice after inoculation with proven icterogenic materials suggested that these persons had had previous experience with A–1 virus and that this might account for the failure of clinical disease to appear.

Paired serum specimens from 40 persons receiving multiple blood transfusions failed to show the development of A–1 virus antibody. Only one of these 40 patients developed jaundice within a 3-month postoperative period; serum specimens taken from this person up to 3 months after onset of jaundice did not contain A–1 virus antibody.

Tests on 400 convalescent serum specimens from patients in outbreaks of infectious hepatitis revealed that 18 or 4.5% contained A–1 virus antibody. This antibody was also found in 4 of 44 specimens from persons who were considered to

have serum hepatitis. However, of the 40 specimens in the latter group which contained no A–1 virus antibody, 12 were taken at a time too early for antibody to have appeared and 26 were taken 9 or more months after jaundice. Previous findings had demonstrated that this antibody usually decreases to undetectable levels within 9 months. Data were unavailable regarding the phase of the disease at which the remaining two specimens were drawn. Thus, A–1 virus antibody was found in four of six specimens in which it might have been expected to have occurred.

Continuous Cell Culture Lines

The BS–C–1 cell line, initiated in March 1960, from kidney tissue of *Cercopithecus aethiops* by Mrs. Hopps of the Section on Basic Virology and Rickettsiology, is now in its 125th passage and is considered to be an established cell line. Cultures have been sent to the Cell Bank Committee (sponsored by NCI) for characterization and subsequent storage at the American Type Culture Collection. The BS–C–1 line is unique in that this is the only reported continuous culture whose chromosomal pattern and virus susceptibility have been studied in concert since early passage levels. Findings indicate that while the chromosomal pattern of the cells changed from diploid (30th passage) to subdiploid (at 41st passage) to heteroploid (111th passage), the cells have not changed their sensitivity to SV–40 virus. Moreover, at the 108th passage they were still sensitive to attenuated measles virus. Further evidence of their stability with regard to virus proliferation was noted in studies with influenza A and B and adenoviruses 3, 4 and 7. The BS–C–1 line was refractory to these agents at low passage and still failed to support their growth at the 95th passage level. In addition to those viruses already reported to multiply in BS–C–1 cells, i.e., SV–40, measles, and attenuated polioviruses 1, 2 and 3, it has been shown that this line will support growth of Rift Valley fever virus, RS virus, O'Malley's A–1 agent, Coxsackie A9, and simian viruses 1, 4 and 5 but not SV–2. Moreover, preliminary data suggest that the line may be useful in basic studies with epidemic typhus rickettsiae. The cell line has come into general use in the scientific world; 46 research and industrial laboratories in North America, Europe, Asia and Australia were supplied on request with seed cultures during the year.

Among the numerous technical difficulties inherent in the present RIF assay procedure is the necessity for a continuous supply of known RIF-free chick embryos. The establishment of a RIF-sensitive chick embryo tissue culture line would solve one of the major problems in measles vaccine testing. Selected chick embryos, presumed to be RIF-free, were employed in attempts to develop a useful cell line. In only 1 of 17 attempts has the cell culture survived beyond the 18th passage. This line, BS–CE–17, is now in its 31st passage but its growth rate has decreased and survival of the line is questionable.

Rickettsial Vaccines

Work on rickettsial vaccines continued in the SBVR–SRV during the year but at a slow pace because of diversion of efforts to problems connected with measles and respiratory vaccines.

As a result of the collaborative work undertaken in the military services last year in which groups of marines were inoculated with progressively diminishing doses of commercial vaccine, changes are being made in the primary course of immunization against typhus employed by the military services. Instead of two doses of 1 ml. each, the primary course will consist of a single dose of 0.5 ml. Additional studies using marine recruits are in progress to determine the minimal amount of typhus antigen which will induce a state of immunological preparedness in young adults so that they will subsequently respond in a booster fashion when given typhus vaccine prior to exposure in epidemic areas.

A collaborative field study using a pilot lot of formalin-inactivated Q fever vaccine prepared at the Walter Reed Army Institute of Research is in progress in several areas of the U.S.A. This is designed to determine the value of a course of immunization which hopefully will prevent previously sensitized persons from developing untoward reactions. Draft regulations for the manufacture of Q fever vaccine have been developed in the LVR, circulated to manufacturers and investigators, and are now being prepared in final form for publication. Reference vaccine and antisera for use in assaying the potency of Q fever vaccine are on hand.

Influenza

In the winter of 1961–62, sporadic outbreaks of influenza occurred in Japan, Taiwan, and New Zealand, during which a number of Asian strains were isolated by workers in several monitoring laboratories located in these areas. Some of the strains were sent to the Division for antigenic analysis. The results obtained by Dr. J. A. Morris and his associates in the Section on Respiratory Viruses in hemadsorption-inhibition tests performed in cell cultures and in neutralization tests performed in mice, indicated an extensive shift in antigenic composition in some of the 1961–62 Asian strains away from the prototype A2/Japan/305/57 contained in the present vaccine.

Antigenic analysis of influenza B strains recovered in the winter of 1961–62 in outbreaks of influenza in the United States and Canada indicated a clear, but relatively small, antigenic difference between the recent strains and the older strains contained in the current vaccine.

Thus it appears that a major antigenic change has occurred in recent isolates of A2 viruses and a minor shift in B viruses as compared with strains of previous years. These findings were presented to an *ad hoc* group assembled at the DBS on December 2 to advise on strain composition of influenza vaccine. Following analysis of the data just mentioned and other information provided by those in attendance, it was recommended that one of the new A2 strains and one of the recent B strains be incorporated into the vaccine which will be manufactured for the 1963–64 season. Steps are being taken to determine whether the two strains tentatively selected for inclusion are suitable for production and are antigenic in man.

Arbor Viruses

A member of the LVR organized and chaired a symposium on "Immunization Against Arbor Virus Infections" held at the annual meeting of the American Society of Tropical Medicine and Hygiene. Progress in this field has been reasonably rapid, but except for an inactivated Rift Valley vaccine, prepared from monkey kidney tissue cultures by techniques analogous to those used in manufacturing Salk vaccine, none of the new vaccines or procedures is apt to require serious consideration by DBS as regards the development of regulations and control procedures for license within the next year or so.

Antimicrobial Substances

Dr. C. P. Li and his group in the Section on Virus Biology continue to devote most of their efforts to the study of the antibacterial and antiviral substances obtained from oysters and other mollusks and have extended their survey of antimicrobial substances of plants and animals. Several psoralen compounds which were identified years ago in fruits and subsequently used in man to accelerate suntanning have some activity against several viruses in tissue cultures and in mice.

LABORATORY OF BIOPHYSICS AND BIOCHEMISTRY

Research activities of the laboratory continue to be identified primarily with the application of physical principles to microbiological systems. An extension of an earlier study of photochemical processes affecting survival of viruses has led to the development of a photosensitization technique for differentiating between parent and progeny virus in cell cultures. This technique has made it possible to detect multiplication of poliovirus in nonprimate cells that were previously thought to be refractory to infection. This finding has some bearing upon the developing concepts of the relationship between viruses and their animal hosts. Other aspects of the subject are pertinent to the development and control of viral vaccines, since the opportunity of cultivating poliovirus serially in novel media will add to our information about properties of various strains.

The staff of the Laboratory of Biophysics and Biochemistry has been supplemented by the addition of two scientists during 1962. Dr. Nobuto Yamamoto, a Japanese biophysicist, appointed as Visiting Scientist, is investigating the mechanism of photodynamic inactivation of bacteriophages with the expectation that this information will eventually contribute to our understanding of viral genetics. Dr. John M. Easton, a medical officer with special training in micropathology, has undertaken an electron-microscopic study of the cytopathic effects of simian viruses on monkey kidney tissue culture cells. Dr. Easton's prior experience with the ferritin-tagging technique for

visualizing antigen-antibody complexes in the electron microscope will be an asset in this investigation.

LABORATORY OF BLOOD AND BLOOD PRODUCTS

Research Activities

The research program of the Laboratory of Blood and Blood Products during 1962, with the exception of work on coagulation components, has been at a virtual standstill as a result of the forced diversion of professional staff time to a major investigation of possible violations of the Public Health Service Act. This also caused considerable disruption of routine duties because of the top priority necessarily assigned to the investigation. The objectives of the research program of the Laboratory remain unchanged, though more than a year's effort toward attaining these objectives has been lost. Hopefully the requirements for staff time to assist the U.S. attorney in the prosecution of these cases will soon be much less and the research program can be pursued.

The various fields of interest have as their goal the improvement of testing procedures used for the control of biological products derived from blood; these procedures being designed in so far as possible to insure the safety, purity and potency of the products. The projects cover all phases of blood collection, processing, shipment and storage. Investigations encompass not only improvements for existing control procedures but also are directed toward the perfection of new tests for existing products as well as for new products.

There is at present much interest in the components of the coagulation system. Some of the new products under development have immunological or biological activities for which there are no established test criteria. The urgency imposed by this interest and the lack of testing methods demanded that work continue on coagulation components. This was the only project that received significant attention during 1962 and research on some of the fundamental characteristics of these proteins was continued to gain information on which useful control tests might be based. Further improvements have been made in the method of preparation of a plasminogen free fibrinogen; an important accomplishment since it brings us one step closer to the goal of control tests for the complex coagulation system that are independent of the reagents used.

Other Research Interests

(1) Studies of freezing, storage at various low temperatures, thawing and/or drying of the many elements of whole blood and protein fractions of plasma. Specific investigations within this broad field are undertaken to obtain data on which to base dating periods and stability of products.

(2) For many years, the Laboratory has maintained an active research interest in the effect on the properties of whole plasma and the individual plasma proteins of time, light, container material, temperature fluctuations and other factors. Normal Serum Albumin was placed on such a long-term study 8 years ago. Subtle changes occur which have been taken into account in establishing a dating period schedule for this product.

(3) Immunological studies related to the identification and quantitation of antigens on red blood cells and other formed elements and of the antibodies which occur in blood continue to intrigue the professional staff of the Laboratory. Many control tests still have an empirical basis. An ultimate goal of these immunological investigations in control tests which result is realistic quantitative expressions of identified antibodies or antigens.

Offsite Research

Because of the nature of the control functions of the Laboratory, many contacts are made with scientific staffs of outside organizations for various reasons. It is the duty of the staff of the Laboratory to be aware of advancements in all areas of blood technology. To this end, staff members regularly visit and consult with other laboratories, attend meetings in fields of their specialties and present the results of their own research.

Somewhat related is the participation by members of the staff in blood bank workshops and training programs. This activity, while performed on an informal basis, nevertheless allows more efficient dissemination and illustration of blood banking techniques. Techniques are being explored that will allow greater participation by the staff in these training programs as a contribution to the raising of the standards of achievement of blood banks.

The Laboratory will continue its cooperation with American Standards Association and the In-

ternational Standards Organization in studies of the interchangeability of disposable blood equipment.

As one means of speeding the flow of specific research information and material, the Laboratory is planning to make use of research contracts. Under active negotiation at the present time are contracts designed to yield needed information or reagents on the following subjects:

(1) Evaluation of the antihuman complement component of an antihuman globulin serum for the Coombs test;

(2) Establish more realistic standards for ABO grouping serum;

(3) Prepare anti sera to specific proteins for use as reagents in immunological control procedures and as standards;

(4) Design and build a variable rate feed pump for special applications in chromatography;

(5) Establish the quantitative significance of A and B antibodies in pooled plasma;

(6) Prepare additional blood antigen antiserums for use as reference preparations;

(7) Prepare fibrinogen and thrombin by a specified method for use as reference preparations; and

(8) Prepare antihemophilic factor for use as a reference preparation.

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