NATIONAL INSTITUTES OF HEALTH

Review of

INTRAMURAL RESEARCH

1960

U.S. DEPARTMENT OF

HEALTH, EDUCATION, AND WELFARE

Public Health Service

ISSUED IN A LIMITED EDITION FOR ADMINISTRATIVE USE

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FOREWORD

The National Institutes of Health, one of the five Bureaus of the United States Public Health Service, has been assigned the mission to conduct and to support research, research training, and other related activities. This mission is discharged partially through an extramural support program which, administered from Bethesda, reaches into virtually every institution in the United States engaged in biomedical research, and is rapidly expanding throughout the world. And it is partially discharged through a direct operation, the intramural program, housed in laboratories in Bethesda, and representing something of a microcosm of the total effort. This publication focuses exclusively on our intramural enterprise, describing it in a series of reviews designed to illustrate the compass and flavor of the local research activities of each of our Institutes. Thus, it is not a comprehensive presentation of the program of either the United States Public Health Service or of the National Institutes of Health.

Glimpses of the extent to which the intramural research effort has participated in the broad forward thrust of medical research are provided in the eight Annual Summary Reports comprising this compendium. The reports are protected from editorial interference, and prepared by the originators in accord with only the most general guidelines. This procedure, considered and deliberate, has been adopted to allow the reader to savor the diversity of outlook and attitude which prevails among a group of brilliant scientists loosely knit and almost imperceptibly harnessed for the attainment of common categorical goals. In such a presentation, it is possible to overlook the thread of mission that ties each of these operating research organizations into the broad program of the National Institutes of Health. It is appropriate, therefore, to pursue this aspect briefly.

The mission of the National Institutes of Health as a whole and of its components is to develop the facilities, resources, and attitudes which would be most effective in acquiring new knowledge concerning disease processes, to the end of relieving suffering, bringing about cure and rehabilitation, and assuring the prevention, whenever possible, of disease. Broadly considered, this mission involves providing the wherewithal and cultivating suitable soil for a systematic study of man and his milieu with the ultimate objective of contributing to improved health. From this overall point of view, the NIH does not differentiate between what is done intramurally and extramurally. However, within these conceptions are contained more specific objectives only some of which can be sought for within an intramural research program, while the others may be searched out most expeditiously by support of work in other institutions through the extramural program.

This second Annual Review of NIH Intramural Research provides evidence of the magnitude of the effort—both in breadth and depth—and of the type of achievements that have placed this installation in the forefront of research in the medical sciences.

James A. Shannon M.D.

JAMES A. SHANNON,
Director.

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NATIONAL CANCER INSTITUTE

INTRODUCTION

The staff of the Institute continued to make important research contributions of a very high order during 1960. The wide scope of the activities, their complexity, and the great numbers of important findings make it especially difficult to summarize adequately the work, and much additional knowledge, often at a rather preliminary stage, is not included in the report. Both the collaborative groups and the individual scientists continue to be highly productive, and the most fruitful collaborations are those established by the individual scientists themselves finding a common meeting ground in research problems of joint interest. The attempt to provide a research atmosphere most conducive to sound research programs must take into account the delicate balance between the needs of the Institute as a whole and those of the individual. The ideal balance, particularly in a large organization with its many rules, regulations, clearances and multiple channels of communication, is often difficult, if not impossible, to attain. Nevertheless, the research accomplishments made and a sense that they are often of high significance for society make the considerable effort directed toward this end worthwhile.

At mid year, a number of changes occurred in several key positions in the Institute. Dr. Kenneth M. Endicott assumed the position of Director, NCI, following two years as Associate Director, NIH. Dr. C. Gordon Zubrod, who has been the Clinical Director of the NCI since 1954, assumed responsibility for the clinical programs, including the Surgery, Endocrinology, General Medicine and Radiation Branches. Dr. Carl G. Baker, who had been Assistant Director with Dr. Heller for 2½ years, assumed responsibility for the nonclinical aspects of the intramural program, including the Laboratories of Biochemistry, Biology, Physiology, Chemical Pharmacology and Pathology, and the Pathologic Anatomy Branch.

These organizational changes and the impending retirement of Dr. Howard B. Andervont as Chief of the Laboratory of Biology brought about extensive discussions of the direction the Institute should take, of the areas of emphasis and de-emphasis, and of intraorganizational relationships. One change is the expectation that scientists on the intramural staff will participate more actively in some of those programs, formerly classified as "extramural" or in the "gray area," for which the Institute staff has clear responsibility as regards quality and execution. In the future, it appears this will be of special importance in the areas of international medical research and, under Field Studies, cancer diagnostic test development and environmental cancer. The major program areas which in the near future will receive most attention throughout the Institute are: viruses and cancer; molecular and developmental biology; environmental cancer; and cancer chemotherapy. The highly competitive market for senior scientific personnel in these areas makes it difficult to proceed as rapidly as one might wish, and availability of facilities also sets limits on the rate of development of program segments.

The relationships with the Board of Scientific Counselors continue to be excellent, and the present Board, made up of Drs. Philip P. Cohen, Chairman, Wendell M. Stanley, E. K. Marshall, Jr., Hugh Roland Butt, Jacob Furth and J. Englebert Dunphy, exhibits the same high interest, helpful advice and general support as has become customary. At the April meeting of the Board, a program of research studies by the Clinical Associates was presented by these young men, and at the fall meeting, a program was held on broad aspects of cancer research, its state of development, its future coast, and the strategy involved. The Laboratory and Branch Chiefs discussed their programs as they relate to these broad subjects.

The Board continues to express confidence in the research staff and its programs. The frank discussions characteristic of past meetings con-

tinued, and selected problems facing the Institute were discussed with a free exchange of ideas and suggestions. Not only do the members of the Board provide the Laboratory and Branch Chiefs, and, indeed, individual staff members, the opportunity to discuss scientific and organizational problems with them, but as disinterested parties, very real assistance is given to Dr. Endicott and his immediate staff.

The NCI was visited by a delegation of outstanding cancer investigators from the Soviet Union, September 26–28, 1960, in accordance with implementation of the Lacy-Zarubin agreements and subsequent detailed plans developed between the Public Health Service and the Ministry of Health, USSR. The group consisted of:

Professor N. N. Blokhin, Director of the Institute of Experimental Pathology and Therapy of Cancer, Moscow, President of the Academy of Medical Sciences, USSR, and Head of the Delegation.

Dr. L. A. Zilber, Director, Department of Immunology and Malignant Tumors and Scientific Director, Institute of Epidemiology and Microbiology in the name of Gamalei, Moscow.

Dr. L. F. Larionov, Head, Laboratory of Experimental Chemotherapy, Institute of Experimental Pathology and Therapy of Cancer, Moscow, and an editor of "Pathologicheskaya fiziologiya i experimentalnaya terapiya."

Dr. M. M. Mayevskiy, Head, Laboratory of Experimental Biotherapy, Institute of Experimental Pathology and Therapy of Cancer, Moscow, and an editor of "Zhurnal mikrobiologii epidemiologii i immunobiologii."

V. V. Gorodilova, Candidate in the medical sciences, Head, Laboratory of Virology and Acting Director, Central Institute of Oncology in the name of P. A. Gertzen, Moscow.

Cordial relationships were maintained throughout the visit, and since the main interests of the visitors were virology and cancer, immunology and cancer chemotherapy, most of the presentations by NCI staff members were in these scientific areas. Little new information on cancer research in the Soviet Union was obtained from the visiting scientists. However, two talks were presented: Dr. Larionov spoke on "Complex Alkylating Metabolites as a New Type of Antitumor Drug" and Dr. Zilber spoke on "Susceptibility of Rats and Rabbits to Rous Sarcoma Virus." It is planned that a group of American cancer investigators will make a return visit to the Soviet Union in May of 1961. This group will probably consist of Drs. Endicott, Andervont, and Hertz from NCI, and from the Sloan-Kettering Institute, Drs. Horsfall, Stock, Dalldorf and Randall. As part of the program for the exchange of individual scientists who will spend longer periods of time working in laboratories, Dr. J. Edgcomb may spend 6 months or longer in the Soviet Union, and two Soviet scientists are scheduled to spend a year at the Sloan-Kettering Institute.

Several members of the staff have received outside recognition for their accomplishments. Dr. Murray Shear is currently President of the American Association for Cancer Research and Chairman of the Finance Committee and of the Chemotherapy Committee of the International Union Against Cancer. Dr. H. L. Stewart is Chairman of the Geographic Pathology Committee of the Union as well as a member of its Executive Committee. Dr. Thelma Dunn is Vice President and President Elect of the American Association for Cancer Research, assuming the office of President next year. Dr. Roy Hertz is Vice President of the Endocrine Society. Dr. Eugene Van Scott is a member of the Board of Directors of the American Academy of Dermatology and Syphilology. Drs. Herbert A. Sober and Carl G. Baker are members of the Executive Committee of the Division of Biological Chemistry, American Chemical Society, and Dr. Baker represents the Division as one of two Divisional Councilors on the American Chemical Society Council. Dr. Harold F. Blum received a Sigma Xi award for his article "Quantitative Aspects of Cancer Induction and Growth: as Illustrated in Carcinogenesis by Ultraviolet Light," American Scientist *47*, 250–260 (1959). Drs. John Weisburger and Vincent E. Price became members of the Editorial Board of "Cancer Research." Dr. A. J. Dalton was elected a Director of the Electron Microscope Society of America and was appointed to the Council of the American Association for the Advancement of Science. Many members of the staff continued to give invited

lectures and serve in consultative capacities throughout the country.

As an honor to Dr. Wilton Earle and his associates, the Tissue Culture Section was asked by the Cell Collection Coordinating Committee to supply clone NCTC 929 of Strain Z mouse fibroblasts as the first selection to go to the repository for characterized tissue culture cell lines of the American Type Culture Collection.

CLINICAL INVESTIGATIONS[1]

The clinical branches of the intramural research effort of the National Cancer Institute have enjoyed a productive year. This is attested to not only by the high quality of research publications on major problems of clinical cancer, but also by continual calls upon the staff for advice, invited lectures and service upon national committees related to cancer research. In addition to the honors listed above, a few others should be mentioned. Dr. Roy Hertz was called upon to advise Secretary Flemming on problems of carcinogenesis. He was also elected to the American College of Physicians. Dr. Nathaniel Berlin was elected to the American Society for Clinical Investigation. Dr. John Fahey spent a year as a visiting scientist at the National Institute for Medical Research, Mill Hill, England. Dr. Mortimer Lipsett is now a visiting scientist at the Karolinska Institute. Dr. Kurt Kohn is working for a period of several years at Harvard with Dr. Paul Doty.

There have been a number of changes among the senior staff. Dr. Donald Watkin, General Medicine Branch, after nine years at NIH, left for a year's work with the Institute of Nutrition of Central America and Panama, surveying nutritional problems of the Caribbean. He will soon assume his new post of Associate Professor of Nutrition at Massachusetts Institute of Technology. Dr. Montague Lane, General Medicine Branch, became Assistant Professor of Pharmacology at Baylor University. Dr. Robert Madden, Surgery Branch, left to become Assistant Professor of Surgery at New York Medical College.

Dr. Thomas Butler, Professor of Pharmacology, University of North Carolina, spent eight months as visiting scientist with Dr. David Rall working on problems of intracellular pH. Dr. Tatuo Saito, Associate Professor of Internal Medicine at Tohoku University Medical School, Sendai, Japan, completed a year as guest worker at the National Cancer Institute. He worked with Dr. David Rall on the transfer of glucose from blood to the central nervous system. Dr. Myron Karon joined the Chemotherapy Service as the first full time pediatrician at NCI. Dr. Paul Carbone transferred from the Hospital Division, to the Chemotherapy Service. Dr. Sherman Weissman, a former clinical associate, returned as a senior staff member of the Metabolism Service. Dr. Jacqueline Whang has accepted a fellowship on the Chemotherapy Service for cytogenetic studies of leukemic patients. The Radiation Branch has Dr. Roger Berry and Dr. Lawrence Draper as new staff members. Dr. Berry is working on the biological effect of neutrons, and on chemical enhancement of irradiation. Dr. Draper's interests concern immunological phenomena as altered by irradiation. Two new senior members have joined the Surgery Branch: Dr. John T. West, who transferred from the Hospital Division, and Dr. Everard Cox, who came from the University of Maryland. In the Endocrinology Branch, Dr. Griff T. Ross has accepted major responsibilities after a number of years at the Mayo Clinic, and Dr. William Odell has joined the laboratory group working on pituitary hormones.

These yearly comings and goings of senior staff raise the questions of what it is that attracts clinical investigators to NIH, why some elect to stay, why some leave. One of the obstacles to full staffing is posed by the nature of the "Cancer Hospital." Some types of research can be justified only in those patients whose disease is not amenable to cure. Thus many of our patients have relatively far advanced cancer, often with the stresses of pain and physiological impairment, occasionally disfigured from previous surgery, with depleted nutritional and emotional reserves, and death often not far away. One or two of these patients can almost paralyze the usual medical service. Our staff must be prepared to care for over a hundred such patients. To do so is costly—in resources, in personnel, in demands upon the moral courage of our staff. For

[1] Prepared by C. G. Zubrod, M.D., Clinical Director.

such care we must have physicians who are compassionate, skillful, wise and yet good investigators interested in patients with cancer. Combinations such as these are uncommon, perhaps rare. There are 8,000+ graduates from medical schools in this country each year. NIH receives inquiries from about 1,500; 400 are considered seriously; 100 are interviewed by NCI; 18 are appointed as Clinical Associates. From each eighteen in some years, we find one who is qualified to stay on. For these physicians and for all our staff, efforts of the Institute must be intensified to make their careers here rewarding, effective and satisfying.

The content of clinical investigation of cancer requires one comment. No useful purpose would be served by attempting to study all phases of human cancer, just as a century ago it would have been fruitless to arrive at an understanding of virus diseases by studying intensively the mechanisms and control of fever. The leads in cancer research must come from a vigorous nonclinical program. It is the task of the clinician to identify in the clinic those situations which will permit him, in a highly controlled fashion to validate the leads of the laboratory scientist. The clinical program is based on this assumption and the review of the past year's efforts provides many examples. The review will be divided into three sections: Origin of the Cancerous State, Development of Clinical Cancer and Management of Cancer.

I should like to call attention to several developments which can be regarded as of major importance:

The description by Dr. Roy Hertz and his colleagues of 5-year survivals of patients with metastatic trophoblastic disease following methotrexate treatment.

The development by Dr. Eugene Van Scott of ways to study cellular differentiation in man.

The definition by Drs. Robert Smith and John T. West of the limitations of sampling of tumor cells in wounds and peripheral blood.

The development by Dr. Emil Freireich and Dr. Allan Kliman (DBS) of a practical way to treat thrombopenic hemorrhage by platelet replacement without the development of immunity.

The emergence of chemotherapy studies under Drs. Frei, Rall and Goldin into a new phase which allows study of the specificity of animal tumor systems for prediction of drug activity for particular tumors of man.

The maturation of the NCI registry of patients to the level that all major studies report 100 percent followup.

The recognition by Drs. Ketcham, Hoye, and Berlin of the magnitude of blood loss during and following surgery, and the failure of the hematocrit to provide a guide for full replacement.

The demonstration by Drs. J. R. Andrews and Brace that radiation therapy of some cancers can be safely and adequately given in a single dose.

Origins of the Cancerous State

There is now a large body of opinion which holds that a normal cell becomes a cancer cell because of some damage to the DNA replicating mechanism. This damage can apparently be initiated by a variety of "causal factors" such as irradiation, chemicals, genetic defect, viruses or metabolic abnormality. It has been difficult for the NCI intramural clinician to identify clinical situations worth study. Most of the leads come from epidemiological considerations, and here our clinicians have had few opportunities. Perhaps the newly formed Field Studies Group under Dr. Shimkin will create the openings for the clinician to study carcinogenesis. (One such opportunity developed recently when the uranium miners' study revealed eight men with suspicious cytology, but lack of adequate surgical staff made admission unwise.)

Another reason for inability to initiate program has been the status of the art of uncovering oncogenic viruses. Drs. Manaker, McLaughlin, and Couch studied 27 primary tumors from NCI patients, by tissue culture techniques, designed to unmask such viruses, without positive result. Vigorous pursuit of human oncogenic viruses awaits new discoveries on how to uncover them.

Several illustrations may serve to emphasize how indirect must be the approach of the clinician to carcinogenesis. Drs. Van Scott, Reinertson, Eisen and Rothberg studied patients with psoriasis as a model of skin hyperplasia, and tried

to relate the hyperplasia to biochemical abnormality. Recognizing psoriasis as a systemic abnormality with arthritis and hyperuricemia they have shown in work with Dr. Seegmuller, NIAMD, that in this disease C–14 glycine is over-incorporated into uric acid; that the uric acid pool is increased in size, not to the degree seen in some cases of gout, but generally greater than that noted in chronic myelocytic leukemia. Drs. Rothberg and Van Scott have also shown that C–14 labelled glycine has a much more rapid turnover time (3–5 days) after incorporation into protein of psoriatic scales, than after incorporation into normal skin (28 days). The reasons for these phenomena are unknown, but the model exists for further study of the relation of hyperplasia to disturbances of nucleoprotein and protein metabolism.

The observations of Dr. John L. Fahey on plasma-cell tumors in man provide another possible approach to clinical carcinogenesis. Continued work with Dr. Michael Potter on eight plasma-cell tumors of mice, showed that the proteins formed by these cells have distinctive electrophoretic, ultra-centrifugal and immunologic properties. The individuality of the myeloma proteins suggests that each neoplasm arose from a single plasma cell clone. This observation is in keeping with those of Morris and Van Potter and of Tomkins on the enzymatic activities of rat hepatomas. Dr. Fahey's work on plasma cell tumors in man shows similar specificity and individuality of proteins and thus the clinician is provided with a marker by means of which the behavior of a malignant cell clone can be inferred. In this manner some of the many new observations on the individuality of chemical activities of induced tumors can perhaps be tested in the clinic.

Another way in which carcinogenic influences in man can be followed is by means of cytogenetic changes. Qualitative and quantitative abnormalities of chromosomes have been described as genetic faults, as a result of radiation and in association with tumors. Drs. Hertz and Ross have used chromosomal counting as a tool for identification of various genetic growth abnormalities in patients which can then be characterized endocrinologically. Dr. Jacqueline Whang has recently joined the staff and is studying cytogenetic abnormalities in the leukemias and in mongolism. In collaboration with Dr. Tijo of NIAMD, she has simplified the methods for chromosomal morphologic study and enumeration, permitting more extensive survey of changes in disease.

Development of Clinical Cancer

Once established, cancer cells invade and destroy locally, spread into distant tissues and in the process of growth produce varying effects upon the host. The mechanisms of these processes may be studied in the clinic from the following aspects:

1. autonomy of cancer cells;
2. the spread of cancer cells from the point of origin to remote sites, where they become established and grow;
3. the resultant physiologic and biochemical abnormalities.

Autonomy

By autonomy, one means the property of cancer cells by which they no longer respond to those controls which the host normally exerts over cellular proliferation, differentiation, and invasiveness. In the normal situation, for example, the cells of the vermilion border of the lip, although multipotential, remain throughout life as a sharply defined line. Somehow the body relays information to these cells to remain as mucous membrane. Drs. Van Scott and Reinertson have shown that buccal mucous membrane, when transplanted autologously to a skin area, retained its characteristics only when its connective tissue stroma was still attached. When pure mucosa was transplanted subepidermally, it became keratinized epidermis; when placed in the lower corium, it developed ductlike structures. The same pattern was observed after the transplantation of other skin components such as hair roots. These observations have been extended to basal cell carcinoma. Pure basal cell cancer implanted into the lower corium degenerated, keratinized or formed ductlike structures. When transplanted subcutaneously, the tumor always degenerated. The authors conclude that although the range of responses possible for epithelial cells appears to be a property of the cell line, the specific response is determined by the environment in its local connective tissue stroma. They have further attempted to define the environmental influences

leading to keratinization by tissue culture study of epidermal cells. Keratinization is enhanced by the elevation of the pH of the culture medium above normal or the presence of high CO_2 tension. Thus, though the environmental control may be exerted through complex chemical influences, it is also possible that *in vivo* the control may be physical in nature. Drs. Van Scott and Reinertson have developed a model for the study in man of cellular differentiation of both normal cells and cancer.

Autonomy can also be subjected to clinical scrutiny in the classic situation of the study of hormonal control of sensitive tissues. Dr. Roy Hertz and his colleagues have continued their observations on the mechanisms by which hormones limit or enhance the growth of normal cells and cancer. Drs. Hertz, Griff T. Ross and Mortimer Lipsett in a study of five men, found breast cancer to be remarkably sensitive to orchiectomy. Four of these, after recurrence, responded to estrogen therapy. There were no abnormalities of thyroid, pituitary or adrenal function. Thus they have identified another highly sensitive system for studies of hormonal control. In studies of breast cancer in women, they have found neither exacerbation or regression of disease after the administration of human or sheep follicle stimulating hormone, human growth hormone, or ovine prolactin. The latter two materials caused no exacerbation of prostatic cancer. Growth hormone was studied extensively for its metabolic effects. It did not cause nitrogen retention in four primordial dwarfs. Reports of others on the activity of chymotrypsinogen treated beef growth hormone could not be confirmed. In addition, although testosterone caused nitrogen retention in active acromegaly, growth hormone did not. Dr. J. M. Van Buren, NINDB, in a collaborative report with the late Dr. Delbert Bergenstal, evaluated 12 patients with breast cancer treated by hypophysectomy. Seven of the 12 had remissions lasting 3–9 months. Serial sections of the hypophysis at autopsy indicated the degrees of completeness of pituitary removal. Twenty-nine to 0.01 percent of the gland remained. Neither the degree of depression of endocrinologic function nor the extent of regression of breast cancer was in any way correlated with the residual amount of pituitary. If less than 10 percent of the gland remained, the patients were permanently cortisone dependent. On the other hand pituitary stalk section in another 12 patients with breast cancer showed little effect upon tumor, and several patients showed persistence of thyroid function.

In studies of the hormonal control of prostatic tissue, Dr. William W. Tullner has shown in castrate male rats that ACTH or ACTH plus prolactin produced growth of the ventral prostate only in the presence of the adrenals and hypophysis. He concludes that pituitary growth is, in part, dependent not only on adrenal androgens but also on unidentified pituitary factors.

Spread of cancer

Studies in man by several investigators including Dr. Robert Smith and his colleagues at NCI in collaboration with Dr. R. S. Malmgren and the staff of the Cytodiagnostic Service, have revealed that microscopic fragments and even single cells from a cancer can be found in wound washings after surgery or in the circulating blood. The recent report by Dr. George Moore that ThioTEPA given at the time of radical mastectomy has substantially reduced recurrences compared to controls, emphasize that an understanding of tumor spread is the most important problem facing surgical research. Continued study of cellular detachment, spread and growth in patient and animal during the past year has helped further to define the problem.

Wound Washings. Drs. Smith and Marvin Arons have followed 100 percent of 111 patients who had excision of primary cancer between September 1953 and December 1956. The followup period since operation was 44 to 83 months. Twenty-six percent of the patients had positive wound washings; 60 percent were negative and 14 percent suspicious. There were no correlations between the results and local recurrence, lymph node or distant metastases, or survival. The lack of prognostic significance of positive wound washings might well be due to sampling problems. Dr. Seymour C. Nash has attempted to obtain more complete wound samples by inserting a suction catheter into the wound for several days. At intervals, samples of the wound washings, trapped and iced in heparin and streptodornase-streptokinase, were removed for counting. Tumor cells were demonstrable in post-operative drain-

age fluids as long as 43 hours after operation, and sometimes when the washings at the time of operation were free of cells.

Cancer Cells in Circulating Blood. Although other investigators have found no correlation between cells in the circulating blood and prognosis, it is doubtful whether adequate experimental design of a study has yet been attained in man. Dr. John T. West and Dr. Malmgren have continued to validate methodology, and still face problems of identification of cells. An equally serious problem is that of sampling, as shown in their study of 377 patients, 26.3 percent of which were positive for circulating tumor cells. There was a linear relation between the number of examinations and the percentage positive. Of 183 patients with one examination, 10 percent were positive. Percentage positives increased with repeated examinations until, with seven or more examinations, 75 percent of 45 patients were positive. Prognostic significance cannot be judged until sampling comparability is achieved. It can be inferred that the present technique is less than 10 percent efficient. The limiting factor is probably the small amount of blood which can be processed with present techniques. If a method can be found permitting greater efficiency of recovery, then it will be possible to examine the influence of manipulation, exercise, and other factors in releasing cells into the circulation. One modification in the millipore technique has been introduced by Dr. Marvin Romsdahl who substituted dextran sedimentation for lysis of erythrocytes. This permitted the adaptation of quantitative recovery from blood of the cells of various mouse tumors.

Spread of Tumor in Animal Models. The animal models of tumor spread developed by members of the Surgery Branch have been ably utilized by Dr. Alfred Ketcham and his colleagues. Dr. Romsdahl injected Sarcoma T-241 tumor suspensions into the hind leg of C-57 mice, amputated the left leg on the next day in one group of animals and on succeeding days in other groups. He found that although circulating tumor cells could be found 24 hours after inoculation, amputation before the seventh postoperative day prevented pulmonary metastases. He concluded that the demonstration of tumor cells in blood is not necessarily equivalent to hematogenous metastases.

Last year it was reported by Dr. David Kinsey that certain tumors will metastasize only to pulmonary tissue, even if the lung is transplanted away from the pulmonary circulation. Dr. Marvin Arons found that metastasis did not occur to a similarly implanted etheron sponge in DBA/P mice with L1210 leukemia, S-91 melanoma or a mast cell tumor. Even though connective tissue would rapidly enter the sponge, tumor would grow around but not infiltrate it.

A number of host factors which influence metastases have been recognized. Dr. Ketcham transplanted several mouse tumors into mice of different ages and found that the growth of the primary tumor was delayed in the older mice. Similar delay was noted if the fluid intake of the mice was limited to 20 percent ethanol, but there was no effect from denervation of the limb into which the tumor was transplanted. Dr. Alan Retik showed an increase in metastases of S-91 melanoma to the previously traumatized liver.

Previous reports have emphasized the failure of drugs to modify the local growth or metastases of transplanted tumors. In the past year Dr. Ketcham has shown accentuation of growth of a variety of transplanted mouse tumors when drugs such as clorpactin, thioTEPA or alcohol were placed in the wound before the inoculum. When the order was reversed, there was a delay in growth related entirely to the mechanical effects of washing, except with 3,6-diamino acridine. Dr. Nash reported that with a mast cell tumor, this agent locally applied 1 to 1½ hours after tumor inoculation reduced takes to 4 percent whereas saline washed controls had 100 percent take. Similar results were obtained with S-91 melanoma. This is an interesting lead because of the variety of acridines available and their pharmacological property of adsorption to nucleoproteins.

Effects of Cancer Upon the Patient

Clinical cancer has taken the place of syphilis as "The Great Imitator," for it can appear in every conceivable guise. This has always been true but has only recently become fully apparent as the patient with cancer has been recognized as a sick human being, rather than a technical

problem in early diagnosis, surgery, radiation or referral to a nursing home. The bewildering kaleidoscope of clinical cancer is in large part due to the capacity of cancer to infiltrate any organ, with resultant change in function, either as a single organ deficit or in unending combinations with damage to other organs, susceptibility to infection and emotional response. The temptation to study intensively these clinical fireworks is difficult for the good physician to resist. And this is fortunate for it leads to precisely the kind of intimate, compassionate and sophisticated care which is the right of every patient and the duty of each physician. It should be appreciated that this "research," vital as it is to the care of the patient and to the continued development of good doctors, contributes little to the understanding of cancer *per se*. This is perhaps not wholly true since some organ destruction of general character, such as of bone marrow in the leukemias or of lymphoid structures in the lymphomas, may relate to the nature of the disease.

In addition to the direct effects of infiltration, cancer produces a number of indirect responses remote from the site of tumor cells. Many of these indirect effects are brought about by chemical substances such as hormones manufactured by the cancer, and even though these do not bear any more relation to the nature of cancer than anatomic and physiologic alterations, they do provide ready-made labels by which tumor behavior can be followed quantitatively. In addition, there are indirect effects of unknown causation, such as nutritional depletion of the host which, if understood, might give clues to the mechanisms of autonomy.

These are the assumptions upon which we have based the clinical investigation of the behavior of cancer.

Direct Effects of Cancer Growth

Blood Forming Tissues—Platelets. The intensive study of mechanism of hemorrhage in the leukemias has continued. It has always been puzzling why some patients with marked thrombocytopenia should show no bleeding tendency and the question has remained as to the need to postulate a pathogenetic factor other than platelet deficiency. Drs. E. J. Freireich, L. R. Schroeder and L. Gaydos have been able to demonstrate in 92 leukemic patients with hemorrhage a direct relationship between degree of thrombocytopenia and risk of hemorrhage. Working in collaboration with Dr. A. Kliman (DBS), a technique has been developed for the separation and concentration of large numbers of platelets. The technique utilizes repetitive plasmapheresis of single donors. Twenty leukemic children with severe thrombopenia have been treated with these preparations; nineteen showed response with clinically significant increase in circulating platelets and control of hemorrhage. Contrary to expectation, no instance of immunization was seen even though patients received repeated platelet transfusions over a period of several months. The investigators feel that if larger amounts of platelets can be given and thus the treatment made available to adults, the way may be open to the more complete control of thrombocytopenic bleeding.

Dr. Schroeder has continued his search for a "platelet hormone" and has defined some of the conditions for platelet control in rabbits.

Leucocytes and Immunity. The effects of diffuse cancer upon leucocytes are quite complex because of the variety of cell types and functions arising in marrow and lymphoid tissues. The interference in leucocyte production is manifested largely by an increased susceptibility to infection. Studies by Drs. Frei, Paul Carbone and Fahey with the collaboration of Dr. J. P. Utz (NIAID) have helped to unravel some of the mechanisms of diminished host resistance. Thus, infections in patients with acute leukemias were shown to be due to rapid depletion of polymorphonuclear leucocytes without alterations in antibody production. Patients with chronic lymphocytic leukemia were shown to have frequent and severe infections which correlated well with inability to respond to antigenic challenge but only partially with the degree of hypogammaglobulinemia. In a controlled comparison, it was shown that prednisone treatment of chronic lymphocytic leukemia increased the frequency and severity of infections, gave no systematic anti-leukemic response and is contraindicated except for management of hemolytic anemia. Susceptibility to infection in other lymphomas seems unrelated to either above factor and is in some way related to inability to combat virus infections, as in seven adults noted with autopsy evidence of cytomegalic inclusion disease.

Dr. Fahey's approaches to the isolation and characterization of antibodies may help to investigate some of the above problems. Working with antithyroglobulin and antiliver nucleoprotein antibodies, he has shown that some antibodies were solely among the 6.6 S gammaglobulins, some were 18 S and some were present in both.

Gastrointestinal Tract. In addition to varied globulin disturbances, hypoalbuminemia is common in patients with cancer. Dr. T. Waldmann, in collaboration with Dr. R. Gordon (NHI), has developed techniques for studying the pathogenesis of serum protein abnormalities. These have been applied to the elucidation of hypoalbuminemic states relates to gastrointestinal disease as previously reported. New findings relate to hypoalbumineria in lymphoma. Study of 15 patients with lymphoma and hypoalbuminemia showed reduction of synthetic rate of albumin in all. Albumin survival was prolonged in two patients with effusions, while survival was shortened in the remaining 13. Albumin loss into the gastrointestinal tract was not observed.

Bone Metastases. The behavior of metastases in bone can be a valuable guide to the advance or regression of cancer. Unfortunately, changes in X-ray lag months behind biological behavior. Dr. L. Avioli has used Ca–47 in association with external scanning and metabolic balance studies. He has confirmed the marked elevation of bone formation and resorption in patients with lytic or blastic lesions. The external scanning with Ca–37 seemed to reflect regression or progression months in advance of the corresponding X-ray change.

Indirect Effects of Cancer Growth

The production in the host of chemically mediated effects is classically illustrated by endocrine active tumors, myeloma and carcinoid. In addition, there are a number of instances of apparent remote effects where no chemical product has been found and the obverse, in one instance, of a chemical product with no physiological disturbance.

Hormonal Products of Cancer. The endocrine program of Dr. Roy Hertz and colleagues encompasses many aspects of this problem. The resourcefulness of these investigators is shown in the development of quantitative assay procedures, application to the study of hormonal physiology in animals, and rapid extension to disease problems. Examples of this may be seen in their studies of adrenal cortical hormones. Dr. M. Lipsett, with the collaboration of Dr. H. Wilson (NIAMD), has developed methods for the isolation and quantitation of a wide variety of steroid precursors and end-products. Application of these procedures to patients with functioning adrenal cortical cancer has shown that in all patients there was an elevated excretion of delta-5-pregnenetriol, which percentagewise was greater than the increase in dehydroepiandrosterone. As a result of further analysis of the possible points of enzymatic block, they found that 11-beta-hydroxylation was generally impaired, and in two instances the block was complete. Pregnenetriol was excreted in elevated amounts in many of the patients, both with adrenal cancer and with hyperplasia. They also concluded that 21-hydroxylation was variably impeded and 50 percent of patients with adrenal cancer lacked 3-beta-hydroxydehydrogenase. In studies of the increase in dehydroepiandrosterone, they found that the desmolase could not dispose of the amounts of the precursor, 17-hydroxypregnenolone, presented to it; nor could they show that the increased dehydroepiandrosterone arose from exogenous cholesterol. Excess etiocholanalone excreted by adrenal cancer patients was shown to have unidentified precursors, other than androstenedione and dehydroepiandrosterone.

Drs. Lipsett and L. Garren examined a number of hypermetabolic euthyroidal patients with leukemia or lymphoma. Cortisol was metabolized at a normal or slowed rate, but cortisol secretion was increased. They concluded that the effect of hyperthyroidism on cortisol metabolism is due to a specific action of thyroxine and is not related to hypermetabolism *per se*. These techniques are also proving helpful in understanding the complexities of endocrine relationships in patients with pituitary tumors, hypophysectomy, genetic growth abnormalities and Cushing's syndrome associated with tumors of nonendocrine origin.

Dr. Hertz has made a number of observations on another chemical product of tumors—chorionic gonadotropin in patients with trophoblastic tumors. In the women with tumor, he found a

normal pregnandiol excretion indicating that the luteal phase of ovarian function was unaltered. Four patients with high gonadotropin excretion had normal menstrual cycles, reflecting lack of interference with normal pituitary gonadal relations. Not only does chorionic gonadotropin of pregnancy prolong the luteal phase of the normal cycle, it will stimulate the interstitial cells of the testes of hypogonadal males. In two such boys, the interstitial cells were stimulated by pure gonadotropin from patients with trophoblastic tumors. He concluded that the refractoriness of the ovaries in the tumor-bearing patient involves some special mechanism for interference with peripheral gonadal action. This refractory state appears late in the course of trophoblastic disease; at an earlier phase the ovaries show considerable enlargement.

Another difference between placenta and trophoblastic tumor has been found by Dr. D. Kellogg. Tumor tissue contained only a minute fraction of the steroid dependent transhydrogenase and dehydrogenase found in normal placenta. He also found by histochemical techniques that each enzyme had a different locus in placental cells, supporting the view that they are different enzymes.

Dr. S. Genuth has devised a method for the detection of minute amounts of gonadotropin in the rat hyperemia test. This assay is now applicable to the study of substrate influence on synthesis of gonadotropin by cell free systems.

An annoying fuzziness in gonadotropin assays resulted in findings which illustrate the ability of Dr. Hertz and his colleagues to capitalize on chance, as well as the importance of resolving discrepancies. Unexplained uterine stimulation in immature mice led to the discovery by Drs. Hertz and Tullner that an insecticide, used for ectoparasite control, was responsible. The insecticide proved to be a mixture of Methoxychlor isomers, analogues of the adrenocortical inhibitor, DDD. Further work showed that the mixture was uterotrophic for the mouse, even in the absence of adrenals or anterior pituitary, and that in parabiotic rats it inhibited the gonad stimulating effect of pituitary gonadotropin. It also stimulates testicular growth in male rats, but has little effect on the adrenal cortex of dogs. Further work proceeds with the pure isomers. Thus, alert pursuit of a discrepancy led to a new endocrinologic observation.

Indirect Effects on Erythrocytes. The mechanisms of anemia in the leukemias and other cancers have been reported previously by Dr. N. Berlin and colleagues. In the past year their attention has turned to tumors and diseases associated with erythropoietin production. Dr. T. Waldmann, in collaboration with Dr. Wendell Rosse, has found an increased mass and rate of synthesis of erythrocytes in patients with cerebellar hemangioblastoma, hypernephroma and pheochromocytoma. Non-dialyzable factors with marked erythropoiesis stimulating activity were isolated from the cystic hemangioblastoma and from the plasma and tumor homogenate of the patient with pheochromocytoma. Erythropoietin was also demonstrated in the urine and plasma of three patients with refractory anemia of unknown etiology. Autopsy in seven such patients revealed hemosiderosis of the liver, an organ which has been suggested as the site of erythropoietin degradation. Dr. Waldmann found that dogs did not destroy erythropoietin during a single passage through the liver. He has also shown in ingenious experiments with parabiotic rats that the kidney is the major but not the sole site of erythropoietin production. Two other findings of interest in the dog were that cerebellectomy caused no change in red cell mass but administration of desiccated thyroid was associated with a 25 percent increase.

Unexplained Indirect Effects of Cancer. One of the great enigmas of cancer is the mechanism by which the hot tissues are depleted during rapid tumor growth. In order to examine this, Dr. Berlin has developed techniques to determine gross body composition and total caloric expenditure. These methods have been applied to three markedly obese patients and nine with various neoplasms. He concluded that measurement of caloric expenditure in man, even with extensive tumor, is feasible but requires thirty days of observation on a metabolic balance regime. Agreement with caloric expenditure values calculated by the Newburgh insensible water loss method, was found in only one-half of the patients.

Dr. Bartter (NHI) and his colleagues have

published accounts of a hemodilution syndrome with urinary salt wasting occurring in association with mediastinal tumors, possibly related to inappropriate secretion of antidiuretic hormone (ADH). Drs. Tschudy and E. Hellman have found a similar situation in the crises of acute hepatic porphyria, apparently related to the hypothalamic lesions of this disease. Dr. Tschudy's work on the biochemical lesion of porphyria has led to a finding of interest in animal tumor pathogenesis. As noted previously, the livers of most tumor-bearing animals show a decrease in delta-aminolevulinic acid dehydrase, an enzyme on the pathway of porphyrin biosynthesis. However, a possible exception is found in tumors due to polyoma virus, where no decrease was observed until very late in the course of tumor growth.

Dr. E. McLaughlin has found a marked difference in the biological properties of serum of cancer patients as compared to normals. The method involves the effect of serum on the rate of mitotic activity in the regenerating livers of partially hepatectomized Sprague-Dawley rats. Dr. McLaughlin measured the livers for dry weight, mitotic counts and amount of tritiated thymidine incorporated into DNA. Serum from patients without cancer inhibited liver regeneration by all three measurements. By dry weight measurements, less than 50 percent of the liver regenerated. When serum from patients with cancer was injected, in excess of 70 percent of liver regenerated. This property does not fade when a primary tumor is excised or irradiated. Serum from animals with transplanted tumors inhibited regeneration, while that from animals with spontaneous tumors stimulated regeneration.

Management of Cancer

As pointed out previously, cancer in man by virtue of its ability to interfere with all bodily functions can induce physiologic infectional or emotional deficits in any combination. The adequate management of cancer must include their early recognition and correction. This report and previous reports have given many examples of the contributions of our clinical investigations to the further definition and control of such distortions of the bodily economy. One instance will suffice to illustrate the role of research in providing rational medical support to the patient with cancer. Drs. Paul Schwab and Fahey have continued their work on Waldenström's macroglobulinemia. As noted last year, the macroglobulinemia is associated with a marked rise in plasma viscosity. Several patients have been treated by repeated plasmaphereses, with reduction in macroglobulins and plasma viscosity. In addition, papilledema, retinal vessel dilitation and hemorrhages, and in one instance, congestive failure receded as the plasma viscosity was reduced.

Although these types of studies are an essential part of the rational management of the cancerous patient, they deal with remote effects and do not necessarily improve ability to control cancer. The modalities available to the clinician are the classical quartet of surgery, radiation, hormonal manipulation and chemotherapy. Endocrinologic aspects have been previously discussed; a few remarks follow on new information gathered in the other areas.

Surgery

Operative Effectiveness. Programs of curative surgery at NCI have been limited to relatively far advanced cancer of the head and neck, and of the uterine cervix. These studies are of sufficient size that it has been possible during the past year to obtain an estimate of the effectiveness of resection in this unusual type of extensive but still localized cancer.

Drs. Arons and Smith have analyzed the results in 89 patients with cancers of the head and neck, admitted between July 1953 and January 1960. All patients were followed through August 1, 1960, giving a range of followup periods of 6–76 months. Life table analyses were made of the 72 patients subjected to definitive surgery. At 2 years, 23 percent had clinical evidence of distant metastases, and at 3 years, 47 percent showed local recurrences. Forty percent survival at 60 months was calculated from the life tables.

Dr. Robert Hoye studied the metastatic pattern in the 42 patients who were autopsied. Forty-five percent of these patients had disease limited to a potentially curable site. Fifty-five percent of the patients had metastases below the clavicles; the lung was involved in all but one, but this was apparent ante-mortem in less than

half of the patients. This meticulous followup in both studies of head and neck cancer patients shows that although distant metastases are perhaps less common than with some other cancers, it could be demonstrated during life in one-fourth of the patients, and at autopsy in one-half.

Drs. Smith and John Dillon similarly reviewed their experience with 131 patients with carcinoma of the cervix, admitted between August 1953 and January 1959. Only 120 of the patients were proven to have uterine cancer, and of these, 117 were epidermoid cancer. Most of the cancers were relatively late; only three patients were classifiable as stage I. Management was as follows:

29 patients—clearly inoperable on admission
31 patients—explored and found inoperable
17 patients—radical hysterectomy and node dissection
8 patients—anterior exenteration
35 patients—total pelvic exenteration

Operative mortality for the 60 patients in whom resection was attempted was 2.3 percent. As of May 1960, 9 of the 17 patients who had hysterectomy, and 17 of the 43 patients who had exenteration, were alive and free of tumor. Of the 34 patients who have died, 9 were free of tumor. In the 7 patients with hysterectomies and recurrence, 6 were local and 2 distant; in the 24 patients with recurrence after exenteration, 12 were local and 18 were distant. Dr. Smith concludes that the surgeon should do more radical surgery in some cases selected for hysterectomy, but avoid it when disease is clinically extensive.

The two studies contain many valuable observations on the natural history of these common cancers, have helped to define the place of radical surgery, and have resulted in the complete acceptance of these procedures by the local medical community.

Complications of Radical Surgery. Two of the circumstances which sharply limit the safety and completeness of surgery are wound infection and hemorrhage. Studies during the past year by physicians of the Surgery Branch have added vital facts and have provided the means of prevention.

Wound Infections. Drs. Jack Bloch and Ketcham found 41 wound infections in 247 operations. Thirty of these were due to *Staphylococcus aureus hemolyticus*, coagulase positive, and 20 of these occurred in ischemic tissues. Twenty-four of the 30 patients carried staphylococci preoperatively, and where typing was possible, the types were the same—pre- and postoperatively. There was no evidence for cross-infections among patients, personnel or in the operating suite. A prospective study was then designed, with randomization and coding techniques, in which half the patients received chloromycetin (4.0 grams daily) and half a placebo. There was a 14 percent occurrence of wound infection in 43 chloromycetin treated patients, whereas 52 percent of the placebo treated patients had wound infections. The investigators concluded that (1) wound infections in their patients occurred largely as a result of operating in areas which contain some ischemic tissue and are preoperatively contaminated by staphylococci, and (2) under these conditions, prophylactic chemotherapy is required.

Operative and Postoperative Blood Loss. Although the need for replacement of blood attendant upon surgery is well understood, the use of the hematocrit as the measure of repair may lead to substantial under—or over estimates of amounts required. Drs. Ketcham and Hoye, collaborating with Dr. Berlin, have employed two techniques which allow for precise restoration. The first of these is the use of radioactive chromate labeled erythrocytes for the quantification of total red cell mass. Sixteen patients were studied pre- and post-operatively; blood was replaced as indicated by hematocrit. In three instances the total red cell mass was constant; in three patients there was a net gain of about 250 ml. of red cells. In the other 10 patients there was a failure to replace blood varying up to 800 ml. of red cells. In addition, an occasional patient who had been transfused up to a normal preoperative hematocrit was substantially anemic in terms of red cell mass. In the postoperative period, there was a further decrease in total red cell mass, indicating a loss of 160 to 477 ml. of red cells over 14 days; a loss which could not be recognized grossly or by means of the hematocrit. Thus, the repetitive study of red cell mass,

can avoid the dangers related to too many or too few transfusions.

The second technique employed in the operating room has been the utilization of the LaVeen-Rubricus blood loss meter, which by an estimation of blood chloride permits a direct cumulative measure of operative blood loss. Not only did they collect and estimate blood lost by suction, but also the blood on sponges, pads and towels. In nine patients there was a 370 to 2,700 ml. deficit in unreplaced whole blood. In an additional 7 patients, they also measured the blood on drapes and gowns which ranged from 350 to 1,465 ml. Further study during the 14 days after surgery showed further reductions in red cell mass. The surgeons concluded that: (1) the hematocrit is an unreliable guide in estimating operative and postoperative blood loss; (2) 15 to 42 percent of operative blood loss is on the drapes and gowns; (3) following radical surgery, the insidious loss of blood may be substantial, representing 9 to 44 percent of total red cell mass present immediately postoperatively.

Radiation

Radiation therapy, while curative of few histologic types of tumor, remains the principal means of providing prolonged regression of nonresectable cancer. Despite its pre-eminence in palliation, it has not been possible to expand seriously its curative potentiality by use of higher energies or better geometry. Tissue culture and animal evidence that chemical modification of the X-ray effect, or the use of other particles such as neutrons, give greater tumor damaging effects, has yet to be put to clinical trial. The program in the Radiation Branch during the past year has in the clinic continued to examine the usefulness of the 2-million volt external X-ray beam in the management of cancer, either alone or with chemotherapy, and its effects upon bone marrow and renal function. In experimental animals, studies have centered about the physiological processes during recovery from radiation injury and the effect of X-rays upon immune response.

Clinical Studies. Drs. J. R. Andrews and K. Brace have long been interested in time as a modifier of the radiation response. Clinical radiation therapy has classically been delivered during a 4-to-6-week period. Previous reports have shown that no advantage was apparent when the dose was given during a 100-day schedule. During the past year they have studied the effects at the other end of the scale, namely 2,500 r given in a single dose of 40 minutes' duration. Preliminary observations indicate a good tumor response without complications. While it will be impracticable to make a definitive comparison with standard dosage schedules, it should be possible to make some rough estimate of the effectiveness and safety of a single dose. If feasible, such a schedule would radically affect current practice of radiation therapy as well as permit a study of radiation as adjuvant on the day of surgical resection.

Dr. Bruce has made several studies of the enhancing effect of chemicals upon radiation responsiveness. He has shown that Actinomycin D, which sensitizes cells in tissue culture to X-rays, similarly increased the radiation response of skin. In three carefully studied patients, however, there was no sensitizing effect of this antibiotic on tumors. Similar observations were made on the enhancing effects of HN_2 upon radiation therapy of bronchogenic cancer in a collaborative study with the Eastern Group. Disappointingly, neither survival time nor tumor response was improved by the addition of the alkylating agent.

Drs. J. R. Andrews, Brace, J. Levin and Berlin have studied the effects of total body irradiation on erythropoiesis. Patients studied had either disseminated lymphona or chronic lymphocytic leukemia. They received 85–100 r with 2.5 MEV X-rays uniformly distributed to the entire body. The depression of peripheral counts was profound and reached a maximum for all elements at about 28 days after irradiation. The most sensitive index of bone marrow effect was the plasma iron disappearance rate. It showed maximum depression at 2 days and a return to normal one week after irradiation. There was also a curious second fall at 28 days. The therapeutic responses obtained from total body irradiation were striking;—especially in the patients with chronic lymphocytic leukemia, where normal counts were still present 6 months after therapy.

Drs. L. Avioli and Brace have undertaken a systematic study of the effects of abdominal X-irradiation upon renal clearances. Patients with large X-ray sensitive intra-abdominal tumors have been given 400–500 rad in single doses with-

out consistent change in glomerular filtration rate, renal plasma flow or TmPAH.

Dr. Andrews and his colleagues have continued their studies of the use of S_{35} in the treatment of chondrosarcoma. Two additional patients have been treated during the past year. It is clear that these cartilaginous tumors variably concentrate the S_{35}, presumably in chondroitin sulfate, and that the therapeutic utility is limited by bone marrow depression. Only one of the five patients is still in remission after administration of the radioisotope.

Animal Studies. Dr. Roger Berry, who has recently joined the staff, has adapted a method developed in England for *in vivo* examination of chemical enhancement. He used a lmphocytic leukemia in the ascites form in DBA/2 JN mice. Serial dilutions of the ascitic fluid were injected into groups of mice in sufficient number to obtain a 50 percent take value, thus permitting quantitative estimation of X-ray dose-response characteristics with and without chemical or physical adjuvants. A dose response curve for 3 MEV X-rays has been established. If hydrogen peroxide is given intraperitoneally prior to irradiation, a steeper dose response slope was obtained; the "oxygen enhancement" ratio approximating 2.5:1. In addition, Dr. Berry studied the combined effects of X-rays and 5-bromodeoxyuridine or 5-iododeoxyuridine. Both combinations produced a marked decrease in the number of surviving cells compared to either modality used singly. Using the same system, with the kind cooperation of the staff of the Brookhaven National Laboratory, Dr. Berry has established a dose response curve for fission spectrum fast neutrons.

Dr. W. Smith has continued her survey of the processes involved in spontaneous and induced recovery from irradiation damage. She reports that (1) the time of spontaneous granulocyte recovery varied with age in mice and correlated with ability to survive irradiation, and (2) in granulocyte mobilization studies, the ratio of mobilized to nonmobilized cells was similar for irradiated and normal mice. Endotoxin injection, which improves survivorship, caused a fourfold increase in granulocytes.

Dr. F. Smith has studied the effect of irradiation upon the ability of mice to form hemolysin to sheep erythrocytes. Irradiation diminished antibody production and this was a function of total dose rather than rate administration of X-rays. In rats, he found that altitude acclimatization increased capacity to form antibody hemolysin. Dr. Smith has also studied immune responses in C_3H mice with the plasma cell tumor X–5563, which were given X-irradiation and specific antibody. Return of the immune response to near normal limits was observed in the tumor-bearing mice immunized as early as 24 hours after irradiation.

Dr. C. Maxwell in his long-term studies of the effects of irradiation of amino acids has added a thirteenth compound, glycolic acid, to the list of materials appearing when solutions of glycine are irradiated with X-rays. Dr. P. Riesz has evidence for two kinds of hydrogen atoms differing in chemical reactivity which appear in the abstraction reactions resulting from X-irradiation of water. Dr. H. Andrews found that mice can sense an X-ray beam under some circumstances. This is probably related to ozone production. He has also developed dose response data for swine from the mid-lethal range to several thousand r. Offspring from heavily irradiated parents had a dose response curve identical to those of the parents.

Drs. M. Kelly, R. W. O'Gara and T. L. Loo have continued their studies of chemical protection against X-rays and radiomimetic drugs. Previous studies have shown that amino-ethyl-isothiuronium (AET) protects against both and that those tissues which highly localize the drug receive the most protection. C_{14} labelled AET has been used to study its intracellular localization by microautoradiograms. It was found distributed uniformly throughout the cytoplasm, without intranuclear incorporation. Compounds which generate free radicals have been found to be radioprotective. Di-t-butyl peroxide or t-butyl perbenzoate was injected intraperitoneally in mice or rats 30 minutes before HN_2 or whole body X-irradiation. These agents reduced the lethal effectiveness of HN_2 or X-ray by about 50 percent. In studies with Dunning leukemia, neither drug interfered with the therapeutic effectiveness of HN_2.

Chemotherapy

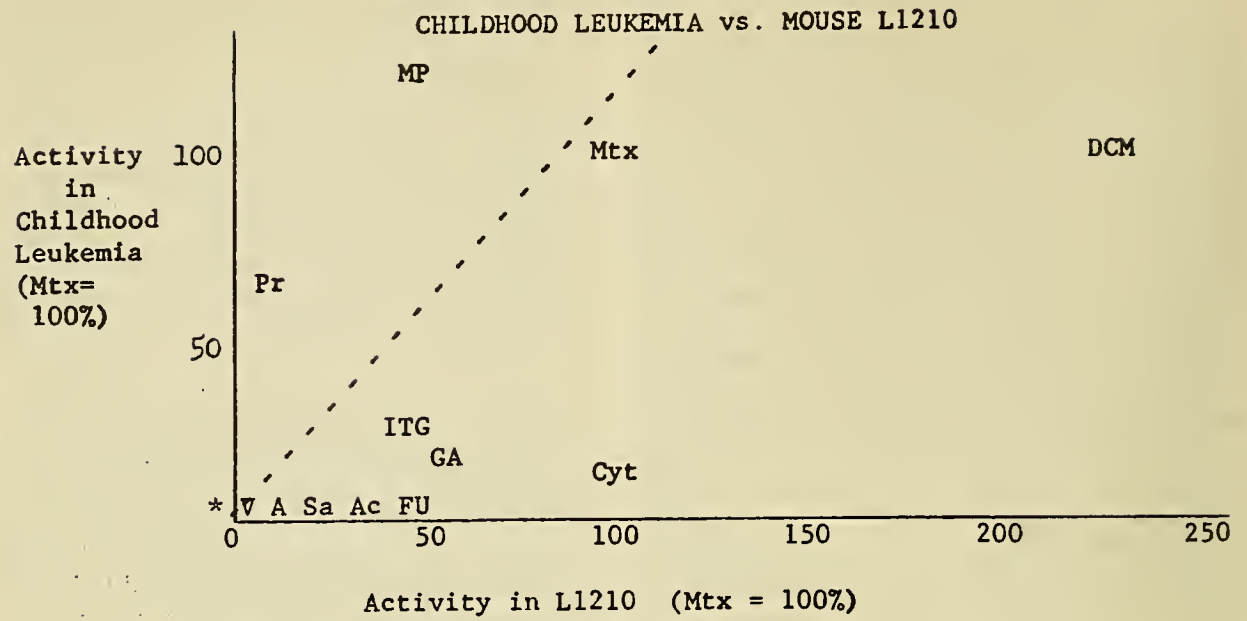
General Remarks. Cancer chemotherapy has reached a new phase, and it is time to adjust attitudes and planning to this perspective. From the observations of Dr. Hertz and his colleagues on trophoblastic tumors, it is clear that a drug can induce at least 5-year survival of patients with cancer. This means that a drug, in one type of cancer, not only assumes a place with surgery and radiation as primary therapy for cancer, but also can accomplish what surgery and radiation could never attempt, the control of diffuse metastatic disease. One problem then is the exploitation of this success, to identify the screening system which could have predicted it. This concept should be extended to the whole search for chemical control of cancer—namely, the task of proving the value of a biological system which can predict drug responsiveness for each of the many diseases known as cancer. Five years ago there was confusion in drug screening because of the number of variables and the lack of quantitative data for either man or animal. Due to the efforts of CCNSC, the cooperation of scientists throughout the country, and I believe the leadership of several NCI scientists and physicians, quantitative data have been gathered and the beginnings of such screening correlates are within our grasp. Predictability seems highly probable for acute leukemia, possible for choriocarcinoma, and other screens, both transplanted animal tumor and tissue culture, are partly categorized for the compounds they find or miss. Finally, the easy convenience of transplanted rodent tumors has been in part abandoned for exploration of the more difficult screening techniques of spontaneous tumors and those induced by viruses, carcinogens and radiation. For the above reasons, it is possible to abandon in large part any random search for the panacea, and to focus on particular cancers. This means that the start must be made with man and that the basis for establishment of screening specificity should be the chemicals which control specific cancers in man.

The Intramural Chemotherapy Program of NCI. The intramural program has been developed on the assumption that it was an integral part of the national effort in cancer chemotherapy, and yet had resources and interests of its own. It was felt that thus our scientists could actively share work load and data with others similarly motivated, and still be able to pursue imaginatively their own research ideas. Since it was undesirable to duplicate expensive facilities, the intramural group developed no synthetic program or primary screen of its own, but depended entirely upon CCNSC. As its share, the intramural program pioneered with quantitative screening, developed data on toxicity and pharmacology of new agents and was responsible for the leadership of two of the clinical groups. The ground to be covered was so vast, the objectives so uncertain, that several years of trial and error were required to develop rational methodology. During the past 5 years many of these methodologic puzzles have been solved, and the intramural program is ready to plan the next phase, that of utilization of calibrated techniques for the uncovering of chemical control of some specific cancers.

Specific Anti-Tumor Agents. (1) *Antifolic Compounds*: Drugs with antifolic activity were among the first antitumor agents to be discovered. Synthesis of these compounds was extraordinarily difficult, and after the first few compounds failed to show increased activity in microbiological systems, chemists turned with relief to the newest drug, 6-mercaptopurine. Despite the ease of synthesis of the antipurines, 6-mercaptopurine has proved of narrow utility. Methotrexate, an antifolic, not only induces complete remission in acute lymphocytic leukemia and metastatic trophoblastic tumors, but has been shown by Dr. Van Scott to cause regression of basal cell cancer, and by Dr. Frei and his colleagues to cause regression in several lymphomas. NCI, thanks to CCNSC and intramural joint efforts, has been able to undertake a broad structure-activity synthesis program with Southern Research Institute.

One of the reasons for the interest in antifolic synthesis, has been the discovery by Dr. Goldin and colleagues of the increased activity of antifolics brought about by halogenation in positions 5 and 6. As previously reported, he found in advanced L1210 leukemia in mice that dichloromethotrexate or bromochloro-methotrexate as compared to methotrexate, effected a threefold

COMPARISON OF THERAPEUTIC INDICES[2]



*Azauracil, Nitrosoguanidine, Narcotine,
V=Vincaleucoblastin, A=Actinomycin D, Sa=Sarcolysin, A=Azauridine,
FU=Flourouracil, Pr=Prednisone, MP=Mercaptopurine,
Mtx=Methotrexate, ITG=Imidazolylthioguanine, GA=Guanylhydrazone,
Cyt=Cytoxan, DCM=Dichloromethotrexate

increase in survival time. This quantitative screen has developed into a predictability system of considerable value for acute lymphocytic leukemia as shown in the accompanying chart developed by Drs. Frei and Goldin:

As a working hypothesis, those compounds with greater than 50 percent of the activity of methotrexate can be expected to be active in acute lymphocytic leukemia. It has been disappointing to recognize that the most active compounds yet found, the halogenated antifolics, have not been similarly more effective in leukemia. Dr. Frei and colleagues, working with Leukemia Group B, found that oral dichloromethotrexate was no more active than methotrexate in acute lymphocytic leukemia. The reason for this was thought located when Dr. Goldin reported that in L1210 oral administration of dichloromethotrexate was associated with sharp loss in therapeutic effectiveness. Repetition of the clinical study by Leukemia Group B, using parenteral drug, did not appear to increase activity in acute lymphocytic leukemia. Intensive studies of the pharmacology of dichloromethotrexate by Drs. Oliverio, T. L. Loo, J. D. Davidson, D. Rall, and Mr. R. Dion, have provided a clue though perhaps not the full solution, to the discrepancy By the use of DEAE ion-exchange columns, they were able to separate and qualify various folic acid analogues and found that 40–50 percent of dichloromethotrexate was excreted in the urine in the 24 hours following a parenteral dose. Moreover, one-fifth of the dichloromethotrexate was in the form of a metabolite, characterized as the 4:7-dihydroxy derivative. They also synthesized dichloromethotrexate containing radioactive chlorine, and found that it was rapidly excreted in the bile of rats, mice, dogs and man. Since it is poorly absorbed from the intestine, biliary excretion effectively removes the drug from the blood. The parent compound because of these

[2] The rational use of animal screens as predictability systems for cancer in man requires quantitative data such as these. It has taken 5 years of intensive effort to collect data for this figure. Note that it involves one animal tumor and a single disease. A similar figure cannot at this time be constructed for any other cancer in man. The magnitude of finding curative drugs for several cancers in man should not be underestimated.

two processes rapidly lost its effectiveness. This does not explain the discrepancy with the screening animal, since qualitatively at least the pharmacological differences between mouse and man are not great.

Drs. Hertz, Lipsett, Ross and colleagues have now treated 68 women suffering from trophoblastic disease with antifolics. Forty-five percent of the patients are in complete remission after methotrexate treatment. The first few patients treated have sustained their remissions for over 5 years. The possibility that the therapeutic response is related to maternal immunization by fetal tissues cannot be supported. In the first place, methotrexate seriously interferes with many immune phenomena. Secondly, Dr. Hertz in a study of red cells and plasma from 35 couples in the above series, could find no evidence of maternal immunization. The patients who have relapsed have been refractory to nitrogen mustard, cytoxan and actinomycin D. However, 6 of 14 methotrexate-resistant patients have responded to an alkaloid, vincaleukoblastine. Two of these have remained in complete remission for 8 and 10 months respectively. The manner in which this compound was selected is highly pertinent to the previous discussion of screening. Dr. Hertz has been able to establish heterologous growth of human choriocarcinoma in the cheek pouch to the untreated hamster in 6 of 35 attempts. Some of these were established prior to treatment and are methotrexate-sensitive. Others are methotrexate-resistant and are used to screen potential antitrophoblastic drugs. Two active compounds have been found, vincaleukoblastine and podophyllotoxin, and it is of interest that neither has activity in the L1210 screen or in acute lymphocytic leukemia. Thus these two methotrexate-sensitive tumors—trophoblastic disease and acute lymphocytic leukemia will likely have entirely different screens for predicting active drugs.

(2) *Purines and pyrimidine antagonists*: The discovery that 6-mercaptopurine was an effective drug in the leukemias brought a new measure of hope for the chemical control of these diseases. Not only was 6-mercaptopurine less toxic than the antifolics, it had activity in the myelocytic leukemias, both acute and chronic, as well as in the acute lymphocytic form. Dr. Frei and his colleagues in collaboration with Leukemia Group B have completed their definitive comparisons of 6-mercaptopurine and methotrexate, as reported last year. They have gone forward in their methodologic studies to examine the usefulness of sequential design in 6-mercaptopurine treated acute leukemias. All previously untreated children with either type of acute leukemia were given prednisone. When remission occurred the patients were placed on continuing 6-mercaptopurine or placebo. The duration of remission was used as the measure of effect and it was quickly shown that a small number of patients could demonstrate the active drug. Moreover this design, since it incorporates initial therapy with steroids, permits the use of new drugs in the study of untreated patients. The safety of this maneuver is further reinforced by the fact that relapse after steroids plus new drug can be fully treated with 6-mercaptopurine or methotrexate. Finally, it has the further advantage that new drugs can be given while the patients are in steroid remission, and there is little danger of confusing toxicity with the complications of acute leukemia.

The screen which is best for indicating antipurine type of activity is CA 755. Dr. Goldin and his colleagues have developed this system into a reliable quantitative measure of comparative activity of thio-purines. The only compound of greater activity than 6-mercaptopurine has been its riboside. Preliminary studies in man indicate little advantage, but the definitive comparison is in the future. Imidazolyl thioguinine, which is somewhat less effective against 755, had less activity than 6-mercaptopurine in 52 patients with acute leukemia treated by Leukemia Group B. Recently, CCNSC held a conference about the thiopurines in which Drs. Goldin and Frei participated. There was general agreement to limit the synthesis of these compounds until 6-mercaptopurine riboside activity in man is fully explored, and more is known about the effect of 9-ethyl, and 9-butyl-6-mercaptopurine in man.

In spite of the failure of the synthesis efforts to produce better drugs, 6-mercaptopurine has provided an excellent model for biochemical studies of resistance. Dr. Davidson has previously reported studies of L1210 leukemia in which resistant leukemic cells showed reduction in inosinic phosphorylase. Studies in the past year

with the isolated enzyme have confirmed the cellular findings of competition between 6-mercaptopurine and hypoxanthine for inosinic phosphorylase. Dr. Davidson also has developed new techniques for the assay of the enzyme in human leukemic cells. Eighteen patients have been studied and all showed some ability to convert hypoxanthine C_{14} and 6-mercaptopurine S_{35} to nucleotides. Four apparently 6-mercaptopurine resistant patients have been studied and two of these had normal amounts of the enzyme, suggesting that their resistance was not the same as that of mouse leukemia L1210.

Other studies of leukemic leucocytes with a pyrimidine antagonist, azauridine, have been carried out by Dr. H. Fallon. Azauridine is known to inhibit orotidylic decarboxylase, essential for pyrimidine biosynthesis. Dr. Fallon has found that the enzyme in leukemic leucocytes is profoundly inhibited by drug. Azauridine causes a sharp fall in the peripheral leucocyte count of leukemic patients and this correlates well with enzyme inhibition.

Finally, intrathecal 6-mercaptopurine riboside is being used for the treatment of leukemia involving the central nervous system. As reported last year, central nervous system leukemia is occurring with high frequency and is a common cause of treatment failure. This is largely because of failure of methotrexate or 6-mercaptopurine to achieve therapeutic concentration in the central nervous system. Dr. Rall and colleagues have continued their studies of the principles of drug transfer from blood to the central nervous system. Working both with dogs and dogfish, they have evidence that the failure of acids, such as sulfanilic and para-amino hippuric, to achieve high concentrations, in spite of continuous high blood concentrations, is due to bulk out-flow of cerebrospinal fluid (CSF), removing fluid and drug at such a rate that inward transfer rates are inadequate to reach a ratio of unity. Drs. Rall and Streicher (NIMH) have for the first time found a compound, thiocyanate, which has a variable CSF/plasma ratio related to plasma concentrations. At plasma thiocyanate concentrations of 8–10 mM/L, the ratio approaches unity, while at 1–2 mM/L the ratio is 0.1. They suggest that this is evidence that the bulk return of CSF into blood is an active process.

Binding to proteins limits passage of drugs into the CSF. Dr. Rall and his colleagues, in studying the protein binding in various species, have noted the absence of albumin in the dogfish with predictable effects on central nervous system transfer of drugs. They also report the absence of albumin from the plasma of sea lamprey larvae (*P. marinus dosatus*). Associated with metamorphosis into the adult, there appears a new plasma protein, with electrophoretic characteristics different from mammalian albumin.

(3) *Alkylating agents:* The first non-hormonal drug shown to affect disseminated cancer was nitrogen mustard. It clearly can cause substantial regression of Hodgkin's disease and other lymphomas, both forms of chronic leukemia, mycosis fungoides, neuroblastoma and adenocarcinoma of the ovary. It has erratic and insubstantial effects in other epithelial tumors and none in the acute leukemias. In the 15 years since the discovery of nitrogen mustard, many other alkylating agents have been synthesized, shown to have activity in a variety of transplanted tumors and a few put into clinical trial. The programs of NCI, both of CCNSC and the intramural area, have undertaken considerable research concerning alklyating agents. Both groups have participated in recent conferences attempting to interpret the vast amounts of information. It is clear that alkylating agents vary considerably in quantitative and qualitative effectiveness in transplanted animal tumors. There is some evidence that this is also true in man—for example: (1) Myleran has much greater activity in chronic myelocytic leukemia than in any other disease; (2) Cytoxan has a modest degree of activity in acute leukemia, whereas the other alkylating agents do not; (3) Cytoxan has relatively little effect on megakaryocytes and platelets as compared to other alkylating agents. Nevertheless the degree of difference found in transplanted animal tumors is not generally reflected in man. I believe the difficulty may represent in an outstanding way what has been said above about screening. The guiding principle of the past has been to find a generally active drug in animal tumors which will be generally active in human cancer. What is needed, before the lead of the alkylating agents is abandoned, is specificity—particular animals systems which can dependenably predict activity in a single human cancer. We have nowhere in alkylating agent research approached the knowledge

we have about L1210 and acute lymphocytic leukemia and Ca 755 and myelocytic leukemia.

NCI intramural scientists are participating in several efforts to find such specificity, using transplanted rodent tumors and induced tumors. With the former, Dr. Goldin has attempted to develop Sarcoma 37 into a quantitative screen. This tumor has been unaffected by all the standard drugs, and it was hoped that it would be an effective screen for alkylating agents of high potency. Recently cytoxan and alanine mustard have been shown to be active, but the meaning of this in the clinic has yet to be determined.

Dr. Leon Schmidt of Cincinnati has undertaken a thorough study of the value of rat transplanted tumors in predicting drugs for specific cancers. He has found that relatively slight alterations in experimental design can change markedly the relative activity of an alkylating agent. With control over these experimental variables, he is now able to rank various major alkylating agents for order of effectiveness and is cooperating with clinicians and CCNSC in trying to relate these to clinical predictability. Because of the mass of data, this will probably be solved only with the help of computers. Dr. A. Pratt of the Physiology Laboratory and several NCI clinicians have worked with Dr. Schmidt in developing mathematical models suitable for the examination of this problem. When more clinical information is available on newer alkylating agents, the specificity (or the lack of it) of these rat systems can be understood for the first time.

Since it is possible that transplanted animal tumors may not provide the required specificity, nontransplanted tumor systems are being developed. Dr. Goldin and his colleagues have developed the Moloney leukemia virus into a partially quantitative system. They have also been attempting to use similarly the spontaneous breast tumors arising in the mouse colony at NIH. In both instances preliminary results with drugs warrant the development of larger supplies of these tumor types.

Drs. M. Kelly and R. O'Gara have been studying drug effects in carcinogen-induced tumors. Chemical carcinogens were given to new-born mice; they developed pulmonary tumors, leukemia and fibrosarcomas. Drug therapy was given from age of 8 weeks to 16 weeks, and the frequency of pulmonary tumor nodules noted. In preliminary observations alkylating agents or cortisone had more effect than antimetabolites.

o, p'-DDD (2,2-*bis* (2-chlorophenyl-4-chlorophenyl)-1,1-dichloroethane)

Dr. Hertz and his colleagues have continued their studies of the effect of this adrenal cortical inhibitor upon adrenal cortical cancer. Eighteen patients have been treated and all but 4 have shown depression of tumor hormone output. Seven patients have had regression of metastatic disease and clinical improvement. Damage to normal human adrenals has also been demonstrated. Pharmacologic studies have shown that much of the drug appears in the stool after oral administration. Nevertheless infusions of the drug in Lipomul did not increase activity. The drug is concentrated in fat depots which rapidly remove circulating compound.

This compound is of interest because it was discovered without the aid of an animal tumor screen, since to date it has not caused regression of animal tumors. Since it does not affect normal adrenal activity of rodents, "screening" for activity of analogues is carried out in the dog, whose normal adrenal cortex is highly sensitive. Dr. W. Tullner has found that duration of treatment and drug dose of o,p' DDD was correlated with degree of cellular destruction in the male dog. He prepared animals in which damage was confined to zona reticularis and zona fasciculata with the zona glomerulosa appearing normal. After cessation of drug, the adrenal secretion of 17-hydroxysteroids was reduced to extremely low levels. Gradual recovery of hormonal output and normal histology appeared in 4 weeks and autopsy showed regeneration of zona fasciculata. Dr. Tullner concludes that the zona glomerulosa is multipotential and even though it produces aldosterone and does not contain the enzymes for hydrocortisone production, it can give rise to fasciculata cells which can carry out this hydroxylation. He has also shown that intravenous difluoro, dinitro DDD was as active in the dog as o,p' DDD, but possessed no advantage over that compound.

Conclusion

In conclusion, I shall indicate several areas where resources not now extant are required. Additional laboratory space in the Clinical Cen-

ter is clearly inferred from the need to have deeper staffing. An "Operations Research" group is required to consider in a formal way clinical data management—its collection, coding, analysis —and the development of mathematical models for computational examination. Several areas are in need of primate breeding facilities for carcinogenesis studies in the new-born as well as for trophoblastic tumor pathogenesis. Resources are in demand for large mouse colonies for spontaneous tumors and for special isolation facilities for chemotherapy and other studies of virus-induced tumors. Personnel and facilities should be planned for a systematic examination of predictability value of animal systems for toxicity of drugs for man. In this regard electron microscopy of drug damaged structures will certainly be needed as light microscopy is non-specific for such pathology. Finally, personnel and resources should be planned for the expansion of clinical cytogenetics.

I should like to extend to the Director, NCI, and Director, Clinical Center, and their staffs my gratitude for the kind and effective manner in which they have met the mercurial demands of the clinical program. May I also express my appreciation to Dr. Stuart Sessoms and his staff for their extraordinary help in working with us in the chemotherapy program. I want to make known my thanks to the Laboratory Chiefs and their staffs for their kindly help to the clinicians. I am grateful to Mr. Walter Magruder, Mrs. Martha Rees and the secretaries of OADR for their cheerful help in carrying out the functions of my office. Finally, it is a pleasure to acknowledge my indebtedness and thanks to the Clinical Branch Chiefs and all their physicians and to Miss Burgess and Mrs. Kilian and their staffs for courteous support in many ways, but especially in bringing to NCI patients the superb medical care which is the beginning of all clinical investigation.

NONCLINICAL INVESTIGATIONS[3]

Members of the staff who participate in the nonclinical programs continue to create new knowledge that enhances our understanding of cancer, entails development of new concepts, and provides a reservoir of laboratory findings important for future application to the problems of cancer and other diseases. The joint presence of this group of research workers and the clinical investigators within one sizable Institute provides the means for an exchange highly beneficial to both groups. The function of the nonclinical intramural program may be viewed as the creation of new knowledge that is well ahead of that required for application to problems of man, and the yields of the program form a wellspring which must be maintained with a vigor that insures a wholesome level. Among the most important ingredients is the continual supply of new concepts since they are the key to bringing new approaches to bear on the cancer problem beyond those receiving current consideration. Another important ingredient is the development of concepts already at hand to a stage sufficient for adequate evaluation. Collaboration by investigators in different disciplines, founded upon mutual interest, understanding, and respect, can do much to bring about new concepts and to speed along their development. In a number of cases, sizeable groups, ably led, provide greatest progress. In other cases, the mature investigator working independently makes his greatest contribution. Oftentimes the investigators who create new concepts and make initial development of them can make their best contribution when they do not carry through the application of the findings, but rather are left free to develop new concepts, understanding and evaluation from the purely scientific standpoint. In other cases, the investigator who initiates a study may effectively carry it through to a point where its applicability to an evident problem is attained.

The summarization which follows of the research progress of the nonclinical intramural staff attests the continuing highly important contributions made by the Institute staff. While all areas of cancer research are not fully represented, the staff is producing research of a very high order in many areas believed to be of significance for the future development of cancer research.

Studies on the Induction of Cancer

Several investigators throughout the Institute continue to make contributions toward our under-

[3] Prepared by Carl G. Baker, M.D., Assistant Director (for Research).

standing of the factors underlying the causation of cancer. Many aspects of this problem warrant increased effort, both in terms of the materials in the environment to which man is exposed, singly or in combination, and the mechanisms within the living organism that are involved in initiation of the cancer process.

Chemicals

Studies progress on the organic fractions of the particulate phase of urban air pollutants, as part of a comprehensive and prolonged program dealing with the relationships between air pollutants and health hazards, by Drs. W. C. Hueper and W. W. Payne in collaboration with Drs. P. Kotin and H. Falk (University of Southern California) and Messrs. E. Tabor and E. Sawicki (Taft Sanitary Engineering Center). Experiments with mice receiving the powerful carcinogen, benzo[a]pyrene, which serve as a reference standard for comparing relative potency of the solvent fractions of air pollutants, confirmed the concept that a low-level recurring exposure to a carcinogen is more hazardous than a single exposure to the same amount. In addition to confirming the presence of benzo[a]pyrene in samples collected by filtration of air from eight large United States cities, the group has also shown carcinogenicity of samples not containing benzo [a]pyrene. Distinct variations in composition of air pollutants from different cities were demonstarated in terms of biological and chemical parameters. The percentage of tumors observed differed with the chemical fraction studied as well as the city from which the sample was obtained. No consistent correlation between the degree of carcinogenic potency, the amount of benzo[a]pyrene per sample, and the lung cancer mortality rate reported for a given city was found.

Preliminary studies on testing eluates of carbon adsorbates from raw river waters for carcinogenicity have produced several cancers in mice receiving samples subcutaneously.

Drs. Hueper and Payne have extended the studies on carcinogenicity of chromium-containing materials. Chromite ore roast, which contains various compounds of chromium with different solubilities, produces tumors in rats, and the list of chromium compounds shown to be carcinogenic has been extended, with the addition now of a trivalent chromium compound along with other hexavalent species. Moderately soluble (water) hexavalent chromium compounds display the highest carcinogenicity.

Prolonged inhalation by rats of dusts of chromic oxide and the chromates of barium, lead and zinc, compounds widely used as pigments and hence in contact with many workers, has produced green deposits, adenomatous changes and squamous cell metaplasia in the lungs of the exposed animals, but no primary cancers.

Because tumor-inducing effects of certain-water insoluble polymers have been interpreted by some investigators as resulting from physical factors, e.g., surface forces, rather than chemical ones, Dr. Hueper investigated the tumor-producing abilities of chemically different polymers of this type implanted subcutaneously or intraperitoneally in rats and mice in different physical forms, such as cube, sheet, disk, film foam, and powder. Although data obtained with polyethylene are consistent with the interpretation that physical factors play the key role in carcinogenesis with polymers, the data obtained with polyurethane are against the concept.

Because previous testing of polyvinylpyrollidones (PVP) containing a wide range of molecular weights gave inconsistent results as to their carcinogenic properties and the variability of the findings might have been due to differences in retention of some of the larger polymer species, Dr. Hueper re-evaluated the carcinogenic properties of PVP preparations containing either small (average molecular weight of about 10,000) or large (average molecular weight of about 50,000) molecular species. Although deposits of homogeneously staining material were seen in various tissues in the rats and rabbits injected with PVP of both large and small molecular species, the cancers that appeared were the same as those seen in the controls (frequency, location and morphological types). The smaller molecular weight PVP molecules were retained by the glomerular filter in the kidneys of the rat, but not in those of the rabbit.

Several investigators have produced cancers of the urinary bladder in mice by surgically implanting pellets of paraffin wax or cholesterol containing a carcinogen into the bladder by a method developed by Jull. However, bladder

tumors have also appeared in the control animals with pellets of paraffin wax or cholesterol alone. Drs. W. Conway, W. Hueper and R. Feldstein and Miss E. Lethco have, therefore, prepared pellets made of synthetic wax (hexadecyl palmitate) and have incorporated into the pellets in addition to the dyes under test, varying amounts of powdered cellulose to provide greater permeability of fluids into the pellets. They have shown that more rapid release of the test substance from the pellet occurs with higher proportions of cellulose, thus providing the means for controlled release of the potential carcinogen under study. With this new technique for evaluating substances for carcinogenicity, several azo dyes are under study.

Dr. Conway and Miss Lethco are also investigating the metabolism of several azo dyes by other techniques. Utilizing sensitive colorimetric and radioactive tracer techniques, they have observed several colored and colorless metabolic products in the urine. The percentage of the dose which appears as urinary metabolites increases as the dose decreases.

Drs. M. G. Kelly and R. W. O'Gara have added to their observations on chemical carcinogenesis in newborn mice. After implantation of either 0.02 mg. dibenz(a, b)anthracene (DBA) or 0.011 mg. 3-methylcholanthrene (MC) into noninbred albino mice during the first 18 hours after birth, 100 percent of the animals developed pulmonary tumors by 16–29 weeks. Subcutaneous fibrosarcomas arising from perimysium appeared at the site of injection of either carcinogen. Mice given 3-methylcholanthrene developed fulminating leukemia 9–16 weeks after injection. In collaboration with Mr. Nathan Mantel, the dose response relations were studied. The smallest single doses producing tumors are: Fibrosarcomas—0.0002 mg. DBA and 0.0004 mg. MC; lung tumors—0.0067 mg. DBA and 0.0037 mg. MC. At high doses of MC, 30 percent of albino mice developed leukemia and 70 percent of C3H male mice developed hepatomas. The production of tumors with such small doses suggests the value of the newborn animal as a test subject for potential carcinogens of greater sensitivity than that available formerly.

In studies aimed at inducing lung cancers, Dr. M. Stanton and Messrs. R. Blackwell and J. Albrecht have produced metastasizing squamous-cell carcinomas of the lung in rats by intravenous injection of a suspension of methylcholanthrene in tricarprylin carried to the lung in an halogenated aliphatic hydrocarbon (hexachlorotetrafluorobutante). The tumors apparently arose in areas of bronchial metaplasia, probably arising from halocarbon-produced infarction. Ultraviolet light microscopy indicated deposition of methylcholanthrene at the tumor site.

Dr. E. W. Chu, with Dr. K. M. Herrold, is engaged in a study of the effects of carcinogens on the female genital tract of Syrian hamsters. Hamster cervices painted with 9, 10-dimethyl: 1, 2-benzanthracene showed atypical cytological changes in the vaginal smears after 1½ months, and definite carcinomatous changes in 2 months. 3, 4-Benzpyrene-painted cervices showed comparable cytohistological findings after a much more prolonged period. Cytological changes have been noted in vaginal smears obtained from animals whose cervices were painted with tobacco tar dissolved in acetone only after 10 months.

Of 400 hamsters that received pellets containing suspected carcinogens surgically implanted into the cheek pouch, Dr. L. Dunham found that 24 animals displayed lesions in the pouch, of which 19 were inflammatory in nature. One squamous papilloma was seen and four examples of epithelial hyperplasia were noted.

Further work on the hemangioendotheliomas of liver and spleen that developed following intravenously injected Thorotrast (colloidal thorium dioxide) has been performed by Dr. R. Swarm. Metastases to spleen and liver occur following subcutaneous transplantation. Dr. P. Mori-Chavez has found visceral metastases with these tumors more frequent at high altitudes (in Peru) than at sea level. Dr. Swarm has also reviewed in several European laboratories tissue specimens from human patients who were given thorotrast.

Dr. M. Potter is investigating mouse strain relationships and the induction of leukemia by skin painting with methylcholanthrene. Approximately 80 percent of one-month-old DBA$^{f}/_{2}$ mice painted with the carcinogen developed generalized leukemia. No leukemias appeared in strain BALB/c mice which had been painted in a similar way. The F_1 hybrids of BALB/c and DBA/2 also did not develop leukemia after skin painting. The animals of the first generation

backcross to $DBA^f/_2$, however, had an incidence of about 25 percent leukemia. Studies are being extended to a family study of second generation backcross mice.

Mechanisms of Carcinogenesis

Dr. H. Sidransky is investigating a number of factors influencing the pathogenesis of rat hepatomas induced by certain chemical carcinogens. Although liver regeneration in partially hepatectomized rats is somewhat inhibited in rats ingesting ethionine in comparison with rats on the control diet, the histologic changes in the liver of the experimental animals are similar to those seen in nonhepatectomized animals ingesting ethionine. However, rats with partial hepatectomies and ingesting ethionine appear to have a delayed induction period for hepatomas. *p*-Hydroxypropiophenone, which inhibits the development of liver tumors induced by butter yellow, was found to inhibit ethionine-induced hepatomas. With Dr. H. Morris, Dr. Sidransky found that male rats show a higher incidence of liver tumors when fed N–2-fluorenylacetamide than female rats. If the survival of female rats is prolonged by removing breast tumors, the incidence of hepatomas can be increased appreciably in the female animals.

Dr. Sidransky, with Drs. E. Farber (Tulane University), M. Rechcigl and T. Baba, is investigating the effects of nutritional deficiencies on the pathogenesis of hepatomas in rats in an effort to learn if other nutritional deficiencies besides chronic choline deficiency can lead to liver cancer. In short-term experiments, young rats were found to develop signs of acute choline deficiency only when the diet contained no choline and 0.12 percent or less of methionine. In long-term experiments, no signs of cirrhosis or hepatomas have yet appeared in rats on an adequate choline but methionine deficient diet for 22 months. Species differences have been observed in determinations of liver choline oxidase activity; among nine species, the rat has the highest activity and human the lowest. Pathologic changes in the liver of the rat similar to those seen in kwashiorkor were observed within a few days after tube-feeding of young rats on certain essential amino acid deficient diets.

Dr. H. P. Morris, with Mrs. B. P. Wagner, continues to add to the knowledge of the carcinogenic activity of certain chemical compounds, structural and metabolic relationships, species differences involved, and dietary effects. Last year, Dr. Morris reported that hydroxylation of N–2-fluorenylacetamide (2–FAA) in positions 1, 3, 5, or 7 reduces its carcinogenic potency, and the *in vivo* hydroxylation that occurs is interpreted by Dr. Morris as a detoxification mechanism or a means of solubilization to facilitate excretion (these compounds occur in the urine in the largest amounts as conjugates of glucuronic and sulfuric acids after 2–FAA feeding). Last year also, it was reported that replacement of the hydrogen atoms on the omega-carbon of the acetyl group in 2–FAA by fluorine led to increased carcinogenic activity. Fluorination of all six omega-carbon hydrogens in 2, 7-fluorenylene-*bis*-acetamide (2, 7–FAA) has now been accomplished, and this new compound shows increased carcinogenicity. Sites in the rat most affected were the mammary and salivary glands. It has also been shown that the weak carcinogen, N–1-fluorenylacetamide (1–FAA), was converted into a moderately active one by substitution of the hydrogens of the omega-carbon by fluorine; activity was again most pronounced in the mammary and salivary glands.

Four papers (with Drs. H. L. Stewart and K. Snell) setting forth a large series of observations on the carcinogenic effects in rats of 2, 7–FAA, including comparisons with 2–FAA, have been accepted for publication. Tumors were produced in the small intestine, glandular portion of the stomach and salivary glands, and neurogenic tumors were noted. 2, 7–FAA also produces atrophy of many organs and tissues and raises questions anew regarding the role of atrophy in tumorigenesis. Of special interest is the gastric adenocarcinoma produced in the rat, for this is the first instance that this important type of cancer has been produced by an orally administered carcinogen.

Animal experiments concerned with the effects on carcinogenicity brought about by blocking the most active hydroxylation sites in a simple aromatic amine such as aniline or acetanilide have been partially completed. Blocking both the *ortho* positions and the *para* position with $-CH_3$, $-Cl$ and $-F$ was studied as well as the *para* position with $-OH$. The 2, 4, 6-trifluoroacetanilide experiments are still incomplete. Liver tu-

mors were induced in rats fed 2, 4, 6-trimethylaniline, and a number of animals showed severe cholangiofibrosis. Enlarged pituitary glands were also noted. In the *p*-hydroxyacetanilide experiment, mammary glands were found to be hypertrophied, and a large number of animals developed enlarged pituitary glands. Enlarged pituitary glands were also seen in rats fed 2, 4, 6-trichoroacetanilide and in female rats fed acetanilide.

One of the hepatomas (Morris hepatoma 5123) recently developed by Dr. Morris is of considerable interest since its enzymatic characteristics are very similar to those of normal liver and are in fact more similar to normal liver than any other transplantable rat tumor studied to date. Some enzymatic activities are actually greater in the tumor than in normal liver. Dr. Morris is collaborating with investigators in several laboratories in studies on this tumor, particularly in regard to Dr. Van Potter's deletion hypothesis. This hepatoma was produced in the Buffalo strain of rat by a slow acting carcinogen, N–(2-florenyl) phthalamic acid (2–FPA).

Dr. H. M. Dyer, working with Dr. Morris and Miss B. Ensfield, has continued her studies on the interference of 2–FAA and related compounds with tryptophan metabolism. It appears that the excretion of increased amounts of urinary xanthurenic acid, kynurenic acid, and kynurenine derivatives after a load dose of L-tryptophan to rats maintained on carcinogenic concentrations of 2–FAA is due to an inhibition of kynureninase activity. Inhibition may result from combination of the coenzyme pyridoxal phosphate with fluorenamine or some other amino metabolite of 2–FAA in the form of a Schiff base. Therefore, a more than ordinary requirement of vitamin B_6 may be needed by rats ingesting aminocarcinogens. A relatively low concentration of dietary B_6 was found to cause an increase in the induction period and/or a low incidence of tumors of the liver induced by 2–FAA. Low dietary levels of vitamin B_6 inhibited mammary tumor development.

Drs. J. H. and E. K. Weisburger, with Drs. Morris and K. Suzuki and Mr P. H. Grantham, continue to make many contributions to our knowledge of the mechanisms of carcinogenesis and the chemistry and metabolism of N–2-fluorenylacetamide and related compounds. Previous work had indicated that monohydroxylation is an important biochemical reaction undergone in the body by compounds such as N–2- and N–3-fluorenylacetamide. The suspicion that the more polar metabolites excreted were dihydroxy derivatives has been confirmed through studies performed with the synthesized 2, 7-dihydroxyderivatives of N–3-fluorenylacetamide. In addition, N–(7-hydroxy-3-fluorenyl)acetamide formed a sizeable portion of the urinary metabolites and was the only product excreted as a sulfuric acid conjugate. The 2, 7-dihydroxyderivative was excreted mainly as a glucuronide, as was also the previously known 2-hydroxy derivative. The polyhydroxylated compounds posses a number of charged reactive groups which make them especially interesting from the biochemical and biological standpoint.

Studies were continued on the protein binding of 2–FAA and its metabolites. It is obvious that this kind of binding represents a complex series of reactions involving a number of tissue constituents and several metabolites of the carcinogen. Last year, reactions were described that involved a few selected metabolites of 2–FAA, namely, the aminofluorenols, which gave rise to rat liver protein-bound radioactivity as an artifact. Through use of *in vitro* and *in vivo* experiments involving proteolytic enzymes, it has been shown that the aminofluorenols form only a small part of the metabolites of 2–FAA and that the artifact type of binding plays only a minor role in the binding noted in *in vivo* experiments. Studies are continuing on this complex problem.

Protein binding of 2–FAA was studied in rats bearing the Morris hepatoma 5123 with radioactivity labeled carcinogen. Since this tumor resembles normal liver in many respects, it was of interest to find that proteins derived from this tumor showed only about 10 percent of the radioactivity of proteins derived from normal livers.

Drs. E. K. and J. H. Weisburger have performed considerable synthetic organic chemical work to prepare compounds suspected of being intermediates in the metabolic sequence, labeled carcinogens and their metabolites for distribution studies, and carcinogens and derivatives for feeding experiments. Not only is knowledge increased in a segment of organic chemistry through such studies, but several new reactions have been developed during the course of these

syntheses and many valuable compounds prepared have been made available to several investigators, both within NIH and elsewhere. In addition, physical-chemical data have been acquired by means of ultraviolet and infrared spectra and by measuring ionization constants of aromatic amines. Polynuclear aromatic amines have been synthesized and some show metal chelation.

Last year Dr. H. F. Blum reported on a model that is consistent in a quantitative sense with the existing data on cancer induction by ultraviolet light (a book by Dr. Blum on this subject has been well received by cancer investigators and other scientists). Dr. Blum has now formulated a quantitative model for carcinogenesis by chemical agents. This model, although containing the same fundamental assumptions as the former one, entails equations of a different form, explainable on the basis of temporal relationships of the carcinogenic agent. Preliminary examination of the goodness of fit of the model for data in the literature indicates good approximation. The model also points up difficulties in testing the "specific" effectiveness of a given carcinogen, since multiple factors, which need evaluation in testing, are involved, some of which are difficult or impossible to measure by the usual testing methods. In particular, the setting of tolerance levels based on the usual type of data obtained in toxicological testing would likely lead to a false sense of security. The new model, like the older one, describes a cumulative, essentially irreversible and essentially nonthreshold process.

Viruses

The Virus Oncology Section, Laboratory of Biology, has an integrated program of research on viruses and cancer which *in toto* has a wide variety of experimental approaches aimed at elucidating the role of viruses in human cancer and which, at the same time, permits the individual senior investigators to work with model animal systems of particular interest to each of them.

Virological Aspects of Human Tumors. From knowledge of the tumor viruses of laboratory animals, it appears that human tumor viruses, if they exist, are likely to be species specific under most circumstances. Six major approaches, designed to take into account this difficulty, are under way in the attempt to obtain data relevant to the question of the viral etiology of human cancer: (1) Attempts to detect cytopathogenic or other changes in tissue culture following inoculation of cell-containing and cell-free preparations of human cancer tissues into cultures of cells from human beings and animals; (2) Attempts to detect changes in the chorioallantoic membranes and allantoic cavities of eggs inoculated with cell-containing and cell-free preparations of human cancer tissues, and study of the phenomenon of viral interference in embryonated eggs utilizing known chicken or human viruses that grow readily in such eggs; (3) Primary inoculation into newborn mice of microsome fractions of human cancer tissues prepared by the methods found most suitable for the Rous sarcoma virus; (4) Tests in newborn mice on nucleic acid fractions derived from human cancer tissues; (5) Procedures for producing immunological tolerance in tests for human viruses involving the inoculation of other animal species; and (6) Electron microscope studies (in collaboration with Dr. A. J. Dalton).

The tissue culture studies, which have been in progress for about two years, have yielded no evidence thus far of a virus of any type being associated with human cancer tissue. A total of 75 separate cancers have been investigated in tissue culture. With the other approaches, insufficient time has elapsed to expect definitive information. It is of interest to point out that 10 cancer specimens have been employed in coordinated experiments involving all or most of the various approaches listed above.

Because it appears to be of great importance in studies on the possible viral etiology of human tumors to be able to manipulate artificially a virus or host to permit the growth of a foreign virus, Dr. M. A. Fink has been attempting to induce in mice a recognizable, reproducible change by the injection of a known, alien tumor virus (the avian Rous sarcoma virus) in the hope that a useful model system might be developed. Although hundreds of mice have received injections by a variety of routes, no effects have been noted. When attempts to prevent an antibody response in later life were made by injecting the neonatal mouse with a chicken tissue component, with cortisone, or with both, it was found that cortisone, but not the neonatal injection of chicken protein, could completely suppress the

formation of antibody to chicken protein. Preliminary results with administration of Rous sarcoma virus to cortisone-treated mice suggest that the virus is affecting the animals adversely, but more careful control of cortisone dosage is needed in further work.

Extracts of 45 human tumors have been inoculated into cultures of human cells and into newborn and fetal hamsters by Dr. A. Rabson, and, thus far, no tumors attributable to the inocula have been observed. In one case a cytopathic effect was seen in culture, and the agent producing the effect was adapted to grow in HeLa cell cultures where cytopathic effects were also noted. Attempts at identification are under way.

Dr. S. Stewart is also studying cell-free preparations from human neoplasms. Hamsters were injected when newborn either with fluids from embryonic tissue cultures which had received neoplastic material or with fluids from noninoculated control cultures. A wide variety of tumors resulted in a high percentage of animals (many animals had more than one tumor) not only in the experimental group, but surprisingly also in the control group (essentially the same tumor types with the same incidence). Liver cysts, spleen atrophy, enteritis and polyps of the bowel were also seen in many of the animals. Seven percent of the females developed a trophoblast-like lesion of the uterus. Results obtained in mice which were inoculated for another purpose were similar. Of 276 Swiss mice over 12 months of age that had been inoculated when newborn with culture fluids from control mouse embryo cells, 56 percent developed several types of tumors. Of 103 litter mates that received culture fluids plus rabbit antisera (containing antibodies to mouse embryo cells), however, essentially no unusual tumors were seen. Dr. Stewart postulates that the high incidence of neoplasms resulted from an induced tolerance to embryonic cells brought about by the tissue culture inoculum.

Moloney Virus. Dr. J. B. Moloney continues to add to the knowledge of the murine lymphoid leukemia virus which he isolated from a transplantable Sarcoma 37 that had been transferred to BALB/c mice after 195 tumor generations in strain A/Ln mice. He has now isolated the virus from two other transplant lines of Sarcoma 37, the original tumor line in A/Ln mice and the sarcoma line from which this arose, now being carried in ZBC mice. Further improvements in concentration and purification of the virus have been made by means of protamine precipitation, followed by trypsin digestion of the protamine-virus complex. Regardless of the route of inoculation, the neoplasm produced is always of the generalized type with no tumors at the site of inoculation. The intravenous route produces leukemia deaths in weanling and adult mice about one month earlier than those produced by other routes. The virus is capable of eliciting a 100 percent leukemia response in BALB/c mice inoculated as late as 8 months of age. The potency of the virus has increased with successive passages through newborn mice; after 12 passages the average latent period for 100 percent induction of lymphocytic leukemia has fallen from 6.4 months to 2.5 months period. A high percentage of animals develop the disease as early as 5.5 weeks after inoculation.

Recent studies by Dr. Moloney indicate the following important results: the viral induction of *lymphoid* leukemia in the rat; a lymphoid leukemia virus that can cross the *species* barrier; a virus that is not rat strain specific. The agent has not acquired host specificity after repeated virus passage in the rat or mouse, since virus from either rat or mouse passages will induce the disease in both species of animals.

Either thymectomy or splenectomy delays the onset of leukemia in the inoculated hosts although all eventually die with the disease. Thymectomy of the host followed by inoculation results in the induction, in a high percentage of cases, of a *Hodgkin's diseaselike lesion* which is a reticulum cell neoplasm of Type B as described by Dr. T. Dunn. Splenectomy of the host followed by inoculation results, in a few cases, in the induction of *myeloid* leukemia which can be further differentiated as chloro-leukemia, an extremely rare tumor for this strain of mouse. Cell-free material was prepared from the induced myeloid leukemia and inoculated into intact newborn BALB/c mice; all the mice died with *lymphoid* leukemia.

Preliminary results give good evidence that the agent is composed of RNA-containing particles. Dr. Moloney has initiated experiments to learn if the nucleic acid isolated by the method of

Gierer and Schramm from a leukemic tissue microsome fraction containing active virus can induce tumors.

Preliminary studies by Dr. Moloney indicate that the tumor-producing activity of the leukemic ascites cells, which contain active virus, was completely inhibited by oxidation products of linolenic acid.

Dr. T. Dunn, working with Dr. Moloney, has found that leukemia first appears in the thymus gland in mice inoculated with the Moloney virus. Prior to the development of this lesion there was noted regularly a marked hyperplasia of the spleen and occasionally a localized reticulum cell hyperplasia of the lymph nodes. Transplants of the hyperplastic spleens from mice with no evidence of a true leukemia reproduced the same sequence of lesions observed after inoculation of the virus material, indicating the presence and multiplication of the leukemia virus in a host which showed no overt signs of lymphoid leukemia.

It has been observed earlier that the young mice born to leukemic parents developed "spontaneous" lymphoid leukemias at a high incidence level after a rather extended period of time. Drs. Moloney and N. Ida (M. D. Anderson Hospital, Houston) have undertaken studies to determine the mode of transmission of the Moloney virus to the offspring. Pregnant mice were inoculated intravenously at various stages of gestation. When the virus was given early in gestation (1 to 4 days), a high rate of abortions or resorption of fetuses occurred. A high incidence of leukemia was noted among the young born of the group of mice that had received the virus after the placenta was formed, with average latent periods of 31 to 52 weeks.

Dr. A. J. Dalton, with Drs. Moloney and W. W. Oppelt, has found by means of electron microscopy that in an analysis of Moloney leukemia the primary site of formation and apparently the major source of virus are the megakaryocytes of the bone marrow and spleen. The majority of particles in any one megakaryocyte were in the same stage of development, the stages varying from one cell to the next. Platelets from rats rendered leukemic with the Moloney agent were examined. Many platelets were found which contained particles, occasionally centrally located. Megakaryocytes and platelets of control rats were found to be negative for the particles.

Dr. H. Kahler and Mr. B. J. Lloyd, in collaboration with Dr. Moloney, utilizing density gradient centrifugation and electron microscopy techniques, observed that the highest concentration of Moloney virus particles of the 100 millimicron diameter size was found at the level in the centrifuge tube where the density was calculated to be 1.24.

Gain in knowledge of the murine tumor causing viruses would be greatly facilitated if more rapid methods of assay not requiring long latent periods of direct observation in animals were available. Dr. F. J. Rauscher is seeking to improve assay methods by attempts to increase the potency of the viruses under study, reduce or remove inhibitors present in some assay systems, and develop simplified assay, systems, e.g., in embryonated chicken and Japanese quail eggs. One approach showing success is an assay of the Schoolman-Schwartz mouse leukemia virus involving the virus interference phenomenon. The leukemia virus has been serially passaged on the chorio-allantoic membrane (CAM) of embryonated hens eggs. No discrete lesions were observed grossly or histologically; however, when eggs were inoculated with CAM extracts of the first and sixth serial passages and then challenged with influenza virus, a 90 to 99 percent reduction in the titer of the challenge virus was noted. The same CAM extracts when heated to 60° C. for 30 minutes, or extracts of CAM's from eggs inoculated with normal mouse tissue failed to depress the titer of influenza virus. Extracts of CAM's showing this interfering activity have not, however, produced leukemia in mice, as of an approximately 2-months' postinoculation period.

Similar studies are under way with the Moloney virus. It is hoped that these methods can also be adapted to studies with human material as further successes are achieved.

Dr. R. A. Manaker has made additional advancement in the *in vitro* propagation of the Moloney virus. With improved culture media and primary mouse spleen cultures, he has found that the fluids overlying the cultures are infective for both animals and fresh spleen cultures, provided the cultures are maintained at least two weeks following addition of cell-free extracts of

spleen, thymus and lymph node pools obtained from leukemic mice. The virus does not proliferate as rapidly when inoculated into *long-term* cultures, but will do so sufficiently to produce leukemia in test animals if maintained for as long as three months. Tests for infectivity must be evaluated in terms of the tumor-inducing ability in animals since no cytopathogenic effects are seen in the cultures, even if maintained without transfer for as long as three months. Tissue culture fluids containing the virus may be stored at −70° C. and used to infect fresh cultures at a later date. Leukemia has not been observed in mice inoculated with control spleen culture fluids. On the other hand, leukemia has been observed in uninoculated mothers and uninoculated litter mates of mice infected with active virus material, suggesting that the virus may be contagious. Dr. Manaker's successful propagation of the Moloney virus demonstrates that its *in vitro* behavior is similar to that of other animal viruses, and the findings add support to the argument that aside from the type of response they elicit in the host, the tumor viruses are not basically different from other viruses.

Drs. Fink and Moloney are investigating the immunological properties of the Moloney virus and have produced the following homologous and heterologous sera: normal mouse and normal rabbit; mouse antinormal spleen; rabbit antinormal spleen; mouse antivirus (formalin-killed); and rabbit antivirus (purified). Only the mouse formalin-killed virus antiserum proved to be effective in neutralizing the leukemogenic activity of the virus. With this antiserum, an indication has been obtained that complement fixation does occur in low titer.

Polyoma Virus. Dr. L. W. Law, alone and with collaborators, has been particularly active in studying biological parameters of polyoma viruses. With Dr. H. Kahler and Mr. B. Lloyd, Dr. C. Dawe and Drs. W. P. Rowe, and J. Hartley (LID, NIAID), Dr. Law has found four strains of virus to be highly variable in a variety of biological properties, including tumor induction, a result not unlike findings obtained with other viruses such as the Rous sarcoma virus and the mouse mammary tumor agent. A highly virulent virus strain required only 20 to 30 particles for tumor formation; on the other hand, another required more than 1 million particles. Different dose levels of the highly virulent strain give different patterns of tumor induction. Dawe's strain, adapted for growth on P–388 cells in high-serum medium, undiluted and in 10^2 dilution produces thymic epithelial tumors while in 10^4 and 10^6 dilutions produces unilateral parotid tumors. The weakly oncogenic strain of Rowe (397), on the other hand, produces parotid tumors only in the undiluted extract.

C57BL/$_{Ka}$ strain mice show a pronounced resistance to tumor induction by a virulent strain of polyoma virus. After an observation period of 6 months, 92 percent of C3Hf/$_{Bi}$ mice have developed thymic epithelial (and other) tumors while none of the C57BL/$_{Ka}$ mice has developed tumors. Only 10 percent of F_1 mice have been tumorous.

Drs. Law, Dawe and Rabson have found differences in infectivity and oncogenic potentialities between polyoma grown on P–388 cells cultured in milk medium and polyoma grown on P–388 cells grown in human serum medium. The former retained high infectivity for mouse and for tissue culture but was nearly nononcogenic, whereas the latter, with a reduced infectivity, remained highly oncogenic. Nevertheless, the virus grown on the milk-adapted cells retained its oncogenicity for hamsters.

Drs. Law and Dawe have observed the appearance of several neoplasms in mice into which polyoma infected fragments of cultured salivary gland tissues (30–50 days on a sponge-matrix system) had been placed, both in X-rayed adults or newborn animals. In extensive studies of three of the tumors, no virus could be detected in two fibrosarcomas, even through eight transfers of tissue in mice, but it was found to be replicating in an epithelial tumor. The results are similar to those reported by Dr. Dulbecco (Cal. Tech.) and by Dr. Sanford (NCI) in their studies of fibrosarcoma development *in vitro*.

Dr. Law, also with Dr. Dawe, has investigated the effects of X-irradiation, administered prior to introduction of polyoma virus into adult C3Hf/$_{Bi}$ mice. Thirty-eight and 43 percent of the adults presented single or bilateral parotid gland tumors following intravenous introduction of thymotropic polyoma virus, a result strikingly different from that of the same strains infected in the neonatal period. Thymectomy of adults

had no influence on the carcinogenic response and virus or X-rays alone were ineffective. X-irradiation plus natural infection (at 3 to 5 months of age) did not induce neoplasms characteristic of this virus. Thymic tumors induced by the virus seldom grew when transplanted into suitable hosts, either in young mice or in X-rayed adults. One of the tumors that did develop was strikingly hormone dependent.

Dr. C. Dawe has obtained additional information as part of the overall study on influences of host factors on the development of cytolytic and proliferative responses to polyoma virus *in vitro*. With Dr. Law, he has found that cultures of salivary gland tissue from adult mice showed an epithelial proliferative response greater than that seen in cultures of this tissue from newborn mice. At the 30-day interval after infection, cultured salivary gland rudiments from 13- and 14-day old mouse embryos, however, showed less epithelial proliferative response than that seen in cultures from either newborns or adults. No proliferative response by mesenchymal cells was seen, and necrosis of these cells was observed in glands of all ages. As indicated above, nearly half the adult mice receiving polyoma virus following total body radiation develop salivary gland tumors, even though no such tumors developed in animals treated with X-ray alone nor in adult nonirradiated controls. This information, coupled with early results on the effects of specific antibodies on cultures obtained in collaboration with Dr. W. Rowe (NIAID), plus the knowledge that antibody response is delayed in newborn mice, indicates that the high susceptibility of newborn mice to polyoma virus tumor induction depends primarily on the weakness of the immune response at this age and not on the presence of "primitive" cells or on a higher susceptibility of rapidly growing cells.

Dr. Dawe has shown that the response of salivary glands from rats, hamsters, mastomys and human fetuses grown in culture following exposure of the excised tissue to polyoma virus was negative, the changes previously reported in similar mouse salivary gland cultures not being observed.

Dr. S. E. Stewart, in collaborative studies with Drs. C. and R. Leuchtenberger (Children's Hospital, Boston), has confirmed and extended previous observations with polyoma virus indicating two main processes in tissue cells of the kidney: (a) a degenerative process in the epithelial cells associated with the viral activity leading to destruction of cells, and (b) a proliferative process in the stroma without evidence of viral activity, but associated with cellular activity leading to further propagation of abnormal cells resulting in tumors. The epithelial cell changes preceded the proliferation of the stromal cells. It was concluded that only the stromal cells become malignant and, on the basis of histochemical determinations of DNA, that viral proliferation occurred only in the epithelial cells. This result appears to be in accord with Dr. Stewart's previous observations since no renal carcinomas have been found in mice injected with polyoma virus, but renal sarcomas are common. Dr. Stewart suggests that a cell responds to the virus in one of two ways: (a) in one instance the cell is used by the virus for its replication, suppressing cell mitosis and finally killing the cell; and (b) in the other instance the virus or its products acts as a stimulating agent provoking reduplication of cells resulting in malignancy.

Drs. R. Love and A. Rabson and Miss F. Legallais have determined a sequence of changes in polyoma-infected $P388D_1$ cells grown in culture by means of cytochemical techniques. The earliest abnormalities seen consist of enlargement of the nucleus, nucleolus and nucleolinus and the appearance of intranuclear vacuoles in which normal chromatin and ribonucleoprotein are replaced by abnormal histone-free deoxyribonucleoprotein. Other changes in nucleoprotein become evident in the nucleus, and terminally, alteration in the cytoplasm is observed.

Drs. W. Banfield and C. Dawe have found that virus-like particles can be seen easily in polyoma-induced mouse tumors kept in a maintenance media in tissue culture for as little as 24 hours, but cannot be found after a reasonable amount of searching in tumors examined directly. Several human tumors held in tissue culture from 24 hours to 2 weeks produced negative results. With Dr. W. Rowe (NIAID), initial electron micrographs were made of mouse adenovirus (morphologically in the adenovirus group) and mouse thymic agent (features in common with the Lucké, salivary gland, herpes simplex and herpes B viruses).

Dr. M. Stanton has studied the histopathologic

characteristics of the polyoma-induced osteogenic tumors in Strain A mice. These tumors, which are especially amenable to progression studies because of slow, step-wise changes, are of interest since Strain A mice receiving polyoma virus develop them without developing the variety of tumors observed in other strains and without affecting the incidence of spontaneous pulmonary alveologenic tumors.

Dr. A. Rabson has succeeded in producing tumors of the lung in hamsters by direct instillation of polyoma virus in the trachea. In 6 of the 16 animals autopsied lung tumors with histologic features of bronchiolar carcinoma, alveolar cell carcinoma and squamous cell carcinoma were found. The squamous cell carcimona, which appeared to arise in bronchioles and intra-pulmonary bronchi, has been established as a transplantable tumor and is now in the 16th transplant generation. Attempts to isolate polyoma virus from this transplantable tumor and from one of the primary tumors have been unsuccessful, an experience similar to that of other investigators who have encountered difficulties in isolating this virus from the sarcomatous lesions of hamsters.

Newborn Mastomys (*Rattus natalensis*) inoculated with polyoma virus by Dr. Rabson developed tumors in 14 of 17 animals autopsied (sarcomata in kidney, heart and subcutaneous tissue and angiomatous tumors in liver). Thus far, no parotid gland or thymic tumors similar to those produced by polyoma virus in mice have been observed, however. Polyoma virus was isolated in tissue culture from suspension of one of the renal sarcomas that developed.

Mammary Tumor Agent. In a study designed to elucidate the factors responsible for the sudden disappearance of the mammary tumor agent from a few strain RIII mice, Dr. H. B. Andervont has obtained further evidence that the virus carried by RIII mice is much weaker in tumor-inducing capability than that carried by C3H mice, thus being one factor of probable significance in the disappearance of the agent. The observation that an appreciable incidence of tumors occurred in F_1 hybrids derived from agent-free (RIII-) females and agent-carrying (C3H+) males was of interest.

RIII virus has been introduced into C3H mice and C3H virus into RIII mice by fostering nursing, and the animals have been followed now for several generations of inbreeding. The C3H mice had a low incidence of tumors, and no definite signs of increased potency of the weak RIII virus were seen. With the RIII mice carrying the C3H virus, observations indicate that a single strain of virus in hosts of the same genetic constitution shows a pronounced variation in its ability to induce tumors. Even within a highly susceptible family, an occasional RIII mouse is very resistant even to the potent C3H agent.

In some cases, tumor-causing viral agents can escape through certain sized pores in the membranes of diffusion chambers placed in the peritoneal cavity of mice. For example, Dr. R. Merwin has shown that the mammary tumor agent passes through pores 0.45 micra but not 0.10 micra in diameter, and that the Moloney virus passes through 0.45 micra pores, but no smaller ones. Even with 0.45 micra pores, no tumors appeared in mammary tumor agent-free BALB/c hosts when mammary tissue from agent-free C3H donor mice was placed in the chambers, a finding providing additional evidence that the virus is indeed absent from so-called virus-free mice.

Rous Sarcoma Virus. In attempting to determine the ultimate limit to which the concentration of Rous sarcoma virus in sarcoma tissue can be increased through continued selective serial passage in chickens, Dr. W. R. Bryan has made such passages, selecting the tumor first to appear in each batch of 40 inoculated chickens, for the past 5 years at biweekly intervals (except during the past year the interval was changed to monthly). By this procedure the virus yield of tumors has been increased about 100-fold, but during the past year little increase was observed. It appears unlikely that the yield of virus will be increased further by this technique, but the virus concentration already achieved justifies resumption of studies on purification of the virus.

Drs. Bryan, H. A. Sober, and F. J. Rauscher and Mr. J. P. Kvedar are attempting to purify further the Rous sarcoma virus obtained from "high potency" tumors by means of differential ultracentrifugation and chromatography on cellulose ion-exchange columns. An increase in

virus activity, similar to the 100-fold increase obtained by the selective passage procedure, is present in partially purified fractions separated by differential centrifugation or cellulose chromatography. By both procedures there is a 2- to 3-fold increase in viral activity per gram equivalent of tumor tissue, indicating that an "inhibiting" factor is removed.

Drs. Rauscher and M. A. Fink have studied the effects on the production of infective Rous sarcoma virus (RSV) by conditioning the host with Freund's adjuvant. When the adjuvant was given to young chicks before virus inoculation, extensive granulomatous lesions developed at the site of injection (subcutaneous); when a small dose of RSV was injected into these lesions, the granulomas grew at an accelerated rate and appeared to invade adjacent muscle. Extracts of these growths yielded 2 to 4 logs of virus. The same small dose of virus failed to produce tumors in control birds pretreated with saline, and RSV could not be demonstrated in tissue extracts from these control animals. The incidence and severity of metastases were also increased in birds pretreated with adjuvant. When chicks were inoculated with a dilution of RSV that failed to produce tumors and were subsequently inoculated with adjuvant, 85 percent developed relatively large amounts of anti-RSV antibody in their sera. The sera of control negative birds challenged with saline remained free of significant levels of antibodies.

Although Dr. Rauscher has failed to effect a recovery of infective RSV from RSV-specific antibody complexes by treatment with fluorocarbon (Genetron 113), he has done so by techniques of ultracentrifugation and under certain conditions with simple dilution. Through special techniques involving trypsin he has also made noninfective lesions produced by low doses of RSV or noninfective lesions in Japanese quail eggs yield infective RSV.

These techniques that are opening leads for enhancing host response to a virus, for preparing more potent virus, for detecting and eliminating inhibitors, and for measuring responses produced by minimal amounts of virus are particularly important for the study of the initiation and course of viral diseases as they exist in nature and are especially germane in laying the groundwork for investigation of the viral etiology of human tumors.

Other Virus Studies. In collaboration with Dr. J. A. Reyniers (Germ-Free Life Research Center, Tampa, Florida) and Mr. J. P. Kvedar, Dr. Rauscher is evaluating the value of Japanese quail (*Coturnix coturnix japonica*) embryonated eggs and posthatched chicks in virus and cancer research. Their small size before and after hatching, their ease of maintenance, and their rapid generation time makes them especially suitable as an avian host in studies requiring long periods of observation, particularly since Dr. Rauscher has shown that they may be manipulated in a manner similar to chickens and that they are susceptible to at least six different viruses. In addition, certain interesting differences have been noted with the Rous sarcoma virus and with the visceral lymphomatosis virus. Of special interest is the finding of a substance inhibitory to the Rous sarcoma virus growth in eggs that failed to develop gross lesions. The possible relationship of this phenomenon to "interferon," to the resistance-inducing-factor of Rubin, and to lymphomatosis virus is under study.

More knowledge is needed on the indigenous mouse viruses because, among other reasons, they may interfere with the expression of tumor-causing viruses under study, they may produce disease in colonies of study animals thus invalidating experiments in progress, and they may, if sufficient information can be gained, serve as models of tumor virus behavior involving latent infections. Dr. R. A. Manaker has discovered three such viruses (two that produce hepatitis and one that produces pneumonia) and has determined several of their characteristics. Such information is useful in the control of the diseases produced in colonies by these viruses and provides basic knowledge needed for study of the tumor viruses in animals that are potential carriers of these agents.

Drs. C. Dawe and L. Kilham (DBS) did not find a proliferation-promoting effect of rat virus in organ cultures of rat salivary glands, a system analogous to the polyoma virus-infected mouse salivary gland culture system.

Drs. W. Banfield and G. Ramirez and Mrs. D. Brindley, in studies with a spontaneous reticulum

cell sarcoma in a hamster now carried through 12 passages, have shown that while injections of blood, ascites fluid, and brain from tumor-bearing animals produce tumors in the recipient hamster, cell-free filtrates of brain, ascites fluid, and tumor so far have not. Of six animals fed fresh tumor tissue, one developed systemic tumor involvement and another laryngeal occlusion from submucosal tumor growth. More remarkably, of 10 untreated animals caged with 10 animals inoculated with tumor, six died with laryngeal obstruction caused by submucosal tumor growth and two killed animals had systemic tumor involvement. The tumor also arose in one of three hamsters separated by a screen from tumor-bearing animals. As yet, no viruslike particles have been seen upon examination by electron microscopy.

In electron micrographs, Dr. R. F. Zeigel has observed type A "virus particles" of Bernhard budding from the apical plasma membrane of pancreatic acinar cells in the chick. Although the viral nature of these particles has not been established, they possess morphological characteristics closely similar to those described in association with lymphomatosis, Rous sarcoma, myeloblastosis and other avian viral infections. When particles were observed to bud from pancreatic acinar cells, all other tissues examined contained the particles in intercellular spaces and in intracytoplasmic vesicles and vacuoles. When absent in the pancreas, they were not seen in other tissues. In a study of the albumin-secreting gland of the hen oviduct, the secretory product appears to be produced in close association with the ergatoplasm, accumulated in large cisternal regions and released into the secretory lumina.

Mr. B. G. Young and Dr. P. Mora are conducting fundamental studies on bacteriophage and are preparing to apply some of the techniques developed to the tumor-causing virus, polyoma. Such studies are part of a program designed to study interactions of biological system at the molecular level and employ characterized, synthetic positive (basic) or negative (acid) derivatives of polysaccharides with different degrees of branching and charge distribution (*vide infra*). The bacteriophages are being used as model systems to study quantitatively the changes that polyelectrolytes may bring about in the surface proteins of the virus, in order that more can be learned of effects on tumor-causing viruses, particularly in regard to blockage of the infective process.

The extent of inactivation of viability of bacteriophage (by blockage of attachment of the phage to the bacterial cell and/or the injection of DNA into the bacterial cell) was determined by means of incubation of the virus with negatively charged synthetic polysaccharide derivatives. Inactivation of bacteriophage by polyglucose sulfate, which occurs at an acidic pH range in which sulfuric acid does not produce inactivation, was not reversed by further incubation at neutral pH, by increasing the salt concentration, or by adding positively charged macromolecules. Thus, the inhibition is not due to a purely electrostatic interaction with the positive lysozyme-like protein in the tail of the phage. That an ion exchange effect was not producing the inactivation was shown by inactivation even in the presence of high calcium ion concentrations. Furthermore, since polyglucose sulfate, labeled with S^{35}, interacted with the T2 bacteriophage directly and did not penetrate into the *E. coli* cell, the mechanism of inactivation is most likely due to prevention of normal attachment or DNA injection, rather than blocking processes of viral multiplication in the cell.

When the inactivation was only partial, the residual viable phage had a normal attachment rate to the *E. coli*. The attachment of the total phage components also was not reduced significantly. These conclusions were based on measurements done with P^{32}-labeled DNA and S^{35}-labeled protein of the phage. Experiments of shearing off the labeled attached phage from the *E. coli* surface by swirling it in a Waring blendor showed that it was easier to shear off DNA from the inactivated phage than from an untreated virus. No incorporation of labeled P^{32} into the progenies of the bacterial phages was found.

A reasonable interpretation of the above results is that the lysozyme-like protein is blocked by the anionic polysaccharide, but not sufficiently to prevent attachment, only the penetration and injection of the DNA. It is possible that a second inactivation mechanism might also occur, possibly simultaneous with the other mechanism. Negative DNA in the head of the virus is known to be partially neutralized by positive oligopep-

tides such as putrescine (findings of Dr. B. Ames, NIAMD). The DNA may change its shape if the positive putrescine is removed by the negative polysaccharide, making eventual passage through the narrow tail core impossible.

It is of interest to note that a small percentage of the bacteriophage population which remained viable gave progenies that were more resistant to further inactivation, suggesting that inactivation might be a useful tool for genetic studies. Low concentrations of polyglucose sulfuric acid were also found to reactivate some of the bacteriophage that had been inactivated with antibody, indicating a direct interaction between the antibody and the polyglucose sulfate. Such findings suggest possibilities of antibody fractionation and new ways of studying antigen-antibody interactions.

In the course of preparing for studies on tumor viruses, Mr. Young and Dr. Mora are modifying the method of Dulbecco and Sachs, which produces plaques in a monolayer of embryonic tissue culture, for studies on polyoma.

Host—Tumor Relationships

Host Genetics in Carcinogenesis

Genetics of the mouse has reached a state of development where analysis of effects of specific genes can now profitably be pursued. There are at least 87 gene loci of the mouse identified in 19 linkage groups, and they may represent all but one of the chromosomes. Nine of these loci have been shown to be linked with neoplasia. Not only has Dr. W. E. Heston over the years made major contributions to the knowledge of strain differences in relation to cancer, but he has been in the forefront in amassing data on the role of specific genes of the mouse associated with specific types of cancer. He has been particularly interested in two loci that are linked with cancer: the obese gene ob in linkage group XI and the lethal yellow gene A^y in linkage group V. The ob gene results in excessive obesity, and the A^y gene is lethal when homozygous, but when heterozygous causes the coat to be yellow and causes an increase in body size. From studies on the latter a unique system has been developed which will permit new approaches to understanding of the mechanism of gene action in mammals, at both the molecular and higher organismic levels. Since the A^y gene is lethal when homozygous, forced heterozygosis at this locus during the long period of inbreeding in the YBR strain has led to a situation in which F_1 hybrids resulting from outcrosses between mice of the YBR strain and those of any other inbred strain are isogenic in that they are genetically alike except that half carry the A^y gene and are yellow and half carry the nonagouti allele and are nonyellow. Thus, in these F_1 hybrids, analysis can be made of the effect of this specific gene, for any difference in occurrence of tumors in the two color types is known to be due to this gene. A further advantage in these F_1 hybrids is that tissues can be transplanted freely between the two color groups since in Dr. Heston's Laboratory it has been shown with skin transplants that the A^y gene has no histocompatibility effect. This enables investigators, after identifying an effect of the gene on any type of tumor, to try further to identify its path of action through transplantation of tissues or organs.

Dr. Heston is employing this system with transplantation of ovaries to learn if these organs are involved in the mechanism of action of the A^y gene since the gene reduces the age at which mammary tumors appear in virgin females from 15 to 8 months and since breeding eliminates the difference between groups of female mice with and without the gene. Transplantation studies in young adult animals so far have failed to implicate the ovaries; transplantations in day old F_1 hybrids are being done.

In addition to the increased susceptibility to mammary tumors and pulmonary tumors previously reported, last year Dr. Heston found that males with the A^y gene had a significantly higher incidence and average number of spontaneous hepatomas than those without the gene. The feeding of carbon tetrachloride had no detectable carcinogenic effect on inducing hepatomas in these highly susceptible male mice. In these F_1 hybrids the A^y gene increased body weight by about 10 grams in the males and 15 grams in the females. Positive correlations were found in the males between the number of hepatomas and body weight, body length and length of femur.

In similar studies on the ob gene, Dr. Heston has found that this gene decreases the occurrence of spontaneous pulmonary tumors. He previously

reported that this gene reduces the incidence of induced pulmonary tumors. Male mice with the ob gene also show a higher incidence of hepatomas, and administration of carbon tetrachloride gave no differences in occurrence of hepatomas between the obese ob mice and the normal sibs.

Dr. M. K. Deringer previously reported a reduction in the incidence of mammary tumors in mice of strain C3He which she produced by transplanting fertilized C3H ova to pregnant uteri of C57 black mice without the mammary tumor agent and an even lower incidence in DBA/2e mice (transfer of fertilized ova from strain DBA/2 to C57 BL uteri). Although the data are not complete, it appears that forced-breeding raises the incidence of mammary tumors in strain DBA/2e females, but the incidence does not begin to approach the nearly 80 percent figure seen in the DBA/2 strain of breeding females.

In studies of F_1 hybrids resulting from outcrosses of strain YBR animals (carrying the lethal-yellow gene) and strain DBA/2 animals (susceptible to the development of leukemia) in which the skins were repeatedly painted with 3-methylcholanthrene solution, Dr. Deringer has, as yet, found no effect of the lethal-yellow gene on the development of leukemia, but only a small number of leukemias have thus far been observed. To date, tumors of the skin occurred more frequently in the yellow than in the brown females and about equally in the yellow and brown males of the treated groups.

Radiation Studies

Dr. L. Law is continuing his investigation of the role of variables in X-ray induced and spontaneous leukoses. Chimeras developed in C3H mice (both the Law and Bittner sublines) through introduction in the neonatal period of AKR bone marrow showed the same incidence of X-ray induced lymphocytic and other neoplasms of reticular tissue as the nonchimeric controls, but introduction of AKR thymic tissue into the chimeras increased the frequency of tumors in the Law subline, but not in the Bittner subline. Few thymic grafts were involved in the neoplastic response, and the tumors grew in the host strain ($C3Hf/L_w$) and not in AKR mice. The presence of C3H thymus may therefore inhibit the process of leukemogenesis.

Fetal C57 BL mice were resistant to doses of X-ray that induced tumors in 24-hour and 1-month-old mice. This finding is in agreement with that of Upton, who also observed an insensitivity of fetal tissues in another strain, and indicates the desirability of further investigation, particularly during those early periods when other tissues function as hematopoietic tissues.

Total body irradiation increases the incidence of reticulum cell sarcomas and granulocytic neoplasms in thymectomized C57 BL mice. Shielding of a thigh, in contrast to this influence on thymic lymphoma induction, has been observed to be without effect on the induction of these later appearing forms of leukoses. Differences in response to fractionated, whole-body X-irradiation have been observed among three C3H sublines.

Miss D. Uphoff has extended her work on the genetic factors influencing protection against lethal total-body irradiation by a postirradiation inoculation of bone marrow to include the midlethal dosage level. Sufficient data are now available so that generalizations and comparisons can be made as to the effects of the dose of irradiation on the successful transplantation of bone marrow at the midlethal and lethal dosage levels. The postirradiation inoculation of genetically compatible marrow (marrow donor and host share the same histocompatibility –2(H–2) phenotype) will result in protection of the irradiated mice with no apparent immunological response at either dosage level. The available information indicates that these protected animals are chimeras consisting of an irradiated host of one genotype and a hematopoietic system of the donor genotype. The inoculation of genetically incompatible marrow (marrow donor and host of different H–2 phenotypes or of the $H–2^k$ phenotype), gives somewhat different results. Following lethal irradiation incompatible marrow may give rise to a graft versus host reaction ("secondary disease") which may result in death of the irradiated marrow inoculated mice after apparent recovery from the acute irradiation damage. The severity of the reaction and degree of mortality varies with the strain combinations and is probably a function of the antigenic incompatibility of the strains employed. The inoculation of genetically incompatible marrow following exposure to a midlethal dose of irradiation may result in what appears to be a host versus graft reaction which may result in an early death of the treated

mice. Although the exact cause of death has not been determined, it is clearly a function of the phenotype of the irradiated host. It occurs following the irradiation and marrow inoculation of two different strains of the H–2^k phenotype (other strains of the H–2^k group have not been tested). This lethality of the treatment has not been observed in irradiated hosts of the H–2^a, H–2^b or H–2^d phenotypes.

Miss Uphoff has obtained chimeras by exposing mice to *lethal* doses of X-ray and protecting them with suitable bone marrow. Under these conditions the lymphomas arising in the irradiated mice were mostly of the donor tissue, and those of the host genotype were cases where initial protection by donor marrow permitted regeneration of the host's marrow. When mice received a midlethal dose of irradiation and homologous bone marrow, most tumors were of host origin. At this dose level lymphomas arise primarily in genetic combinations in which the host and donor are of different histocompatibility-2 (H–2) phenotypes. In H–2^b or H–2^d combinations in which host and donor are of the same H–2 phenotype, lymphomas have not been observed. It would appear that although the marrow graft is capable of protecting the host against the acute irradiation damage, it does not appear to protect the irradiated mouse against this late radiation effect, possibly because of the regression of the graft as indicated by the lymphomas of host tissue origin.

Miss Uphoff had found earlier that amethopterin may protect mice that would normally succumb to a homograft reaction following lethal, total-body irradiation and homologous marrow inoculation. Such mice have a permanent state of this type of immunologic nonreactivity since it was shown that the mice are chimeras. On the basis that treatment with amethopterin in the presence of specific antigens might lead to a "tolerant" state in the adult mouse, mice were treated with the drug and strain specific antigens in the form of a crude tissue brei of spleen and thymus. Effects were determined by transplantation of tumors of the same strain as the antigens. Preliminary results are suggestive of success in inducing a "tolerant" state since 5 of 15 mice receiving both drug and antigen died of progressive tumor growth; mice treated with antigen alone were resistant to tumor growth, while with drug treatment alone only 1 of 14 had a positive test. Because tumor transplantation studies may be less adequate for evaluation than other types of transplantation procedures and because of the urgent clinical need for a method other than irradiation for the preparation of the host for a homotransplant, other studies are under way e.g., on dogs with Drs. Ferrebee and Thomas at the Mary Imogene Bassett Hospital.

Radiation of Cells in Tissue Culture. Dr. M. Elkind, in an extension of his previous studies dealing with the repair of sublethal X-ray damage, has found certain experimental conditions under which it appears that survival of mammalian tissue culture cells following irradiation may be influenced by the proximity of neighboring cells. Such a result has important implications since one of the basic tenets of radiobiology, the assumption that the site structure in cells lethally affected by ionizing radiation is discrete, requires that a cell in a group of cells should survive as an individual and uninfluenced by the proximity of its neighbors.

In an investigation of the influence of drugs on lethality and recovery in X-irradiated mammalian cells grown in culture, Drs. Elkind and W. Mohler have obtained results with 5-bromodeoxyuridine (BUDR) which indicate: (1) BUDR can increase the X-ray sensitivity of a Chinese hamster culture cell line; (2) the increase is accompanied by alterations in the survival parameters, mean lethal doses (MLD) and extrapolation number (a graphically determined estimate of the loci, or sites, each containing one target with the necessary and sufficiency condition for survival being the viability of at least one locus, in an irradiated cell); (3) the effects on MLD and extrapolation number may be separable under some circumstances; and (4) the alteration of sensitivity is probably not merely the result of nonspecific cell injury since other drugs alter one X-ray survival parameter (MLD) without affecting the other.

Immunological Phenomena and Polysaccharide Studies

Improvements in techniques and increase in knowledge of immunological approaches are opening new avenues of research on host humoral and cellular factors involved in natural immunity to

infection and neoplastic disease. The polysaccharide nature of bacterial endotoxins, their importance in study of immunologic phenomena, and their ability to damage neoplasms (a matter of long-standing interest in the Institute stemming from the early work of Dr. M. J. Shear), plus the increasing knowledge of the production by the body of a number of substances, including antibodies and special enzymes, in response to infection and tumors have led to expansion of programs in the Laboratory of Chemical Pharmacology in the Polysaccharide Section.

Endotoxin Studies. Bacterial endotoxins, polysaccharide materials now prepared by Drs. M. Landy and E. Ribi (NIAID) in highly potent form with no more than 0.5 percent nitrogen and 1.7 percent fatty acid ester, are highly useful materials in the study of immunity, and with these and the highly sensitive immunological techniques being developed by Dr. Landy and his associates, certain phenomena exhibited by the whole organism and by cells that were puzzling in the past are now becoming clear. The nature of the effects of endotoxin on mammals is under study in several laboratories, including the various means by which host susceptibility can be modified. Although intensive investigation is under way on the state of refractoriness or tolerance produced by prior treatment of an organism with endotoxin, kinds of treatment that greatly increase susceptibility are less well defined. These latter are: reticuloendothelial system-blockade, adrenalectomy, administration of BCG vaccine, and tumor implantation.

In preparation for more extensive work designed to learn whether these diverse treatments produce in the host any change in common, Drs. Landy and R. J. Trapani, in collaboration with Dr. E. Suter (University of Florida), have investigated the possibility of additive or synergistic effects of a combination treatment of CAF_1 mice with BCG vaccine and implantation with sarcoma 37. Results were determined following subsequent challenge with *Sal. enteritidis* endotoxin. No synergistic effect was found; the effects were not additive, and the results indicated interference of the action of BCG by the tumor.

In the course of studies on alterations in the somatic endotoxin complex of Gram-negative bacteria that occur during the serum bactericidal process (see last year's report), it was observed that very small amounts of antibody sufficed for bactericidal activity. Dr. Landy, with Drs. J. Whitby (guest scientist) and J. Michael (Visiting Scientist), has developed a simple, specific and objective assay for bacterial antibodies that is much more sensitive than previously developed procedures. Tests with immunologically calibrated antiserum against *Sal. typhosa* showed that the smallest amount of antibody measurable by the method was 0.0002 microgram antibody and that 0.001 microgram provided marked bactericidal effect. On this basis normal mouse serum was estimated to contain approximately 0.01 to 0.02 microgram antibody nitrogen (against *Sal. typhosa*) per milliliter.

In a careful study of the pyrogenic response of the rabbit to endotoxin, Drs. Landy and W. Keene (PHS Hospital, Boston) have also developed a more precise bioassay for endotoxin by means of a procedure requiring administration of amounts of endotoxin that will produce febrile responses that fall on the linear portion of the dose response curve.

The inactivation of endotoxin by blood plasma described in previous annual reports was found to have characteristics of an enzyme-catalyzed reaction. The progress of this reaction was followed by measuring residual substrate (endotoxin) concentration in the pyrogen assay, and the assay was based on the assumption that the residual fever-producing substance in the plasma-endotoxin reaction mixture was unaltered endotoxin. In the previous report, it was suggested that the residual pyrogen of incubated plasma-endotoxin reaction mixture had the pyrogenic properties of endogenous serum pyrogen. With Dr. Keene, Dr. Landy has now demonstrated that animals tolerant to endotoxin are also tolerant to the residual pyrogen. In addition, daily injections of the residual pyrogen induced endotoxin tolerance. Since the most distinctive quality of endogenous pyrogen is that it is equally pyrogenic for normal and endotoxin-tolerant animals, it appears that the residual pyrogen of incubated plasma endotoxin reaction mixtures is not endogenous pyrogen.

Not only is endotoxin inactivated by blood plasma, but tissues are also capable of inactivating endotoxin. As in the case of the inactivating system in blood plasma, the *in vitro*

inactivation of endotoxins by cell-free homogenates of perfused rabbit liver also appears to be an enzyme-catalyzed reaction, but unlike the former is not influenced by calcium ions (studies by Drs. Landy and Trapani with Dr. V. Waravdekar—AFIP). Preparations derived from rabbit liver display good endotoxin-degrading activity, but several other tissues from rabbit showed extremely low or no activity. Extracted cells also yielded clear preparations low in nitrogen and total solids that were potent in destroying pyrogenicity and tumor-damaging activity of bacterial endotoxin. Rabbit granulocyte homogenates were likewise found to be effective by Drs. Landy and A. L. Notkins in activating endotoxin, although other formed elements of blood, including human erythrocytes, platelets and rabbit monocytes were not found to inactivate endotoxin. Administration of large amounts of endotoxin to mice did not lead to elevation of serum lactic acid dehydrogenase, an intracellular enzyme that might be expected to be elevated in the serum if the endotoxin were producing damage to granulocytes as has been claimed in reports in the literature. Furthermore, no release of this enzyme was detected when mouse peritoneal macrophages were incubated with endotoxin.

Drs. M. Woods and Landy have shown that the capacities of a series of endotoxins (from different Gram-negative genera, isolated by different procedures, and having different potencies in eliciting characteristic effects in the host) correlated well with stimulation of glycolysis *in vitro*. Moreover, experiments on the degradation of endotoxin by humoral and intracellular enzymes of the host, or by progressive acid hydrolysis, resulted in a loss of capacity to stimulate glycolysis. Both aerobic and anaerobic glycolysis are stimulated; the lowest effective concentration of endotoxin is about 0.0001 microgram per ml., and 0.3 microgram per ml. generally provides maximal effect. Similar effects could be produced *in vivo* as measured with *in vitro* determinations of glycolytic activity of peritoneal macrophages obtained from mice given endotoxin a few hours previous to the isolation of the cells.

Tumor Cytotoxic Factors in Serum. Systematic investigation of the antibody-complement system in normal serum lethal to mouse tumor cells, which was reported on last year, has been largely completed by Drs. Landy, Trapani, Michael and Woods. The general findings are that normal sera from many animal species exhibited *in vitro* a lethal effect on ascites cells of sarcoma 37 and a considerable number of other tumor cells of mice. This *in vitro* activity of serum on the tumor cells of a heterologous species was manifested by: failure to proliferate in a susceptible host; altered permeability to eosin; morphologic changes; and cessation of glycolytic activity. The system in serum responsible for these effects consisted of a natural antibody and complement. This antibody had specificity for an antigen present in a variety of tumors and lymphoid tissues of mice. In continuation of the study, the failure of fresh human serum to exert a comparable effect on tumor cells *in vivo* was shown to be due to uptake of the antibody by normal lymphoid cells of the mouse. In tests for this cytotoxic activity, absence of the cytotoxic effect was found in 7 of 17 species studied. Further evaluation indicated, however, that the class of "inactive" species did in fact possess a functional antibody and complement against a bacterial component. Furthermore, sera from two "inactive" species, such as hamster and guinea pig, when combined would give an active preparation as measured by red cell lysis in an appropriate system. It is therefore evident that serum from various species may differ strikingly in the properties of complement they contain.

In collaboration with Dr. R. Smith, the levels of cytocidal activity (mouse ascites tumor test systems) in the serum of cancer patients was compared with that in normal human serum by Drs. Burk and Woods. The action of such sera (active and 52° C.-inactivated) on the patients' own cancer cells was also investigated. Preliminary results indicate somewhat higher than normal levels (2- to 3-fold) of cytocidal activity in the sera of cancer patients.

Antigens Against Human Carcinomas. The complexities encountered in the study of host humoral and cellular factors in natural immunity to infection and neoplastic disease and the critically sensitive techniques required for such investigations are well illustrated in the experiments summarized above. Perhaps for these reasons more than any other, progress in immunology and cancer has been slow despite a vast amount of

effort expended in this direction over the past 50 years. With such modern procedures, Dr. B. Björklund (State Bacteriological Institute, Stockholm) has conducted experiments which led to a recent report that human carcinomas have antigenic properties different from those of normal human tissues. By prolonged immunization of horses with preparations of pooled human carcinomas, an antiserum cytotoxic for HeLa cultures was obtained. This cytotoxicity, which was not affected by absorption with normal human tissues, was removed by absorption with human carcinoma tissue or by HeLa cells.

Drs. Landy and Trapani, in collaboration with Dr. Björklund have initiated investigations which not only sought to confirm the principal findings of Björklund, but also were designed to explore the possibilities of developing serological procedures for more extensive work with such antigenic preparations. Their serological experiments have indicated that it is possible, under appropriate conditions, to "coat" tannic acid treated erythrocytes with preparations extracted from HeLa cells with buffer at pH 8. The treated erythrocytes were agglutinated to high titer by horse antiserum against pooled human carcinomas. It was suspected that the antiserum also contained antibodies against normal tissue antigens. The presence of such antibodies was demonstrated with the use of tanned erythrocytes coated with pooled normal human serum. These cells were also agglutinated to high titer by the horse antiserum. Practically all of the antibodies reactive with human serum coated erythrocytes could be removed from the antiserum by absorption with cells suspensions of normal human tissues. However, the reactivity of such absorbed serum with HeLa antigen-coated erythrocytes, and its cytotoxicity for HeLa cultures, were not discernibly affected. On the other hand, analogous absorption of the antiserum, with Björklund's antigen preparations derived from human carcinomas, removed cytotoxicity for HeLa cultures and reactivity for erythrocytes coated with HeLa antigen; it is noteworthy that reaction with erythrocytes coated with normal human tissue antigens remained unchanged. The absorption technique was controlled by parallel treatment of an antiserum prepared in rabbits by immunization with normal human serum. This antihuman serum was cytotoxic for HeLa cells and agglutinated erythrocytes coated with HeLa antigen or with preparations from normal human tissues. However, these properties were no longer evident after the serum had been absorbed with normal human tissues.

Immune Tolerance Induced by Breeding. Last year, Drs. M. K. Barrett and E. J. Breyere (Research Fellow, reported the interesting finding that a state of immunological tolerance can be induced in female mice by breeding with males of another strain which makes them susceptible to transplants of tumors and skin from the strain of the male with which they were bred. Further details have been developed, and, to date, the effect has been observed in three genetic systems with three transplanted tumors and with skin homografts in two combinations. The H–2 histocompatibility locus does not appear to be involved. This tolerance is highly specific and depends upon a genetic concordance between the transplanted tissue and the sire of previous litters. Tolerance was not found in females parous from intrastrain matings, nor in virgins, nor in those parous by males of still another strain unrelated to the graft. Experiments under way have thus far shown the following: (1) Tolerance increases with multiparity. (2) It is effective both in the case of "natural" and induced resistance. (3) It is long lasting, i.e., at least 150–200 days in the mouse. (4) A previously induced immune state can be partially abrogated by specific parity. (5) This tolerant state is resistant to the usual immunization procedures, i.e., immunity cannot be induced in tolerant females by an inoculation of homologous blood and a degree of tolerance remains even after the rejection of a homologous skin graft. (6) The effect of parity is not due to mere exposure to the male in caging, and (7) the presence or absence of the mammary tumor milk agent in the male had no detectable influence.

Polysaccharide Investigations. The *in vivo* production of cytoplasmic vacuoles in ascites tumor cells following the intraperitoneal administration of polysaccharide was reported previously by Dr. M. Belkin. The phenomenon, which occurs only *in vivo*, appears to be the result of an antigen-antibody reaction. In collaboration with Dr. Dalton in studies of treated cells of three

ascites tumors, the following results have been observed with electron microscopy:

(1). In sarcoma 37 cells the vacuoles appear to arise primarily at the site of virus particle formation; but others arise also from preformed small vacuoles and "inclusion bodies" which are occasionally present.

(2). Vacuoles develop in Yoshida ascites cells preformed small vacuoles and membrane-enclosed, electron-dense inclusion bodies.

(3). In Ehrlich ascites cells, few preformed vacuoles are present in untreated cells, and the vacuoles develop more slowly from membrane-bound, electron-dense "inclusion bodies"; the cells of this variant of Ehrlich ascites tumor were rarely found to contain virus particles.

The programs of the Macromolecular Chemistry Section, Laboratory of Chemical Pharmacology, have as their over-all aim study of the interaction of biological systems on the macromolecular level, with special emphasis on the forces that initiate the interactions and that orient the interacting biological systems in a manner of high efficiency. Included in these programs are studies on polysaccharides, and important information about these and related compounds complement the results of investigations made by Dr. Landy and his group, often obtained in collaboration.

The extensive collection of polysaccharides synthesized by Dr. P. T. Mora and Mr. J. W. Wood, which includes polymers of various sugars and their substituted derivatives with negative (acid) groups (such as polyglucose sulfate or carboxyl derivatives), has been enlarged now to include derivatives substituted with positive (basic) groups. By synthesizing amino derivatives, they have obtained polymers with graded substitution of positive groups. In the course of the work, new methods were developed which led to the introduction of unprecedentedly high amounts of amino groups into polysaccharides: (1) The reduction of the tri-*beta*-cyanoethyl derivative of polyglucose with lithium aluminum hydride in tetrahydrofurane, and (2) the reaction of 1-N-diethylamino-2, 3-eposypropane with polyglucose in aqueous sodium carbonate solution. Some of the synthetic polysaccharides (polyrhamnose and polymaltose) possess considerable lipemia-clearing activity without demonstrable toxic or anticoagulant activity, and some others inhibit mitosis in tissue culture (findings of Professor R. Meir, Ciba, Ltd., Basle and Dr. A. Di Marco, University of Milan, in collaboration with Dr. Mora). These synthetic, charged polysaccharide derivatives can also be usefully employed in fractionation procedures to obtain at a certain pH a precipitated complex with a biological macromolecule. After centrifugation and separation from other soluble components, the precipitate can be easily dissociated again by changing the pH or salt concentration. Such a procedure is now under study as a means of fractionating the endotoxin detoxifying component of Landy.

Additional information has been obtained on the polysaccharide (P–45) prepared from *Serratia marcescens* by a new, large-scale method of Mr. A. Perrault reported on last year. This polysaccharide endotoxin is an acid polysaccharide complex. In earlier work with Dr. E. Merler, Dr. Mora found 80–100 negatively charged groups with pKa = 2.3 in a 100,000 molecular weight unit. With Mr. B. G. Young, the titration of the negative polysaccharide with positively charged macromolecules, such as polymyxin, protamine and lysozyme, has been studied. There was a neutralization observed at a pH range where the maximum increase in turbidity indicated that strong macromolecular interaction (precipitation) took place. Generally, with the positive macromolecules the tumor necrotizing activity decreased, but the pyrogenic activity remained constant. However, after interaction with lysozyme the tumor necrotizing activity slightly increased, and after prolonged incubation for one or two weeks the fever-producing activity substantially decreased. The interaction with lysozyme was accompanied with changes in the sedimentation: lower molecular weight components appeared. Some fractionation studies were carried out in the ultracentrifuge in collaboration with Dr. W. Carroll (NIAMD). The P–45 preparation was also found to be effectively deaggregated by treatment with the anionic detergent: sodium lauryl sulfate. Sedimentation of the original P–45 in the ultracentrifuge shows two peaks, one $S^{w}_{20} = 4$, and another $S^{w}_{20} = 12$. After treatment with sodium lauryl sulfate, there ap-

pears only one peak at $S^{w}_{20} = 2$. However, up to now, the sodium lauryl sulfate could not be removed without causing reaggregation. The biological activity of the $S^{w}_{20} = 2$ component was unchanged in the presence of the sodium lauryl sulfate. This indicates that the biological activity is retained in a relatively small molecular weight unit of about 10,000 to 20,000.

These studies are of importance in understanding the relationship of biological activity of a macromolecule and the types, number and distribution of active sites on the molecule. Studies have been initiated to differentiate the enzymatic and antigenic sites on an enzymatically active protein (RNAase) by electrostatic complexing and blocking of certain limited portions of the protein surface. Other serum components interfered with the enzymatic assay when impure RNAase-antibody was added to RNAase, and purified antibody is now being prepared (method of Singer). Under study are influences on enzymatic activity of complexes with such antibody and effects of adding negatively-charged macromolecules on enzyme activity, enzyme-antibody interaction, and *in vivo* development of antibodies to RNAase. Enzyme-substrate interactions are also being investigated by similar approaches.

Metabolism of the Tumor-Bearing Host

Body Composition Studies. Drs. J. White and F. Miller and Mrs. J. Toal (in collaboration with Drs. J. Bloch, A. Harris and N. Berlin) continue to add knowledge on the changes in body composition and physiology which occur in tumor-bearing rats. Last year, in a study designed to gain information on the increased requirement for sodium ion by rats bearing subcutaneous, progressively growing Walker carcinosarcoma 256, it was found that when the tumor was more than 10 percent of the body weight pronounced retention of sodium occurred. Analyses of carcasses and tumors have now shown that the amount of sodium in the tumor accounted for almost all of the total sodium retained. When the concentrations of nitrogen and electrolytes in carcasses of rats bearing the tumor were expressed on a fat-free, wet weight basis, it was found that sodium, chloride and nitrogen were increased above normal values and potassium and water were decreased. Samples of muscle and bone showed no increase in sodium and chloride ion concentrations, and, although increases were observed in skin, they were not enough to account for the increases found in total carcass. From studies with undernourished mice, it appears that, with respect to carcass composition, the tumor-bearing rat in positive nitrogen balance resembles a nontumor-bearing rat in negative nitrogen balance. Analyses of tumor composition show higher concentrations of sodium and chloride and lower concentrations of potassium and nitrogen in necrotic tissue than in viable tissue.

Rats bearing the Murphy-Sturm lymphosarcoma intramuscularly were also studied. Carcasses showed changes similar to those observed with the Walker tumor-bearing rats. Total sodium retained by the Murphy-Sturm tumor-bearing rats, however, was much less than that retained by the Walker tumor-bearing rats.

In order to compare the rate of equilibration of water in a variety of tumors with water in blood and other tissues, Drs. Millar and White injected tritiated water intravenously into ether-anesthetized rats and mice bearing different primary or transplanted tumors and obtained samples of cardiac blood, tumor and other tissues at subsequent times. From radioactivity measurements on the water vacuum-distilled from the samples, equilibration values from the tissues were compared with those of cardiac blood samples. With the exception of mouse ascites tumor, all viable tumor tissues reached equilibrium with blood in 5–10 minutes. Tumor equilibration time for the tumors were similar to those for muscle, greater than those for liver, but less than those for skin. Mouse ascites tumor equilibration times were about thirty minutes. The results suggest that the major factor in regulating water exchange in tumor tissue is the blood supply to the tumor. The findings have implications of importance to the study of the rates at which drugs enter tumor and normal tissues, and antitumor drugs will be evaluated.

Previous work by Drs. R. E. Greenfield, Jr. and V. E. Price dealt with the etiology and nature of anemia of tumor-bearing subjects. These studies demonstrated that the anemia of rats with certain tumors could be quantitatively correlated

with the loss of cells by hemorrhage into the area of the tumor. In the course of this work it was observed that rats with marked anemia lost weight more rapidly, became cachectic and died while the tumors were in the range of 20–30 percent of the body weight, whereas in animals without anemia the onset of weight loss and cachexia was much slower, and the tumors frequently reached 50–65 percent of the body weight. However, in further study on the relationship of the anemia to the cachectic process, it was observed that during the period of most rapid tumor growth, and despite the onset of anemia, rats bearing the Lymphosarcoma R2788 gained appreciably more weight than the normal controls although they both had almost identical food consumption.

Dr. M. Rechcigl, Jr. and Dr. Greenfield have done additional experiments designed to elucidate the nature of these findings. Carcass and tumor analyses showed that the lymphosarcoma-bearing animals had an increased percentage of water sufficient to explain their increased weight gains. An equally high degree of hydration was observed in rats bearing Hepatoma 3683 whose body weight gains were lower than those of the normal controls. The combined nitrogen content of the tumor and the carcass of both groups of cancerous rats was similar to or smaller than the body nitrogen of the control animals, while the fat content of the tumor-bearing rats was markedly reduced. In confirmation of these findings, balance studies showed nearly the same retention of nitrogen in rats bearing the Lymphosarcoma R2788 as in the controls, but there was a greater retention of water and sodium, concomitant with the increased weight gains in the tumor-bearing animals.

During the terminal stages, the lymphosarcoma-bearing rats were observed to suffer from anorexia, loss in total body weight and finally died. In this period the animals were in a negative nitrogen and potassium balance, and the water retention was reduced below the normal level while the sodium balance was not appreciably affected. The positive sodium balance together with the decrease in the potassium retention suggest the possibility that, to a considerable extent, the terminal dehydration of the tumor-bearing animals was due to loss of intracellular fluid. In these studies, the onset of anorexia and the negative nitrogen balance was not prevented by increasing the sodium chloride intake of the animals, and indeed a decrease in survival time was observed among the tumor-bearing animals ingesting saline.

Chemically Defined Diets. Drs. M. Winitz and S. M. Birnbaum and Mr. M. C. Otey have extended their studies with chemically defined diets which were reported on last year. Earlier studies have revealed that chemically-defined diets composed of the 10 essential L-amino acids (in levels recommended by Rose *et al.*), 8 nonessential L-amino acids, the requisite vitamins and salts, glucose and ethyl linoleate, when provided to rats *ad libitum* as 50 percent solutions in water, sufficed to meet the nutritive requirements for growth, lactation and reproduction over several generations. In the attempt to develop a diet wherein each of the essential L-amino acids was present at the minimal level consistent with optimal growth, diets were prepared which incorporated each of the essential amino acids (taken one at a time) in an amount ranging from 0 to 120 percent of that previously employed. By variation of the nonessential nitrogen level all mixtures were kept isonitrogenous but were otherwise identical. Such diets were fed to weanling male, Sprague-Dawley rats over a 10-day period, and the minimal level of each essential amino acid commensurate with optimal growth was ascertained. Minimal levels of the essential amino acids, relative to the levels in diets previously studied, were as follows: L-lysine, 60 percent; L-phenylalanie, 40 percent; L-tryptophan, 80 percent; L-isoleucine, 100 percent; L-methionine, 60 percent; L-leucine, 100 percent; L-valine, 80 percent; L-histidine, 60 percent; L-threonine, 100 percent. With these ratios as the basis, it became possible to formulate chemically-defined diets whereon weanling rats grew at a rate of over 4g. per day and with more efficient utilization of nitrogen than had been observed hitherto.

It has been known for some time that certain D-amino acids could substitute for their optical antipodes, some more effectively than others, as essential amino acids required for nutrition, presumably through successive conversion to the corresponding *alpha*-keto acid (catalyzed by D-amino oxidase) and, through transamination, to the L-antipode. Last year studies of this type

utilizing chemically-defined diets were conducted on methionine, leucine and tryptophan. Although the conversion of D-leucine to the corresponding *alpha*-keto acid (catalyzed by kidney D-amino acid oxidase) proceeds at an appreciably faster rate than the analogous reaction with D-tryptophan *in vitro*, the degree of utilization of the latter amino acid *in vivo* exceeds that of the former one. A comparable situation has now been found with D-alloisoleucine, which was reported in the literature to be completely ineffective as a replacement for L-isoleucine in the diet of the rat, but which is, at the same time, very rapidly oxidized by D-amino acid oxidase *in vitro*. In order to ascertain why the ability of the organism to utilize the D-antipodes of the essential amino acids varies so considerably, and why results are sometimes obtained that are in variance with those that would be expected on the basis of *in vitro* enzymatic behavior, experiments were set up in which groups of animals were fed chemically-defined diets wherein the L-isoleucine component was replaced, in three separate diets, with one, two and three equivalents, respectively, of D-alloisoleucine. Although no growth was observed with the first two diets, a marked growth response was noted with the third. The data therefore revealed that D-alloisoleucine could be utilized *in lieu* of L-isoleucine if present in sufficiently high concentration in the diet. Examination of the urine of these animals also indicated that the D-alloisoleucine is excreted at an appreciably more rapid rate than is D-leucine, D-tryptophan or D-methionine and thus provided a plausible explanation for both the ability of D-alloisoleucine to support growth when provided in the diet in relatively high concentration and for the relatively lesser degree of utilization observed with D-alloisoleucine than with the other above-mentioned D-amino acids, despite its appreciable susceptibility to D-amino acid oxidase *in vitro*. The data indicated that the degree of utilization of D-amino acids by an animal organism is conditioned not only by the rate at which they are enzymically converted to their L-counterparts *in vivo*, but also by the extent of their renal excretion.

Previous experiments in the Laboratory of Biochemistry have indicated that satisfactory nutrition could be provided for rats on the chemically-defined diet offered as 50 percent aqueous solutions. From controlled experiments conducted this past year, it has been shown that the 50 percent water soluble diet is adequate for normal growth and well-being of rats, at least for a limited period of time, in the absence of independently supplied water.

In collaboration with Dr. C. I. Jarowski (Chas. Pfizer and Co.), Dr. Winitz has begun an evaluation of the concept that the free amino acid levels in the blood plasma of a given animal species, in the fasting state, might serve as a precise indicator of the dietary amino acid requirements of that species. Studies were done to compare the growth response of rats provided with the most efficient water-soluble, chemically defined diet hitherto devised in the Laboratory of Biochemistry, with that of rats fed comparable diets wherein the essential amino acids were present in the same relative ratios as they appeared in the plasma of the fasting rat. The data demonstrated that the former and latter diets elicited comparable growth rates, despite striking differences in their essential amino acid compositions. Thus, in the rat, the concept appears valid, and, if, after work in other species, the principle becomes established, it would be of considerable assistance in the formulation of diets.

In Vivo Perfusion Studies. In efforts designed to find a means for growing a tumor isolated from the surrounding tissues and connected with the host only by one artery and one vein, Dr. P. Gullino (Visiting Scientist), with Mrs. F. H. Grantham, has developed a technique for growing tumors in pouches developed in kidneys (or in ovaries). A kidney was isolated from the surrounding adipose tissue with the vascular peduncle as the only connection with the host. A tumor was then transplanted into the renal parenchyma, and the kidney enveloped in a bag of paraffin was placed into the subcutaneous tissue. The tumor grew in this bag and destroyed the kidney tissue; the renal vessels remained as the only connection between the tumor and the host (rats, hamsters or mice).

In rats, renal implants grew up to the size of 25 grams (about 25 times the kidney's original weight) and ovarian implants grew up to 14 grams (about 400 times larger than the host ovary). Two transplants of the same tumor, one grown in a paraffin bag and the other grown sub-

cutaneously, showed the same correlation between wet weight, dry weight and total nitrogen and also the same level of anaerobic glycolysis. Animals bearing the "isolated" transplant behaved in the same manner as those with subcutaneous tumors as to their change in body weight, food consumption and water intake. Liver catalase was also equally depressed.

Utilizing this technique, Dr. Gullino, with Mrs. Grantham, has measured blood flow from implanted tumors and has studied the effects of certain drugs on this blood flow. In an adult rat the rate for the ovary and oviduct was determined to be about 12 ml. per hour. The same amount of blood flows out of tumors weighing 6.0 grams or more, namely 200 times heavier than the ovary into which they were implanted. The growth of a tumor implanted in a kidney, which has a blood supply 20 times larger than the ovary, produces a decrease of the total blood flow to the degree that when the tumor has destroyed all the kidney parenchyma, the blood reaches values of the magnitude of those found in ovarian implants. Despite a difference of 20 fold in the blood supply between the ovary and the kidney, equal amounts of tumor implanted simultaneously in both organs of the same rat grew at the same rate, suggesting that a large blood supply does not increase the growth rate of rat tumors. Adrenalin reduces the blood outflow of a tumor and the removal of the coeliac ganglion has the same effect. Choline increases the blood outflow in some instances.

Collagen, which was determined in collaboration with Mr. H. Taylor by measuring the amount of hydroxyproline content of acid hydrolysates of tumors, increases with the growth of the tumor. Preliminary comparative studies of some enzymatic activities in the efferent and afferent blood of the tumor are being done.

Kinetic Studies in the Tumor-Bearing Animals. Last year Drs. M. Rechcigl, Jr. and V. E. Price reported on a method for determining the kinetics of *in vivo* synthesis and destruction of the liver enzyme, catalase, by use of 3-amino-1, 2, 4-triazole. With Dr. R. Hartley, they have evaluated the validity of this method by an alternative one in which catalase synthesis was blocked by the use of allylisopropylacetamide administered intraperitoneally to rats. When synthesis was blocked, activity of the remaining catalase disappeared with a first order constant, K_d, of 0.023, corresponding to a rate of disappearance of 2.3 percent per hour, a figure almost identical to that obtained by the older method.

These methods have been employed in comparative studies with rats on a protein-free diet, on a starvation diet, or bearing a tumor. In the liver of starved animals, synthesis and destruction proceeded at essentially the same rate per gram of tissue as in normal animals for the first 5 or 6 days of starvation. The liver and total liver nitrogen is continually decreasing in size during this period, however, so that there is actually less catalase being synthesized and destroyed in the total liver. In contrast to the picture seen in starvation, animals on a protein-free diet of carbohydrate, fat, minerals, vitamins and a source of fiber showed a decreased rate of catalase synthesis per gram of tissue or per mg. of nitrogen. Although there was less catalase being synthesized, the fraction of catalase molecules being destroyed per unit time was within normal limits, thus resulting in a lower concentration of catalase within the liver. However, in the animals on the protein-free diet, the liver size and total liver nitrogen decreased only slightly.

The effects of tumors are more nearly duplicated by the protein-free diet, for in the tumor-bearing animals there is a marked fall in the catalase activity per gram of liver, and the liver is frequently somewhat enlarged. In both these states the total liver catalase is markedly reduced as it is in starvation, but it is not accompanied by the marked decrease in liver size or total liver nitrogen seen in starvation.

Enzyme Activities of the Tumor-Bearing Host. Comparative studies of catalase activity of various hepatomas and of their effects on host liver and kidney catalase activity levels have also been made by Drs. Rechcigl and Price, in collaboration with Drs. H. P. Morris and H. Sidransky. A fairly high catalase activity was found in the 5123 hepatoma and in the primary and the transplanted ethionine-induced hepatomas of mice. Almost no activity could be demonstrated in the Dunning, Novikoff and the 3683 hepatomas of rats.

Despite the fact that fairly high levels of catalase were found in the tumor, the catalase

activity of the liver and kidneys of animals bearing 5123 and ethionine-induced hepatomas was depressed to a degree comparable with the other tumor-bearing animals tested. The amount of depression was correlated with the size of the tumor and the loss of the carcass weight.

In view of the lowering of the liver catalase of normal animals following the feeding of a protein-free diet, it was of interest to find out whether a comparable decrease in the enzyme activity would occur in hepatoma 5123. The experiments have shown that a protein-free diet produces no significant lowering of catalase in the 5123 hepatoma, even though the catalase of the liver was markedly reduced.

In spite of numerous reports that a substance isolatable from tumor tissue (toxohormone) causes reduction of liver catalase activity by liberation of the substance into the blood stream with local action on the liver catalase, other interpretations of the phenomena may well deserve equal or better support. The reduction in catalase activity observed in bled animals and in those placed on protein-free diet (*vide supra*) suggest other mechanisms, such as a relative nutritional deficiency or an alteration of the homeostatic balance brought about by competing altered metabolic pathways. The kinetic models designed to permit the simultaneous measurement of catalase synthesis and destruction, especially if confidence in their validity can be maintained, for nonsteady states present in tumor-bearing animals as more work is done, are likely to aid in the clarification of this well-known effect on the host by a tumor.

Transplantation of a primary ethionine-induced hepatoma resulted in tumors having two different levels of catalase activity, one of which was twice that of normal liver, whereas the other was about half the normal level. Enzyme level estimations in tumors of later transplant generations of the two lines will be of interest.

Utsugi has recently reported that hypophysectomy will prevent the depression of liver catalase activity. Drs. Rechcigl and Price, with Dr. S. Wollman, however, found a marked fall in the concentration of liver catalase in hypophysectomized animals bearing transplanted Wollman thyroid tumors. In fact, the effects of the tumor growth and removal of the pituitary gland were additive. Starvation is often referred to as pseudohypophysectomy, since the effects of starvation and extirpation of the gland are similar. The similarity was also seen in experiments involving liver catalase measurements in hypophysectomized rats, since the livers were only about one-third the normal size, and the catalase activity per gram of liver tissue was at normal or slightly elevated levels.

The association of elevated serum aldolase and lactic acid dehydrogenase with certain tumors and with certain lesions of muscle has been well-documented in both human and animal subjects. Recently, Riley has reported marked elevation of serum lactic acid dehydrogenase in mice that had received injections of cell-free extracts. Drs. Greenfield and R. Berry have confirmed these findings in mice; however, rats injected with a large series of transplanted tumors had no such elevation, and cell-free extracts of tumors were also without effect in these animals. Preliminary studies suggest that the elevation of lactic acid dehydrogenase as seen in mice is not due to a viral agent since elevation was still obtained following administration of cell-free extracts irradiated with a dose sufficient to inactivate most viruses.

Deoxyribosides of the Tumor-Bearing Host. Investigation of the deoxyribosides of rat tissue and the metabolism of these compounds during liver regeneration and growth is under way by Dr. J. Rotherham. Deoxycytidine, 5-methyldeoxycytidine, deoxyuridine, and unidentified deoxynucleotides were found in the urine of normal rats fed a casein diet. When rats are maintained for 2 or 3 weeks on a diet which contains no source of preformed deoxyribosyl compounds, these compounds are still continuously excreted in the urine, an indication that they are formed endogenously (bacterial action has not been ruled out). Studies with this special diet indicated that the level of excretion fell in hepatectomized animals on the second day after the operation. A fall in levels of excretion occurred in chow fed animals bearing the Novikoff hepatoma when the tumor was growing rapidly, but a similar fall was observed in pair-fed control animals.

Urinary Excretion Patterns of Nucleic Acid Congeners in Leukemia Patients. Further progress continues to be made by Dr. J. Reid

and Mr. W. Bell in the difficult and complex investigation of the urinary excretion patterns of nucleic acid congeners and other substances in leukemia and normal human objects. The methods used are primarily chromatographic with employment of ultraviolet absorption spectra determinations of the eluate fractions; machine methods of data processing are being introduced. Dietary composition exerts an important effect on the normal urinary pattern. The absorption curves were changed in all chromatographic fractions when a freely selected diet was given compared with the results seen when a low-purine, low-ribose control diet was administered. Patterns seen in urines from two untreated patients with acute myeloblastic leukemia and from two untreated patients with acute lymphoblastic leukemia differ from those seen in urine from normal subjects.

Vector Analysis-Computer Techniques. In a study designed to test the feasibility of analyzing multicomponent mixtures (by means of vector analysis-digital computer techniques applied to the ultraviolet absorption spectral curves of chromatographic eluates of ribonucleic acid (RNA) hydrolysates), Drs. Reid and A. Pratt have made considerable progress. With RNA as a model, it is intended to develop the techniques to a point where they can be used to analyze urinary excretion patterns from leukemia patients. RNA hydrolysates consisting of the four major nucleotides and three other components present in smaller amounts have been analyzed by the computational method with a precision equal to or better than that of standard methods and an accuracy about as good. When the spectra of the minor components have been determined, the accuracy will be increased. With the aid of Mr. N. Coffey, automation of the system is being accomplished, thus permitting future practical application of the approach to large numbers of samples.

Energy Expenditure Studies. Further improvements in mathematical formulation and instrumentation utilized in direct and indirect calorimetry studies have been made by Dr. Pratt and Mr. W. White. Particular attention is given to methods because of the need for high precision in evaluating the independent measures of energy expenditures (direct and indirect) of the tumor-bearing and normal animal. From mathematical approaches developed by Dr. Pratt to the solution of the dynamic water pool determination and the determination of the body volume of an animal in the respiratory chamber, it appears possible with the use of tritium-labeled water to obtain samples of respiratory water without disturbing the animal and, if measurements can be made with sufficient accuracy, to estimate the total body water and body volume of the animal in the chamber. By use of density measurements of the dry total carcass and of bomb colorimetric measurements of the calories per gram of dry total carcass, Dr. Pratt is now able to calculate from relationships he has formulated, the nonfat and fat fractions without the necessity of chemical separation. These new approaches provide additional means for evaluating independent measures of energy expenditures, one of the important areas of investigation of metabolism of the tumor-bearing subject.

With Drs. H. Benzinger and C. Kitzinger (Naval Medical Research Institute, Bethesda, Md.), Dr. Pratt is developing techniques of clinical thermometry to ascertain body temperature-energy metabolism relationships. A basic relationship between skin temperature, intracranial temperature and energy production has been found in studies on human subjects. The methods developed should also prove useful in other types of clinical studies.

Tumors and Their Properties

Staff members of the Institute maintain about 200 transplantable neoplasms. This very valuable resource includes many tumors of special characteristics and is a vital part of the armamentarium of cancer investigators, not only within the Institute, but throughout the world.

As part of a long-term study of the effect of age on rats of six of the NIH inbred strains, Dr. K. Snell reports differences among them as to the most frequent sites of spontaneous tumor formation: Osborne—Mendel, adrenal cortex; Buffalo, anterior pituitary gland and adrenal cortex; AXC, testis and anterior pituitary gland; Fischer, testis; M520, adrenal medulla; and Wistar, anterior pituitary gland and mammary gland.

Acetic acid-soluble collagen in hamsters, which

is present in lower amounts in back skin as compared with abdominal skin, has been found by Dr. W. Banfield to decrease in skin with increasing age except for a rise in the amount from abdominal skin between the ages of 28 and 42 days. In the process of maintaining old hamsters while developing this useful tool for study of collagen metabolism, Dr. Banfield has also established 16 transplantable hamster tumors (among which are 2 melanomas, 1 adrenal cortical tumor, 1 myxoma, 1 epidermoid carcinoma, 1 adenocarcinoma of the bowel and 3 reticulum cell sarcomas).

Miss D. Uphoff is attempting to establish a line of mice, C57BL/10D, that develop multiple polyposis of the colon. Although polyps are occasionally found, malignancies have not been observed.

The biologic behavior of the adrenal cortical carcinoma 494 (*i.e.*, rats bearing the tumor develop polyuria, polydipsia, degenerative changes in the kidney, and atrophy of adrenal glands, sex organs, lymphatic tissue, and pituitary glands) has remained the same through 15 transplant generations in studies by Dr. Snell. In a collaborative study, Dr. Johnson (NIAMD) has found that desoxycorticosterone is the major steroid elaborated by the tumor, even though this steroid is absent or nearly so in the adrenal glands of Osborne-Mendel and other strains of rats.

In an attempt to learn of differences between steroid secretions by another adrenocortical carcinoma and normal adrenal cortical tissue of the rate and to obtain additional understanding of metabolic pathways related to tumor-normal tissue comparisons, Drs. A. Mulay and E. Price, Jr. have found that rats bearing the tumor show atrophy of the zona fasciculata and zona reticularis of the adrenal crotex, a sharp reduction in urinary sodium excretion (both in intact and adrenalectomized rats), increased potassium excretion, hypernatremia, and hypokalemia. Since studies on stress in tumor-bearing rats demonstrated that the tumor could protect the animals against intoxication produced by intraperitoneal potassium chloride injection, the tumor apparently elaborates mineralcorticoid secretions. Preliminary findings suggest no production of glucocorticoid secretion by the tumor.

Dr. S. Wollman, as part of a continuing study on properties of transplantable rat thyroid tumors, has found that one tumor line can maintain a concentration of radioiodine elevated above that of blood and nearly as high as that in normal thyroid glands, although this tumor cannot form appreciable protein-bound I^{131}. Impairment in transport of radioiodide from blood to tumor has also been observed. Dr. J. Tijo (NIAMD) has examined the chromosome complement of three lines of thyroid tumors maintained by Dr. Wollman and found that two anaplastic lines had deletions of 4 and 7 chromosomes, respectively, whereas a functional line had a deletion of only 1 chromosome.

Cells from a number of spontaneous and induced mouse tumors have been injected intravenously by Drs. R. Malmgren and E. Chu, and comparisons were made of the nuclear DNA content between the initial tumor and the developing metastases. There is a tendency for the metastatic tumor cells to have a higher nuclear DNA than the primary tumor from which they are derived; however, it appears that the high ploidy cells are not the only ones that are capable of establishing metastatic foci.

Mouse tumors and normal tissues and extracts prepared therefrom when injected into mice were observed by Dr. Malmgren to increase the mitotic activity of liver under certain conditions. The factor appears to be heat stable and can be stored *in vacuo*. Heated normal tissue produces an increase in mitotic activity, but unheated normal tissue appears to contain an inhibitor, since it can block the effect of tumor tissue.

With Dr. E. McLaughlin, Dr. Malmgren has observed an increase in liver mitotic activity in mice and rats that received serum from cancer patients. The effect was not seen when serum from normal human subjects was given. Using tritum-labeled thymidine and isolating DNA from the livers at 72 hours, Drs. Malmgren and McLaughlin observed an increase in new DNA in the livers of partially hepatectomized rats treated with serum from cancer patients compared with rats treated with normal human serum.

Dr. Malmgren found no significant difference between the various cells studied from carcinoma *in situ* and invasive cancer patients with techniques of microspectrophotometry, interference microscopy, and planimetry while he was work-

ing in Prof. T. Caspersson's Laboratory in Stockholm.

In continuing investigations on cytochemical changes in intact living individual cells, Drs. G. Williams and A. Peacock have made further improvements in instrumentation used for ultraviolet-television microscopy and time-lapse cinematography. By use of this system, intact cell functioning can be studied with brief exposure of low-level UV radiation, and they have completed a study of a series of tetrazolium salts utilized to investigate the reaction rates of an enzyme system catalyzing the reduction of tetrazolium to formazan and to obtain information on the cellular localization of the reaction. In liver cells, progressive reduction occurs in the vicinity of mitochondria without appearance of formazan outside the cell or inside near the cell surface. Tetrazolium appears to be freely permeable, and all but 20 percent bound to cellular protein can be easily removed with a single washing of the cells. Improvements in instrumentation include new lenses that permit excellent resolution with UV light at 265 mμ in enlargements at a magnification of about 30,000 diameters and a new electronic shutter with precise opening times from 5 to 75 milliseconds, or 10 millisecond intervals at 5, 10, 15, or 30 cycles or 2- or 3-minute cycles. Exposure of the cells to UV light with this TV arrangement is about 1/100th that required to photograph the activity directly on film. With 10 millisecond exposures at intervals of 15 seconds, human leukocytes tolerate UV light at 265 mμ for 15 to 30 minutes before showing structural and dynamic changes of damage.

Plasma Cell Tumors

Dr. M. Potter has made additional progress in his investigations on plasma cell neoplasms. He has found that BALB/c mice given single intraperitoneal injections of adjuvant-staphylococcus mixtures, develop plasma cell neoplasms in the lipogranulomatous tissue that formed in all mice receiving the mixture. Thirty-one plasma cell tumors have been found thus far, none developing prior to 5 months after injection. Because of the complexity of composition of the adjuvant mixture, studies are under way to determine if single components and other materials will also produce these neoplasms. To date, 4 plasma cell neoplasms have developed following the administration of paraffin oil (Bayol F) alone to 40 mice. No plasma cell tumors have appeared to date in similar studies in three other strains of mice.

Growing transplants of plasma cell tumors produce quantities of serum myeloma globulin in new recipients. Specific myeloma globulins are produced by different tumors. When a tumor is established in transplant, the specific myeloma globulin production is constant for that tumor and remains qualitatively the same throughout the transplant history. Studies on different protein-producing tumor lines derived from a single host would be of interest. One case so far has been found: the primary host of Adj–PC6 gave rise to two different, stable transplant lines, Adj–PC6A and Adj–PC6C. One difference in the behavior of these two cell lines results in precipitation of the myeloma globulins when whole serum of tumor-bearing animals is diluted in low ionic strength media in the case of the former, but not the latter cell line. Of the 24 different plasma cell neoplasms (20 are of strain BALB/c origin), two additional cell lines give this precipitation phenomenon; in such cases isolation of the globulins is simplified.

The primary peritoneal plasma cell neoplasm appears to rise multifocally, for the reactive mesentery contains numerous nodules of seemingly incipient plasma cell tumors. Dr. Potter is using an ingenious method to study the mechanisms of their development by transplantation of wedges of mesentery containing separated nodules into recipient mice. By this method he hopes to localize the time and site of origin of the carcinogenic event and to obtain discrete tumors from which lines can be established for additional immunological and chemical characterization of the tumor products.

Several immunochemical and cytochemical studies are under way with Dr. E. L. Kuff. The microsomes contain material antigenically related to the serum protein myeloma globulins. Ribonucleoprotein (RNP) particles prepared from microsomes by deoxycholate treatment also carry the antigenic groups of the myeloma proteins. Similar antigenic determinants are found on normal immunoglobulins such as gamma-2-globulin (SI) and a certain beta-globulin (SII). Three antimicrosome antibodes have been studied:

Tumor source	*Precipitates formed with normal serum*
MPC 1	SII
MPC 2	SI, SII
Adj-PC 5	SI.

Myeloma globulins are related antigenically to SI or SII, or very rarely to both. The normal SI and SII proteins contain different antigenic determinants. Myeloma globulins may contain different antigenic determinants, or may lack them. Characterization of the myeloma globulins of BALB/c mice will be done, and the degree of genetic control obtainable in such a study will be far superior to that possible with myeloma globulins sampled at random from heterogeneous populations.

Dr. M. Landy and Dr. Potter have sought to determine whether the abnormally high levels of beta and gamma globulins in the sera of mice bearing plasma cell tumors (induced with paraffin oil and bacteria) represented antibody or disoriented protein. The serum of mice normally contains, in low titer, a broad spectrum of individually specific antibodies to Gram-negative bacterial species. As far as can be determined, these antibodies are the result of antigenic stimuli supplied by continuous contact of those animals with Gram-negative bacteria. It was reasoned that in the presence of this persistent, multiple, natural antigenic stimulus, the greatly increased synthesis of serum globulins, usually associated with antibodies, might be reflected in markedly increased levels of antibody to these bacterial species. Employing the sensitive and objective bactericidal assay for antibodies to *Salmonella*, it was found that sera of mice with these plasma cell neoplasms contained no greater amount of *Salmonella* antibody than that present in normal mice. Since the increased level of globulins did not reflect enhanced antibody response to bacteria, the function, if any, of globulins is still obscure.

Using microincineration and Brachet's pyronin-methylene green-ribonuclease techniques, Drs. MacCardle and Potter have found that gamma-globulin secreting plasma cell tumors are generally rich in cytoplasmic RNA and in calcium and magnesium, whereas those secreting alpha- and beta-globulin are poor in cytoplasmic RNA and rich in intranuclear RNA. The calcium and magnesium content of the latter varies. The nucleolus of the gamma-globulin tumors is large and rich in silica.

In collaboration with Drs. Potter and Ruth Merwin, Dr. A. J. Dalton has examined by means of electron microscopy 17 primary and transplanted plasma cell tumors and controls consisting of normal stimulated and unstimulated plasma cells or fibrosarcomas. Maintenance of ultrastructural features characteristic of the plasma cells is related to retention of function (determined by the pattern of specific myeloma globulins elaborated by a particular cell type). The evidence obtained also suggests that particles present in the cytoplasm of cells of all the plasma cell neoplasms examined (but not in normal plasma cells nor in the cells of other neoplasms) are not the morphologic representation of an oncogenic agent responsible for the development of the tumors.

Dr. H. Kobayashi (Visiting Scientist) with Drs. Thelma Dunn and Potter, has demonstrated both osteolytic and osteoplastic reactions by plasma cell neoplasms using techniques involving intravenous injection of the ascites form of tumor (growth within the bone marrow resulted), transplantation of solid tumor tissue into marrow cavities and onto peritoneal surfaces, the use of papain-cleared skeletons, and examination of histologic sections.

Diffusion Chamber Studies

The occurrence of several plasma cell tumors and sarcomas in mice following the placement in the peritoneal cavity of millipore filter diffusion chambers was reported previously by Dr. Ruth Merwin. In further work with Dr. Thelma Dunn, she has found that both tumor types are induced more frequently in the BALB/c than in the C3H strain and that the presence of tissue inside the chamber, stilbestrol infection, and anyone of the chamber parts (membrane, lucite or adhesive) do not appear to be essential to induction of the plasma cell tumors. BALB/c mice react by producing intraperitoneal plasma cell neoplasms which are transplantable and have characteristic protein electrophoretic patterns resembling those of human multiple myeloma. The C3H mouse, however, has not produced these neoplasms, but severe fibrosis and occasional ossification occur.

Dr. Emma Shelton has continued her study of the behavior of normal and malignant cells

grown in double diffusion chambers placed in the peritoneal cavities of mice. Fluids, but not cells, may pass freely through the membranes of the chambers. Growing in the chambers, lymphocytes that were procured from the thoracic duct and lymph nodes behaved differently from those procured from the peritoneal cavity, indicating different potentialities for survival, growth and differentiation. The latter formed organized fibroblastic sheets and became a minor component in the cell population; the former produced few sheets and persisted as major components in the population. Peritoneal lymphocytes grown in the chambers on different physical forms of substrate membranes varied in their capacity to form organized tissue and failed to undergo the transformations observed when grown on Millipore substrate membranes, except when they grew in the fibrous coagulum that sometimes formed. No differences were seen when either basic or acid ion-exchange resins were included in the chambers. Preliminary findings with chemical techniques show an increase in mucopolysaccharide correlated with connective tissue growth. Immunological techniques demonstrated passage of antibody into the chambers. These different responses of cells obtained from different body sites may be of importance in determining the factors responsible for establishment or lack of establishment of metastatic lesions in different tissue sites in the body.

In collaboration with Dr. Virginia J. Evans, Dr. Shelton has found that mouse connective tissue grown for at least a year in diffusion chambers (through 10 to 12 chamber transplant generations) did not acquire malignant properties when evaluated by the technique of intraocular transplantation.

Dr. R. T. Bradley (last year an Anna Fuller Fund Fellow) and Dr. L. Law are continuing studies with a technique that allows one to perfuse substances into the extracellular fluid environments of a designated piece of tissue or population of cells within normal unrestrained experimental animals for periods up to several weeks. One study has utilized this procedure to perfuse substances continuously into intraperitoneally implanted Millipore filter diffusion chambers containing P–388 or L1210 cells. When such chambers were perfused with Eagle's basal medium (with pyruvate, serine and glutamine) for 7 days at the rate of 1 ml. per day, the approximate generation time was 12 hours, compared with 24 to 32 hours in control nonperfused chambers. Other perfused media gave poorer growth rates, and the effects of various substances are under study.

Tissue Culture Studies

The careful technique sustained over the years in the Tissue Culture Section has permitted the cell cultures to be carried aseptically, with an absolute minimal and usually no use of antibiotics. Recent studies have shown that all cultures carried in the section are apparently free from pleuropneumonia and diphtheroid organisms which have complicated the interpretation and evaluation of nutritional, biochemical and other biologic data from laboratories relying on antibiotics.

Comparative studies of normal and malignant cells can be facilitated if additional techniques are developed that will enable cell strains, already adapted to static cultures in chemically defined media, to grow luxuriantly in fluid suspension cultures without addition of serum or other ill-defined components. Agitated culture techniques produce stress on the cells grown in fluid suspension which varies considerably with the conditions employed. Messrs. J. C. Bryant and E. L. Schilling and Drs. V. J. Evans and W. Earle are investigating a number of factors that appear important in growing cells in agitated suspension, such as the shape and size of the containers, the type of surface of the containers, the rate of agitation and the presence or absence of certain components in the medium, particularly polymers of known composition that might play a beneficial role similar to that of serum. Last year it was reported that addition of methylcellulose to the chemically defined medium NCTC 109 produced a protective effect on cells grown in agitated culture. Higher concentrations of the polymer than used last year have led to increased proliferation rates of monkey kidney cells. Additions of spermine or much higher concentrations of folic acid than ordinarily employed did not give improved results (from reports in the literature, spermine has been shown to influence permeability of bacterial cell walls by preventing osmoticlysis and high concentrations of folic acid appear to be required for certain kidney cell lines grown in static culture). In roller tubes, different rates

of tube rotation (from 19 to 2,400 rph.) have been investigated; 1,200 and 600 rph. gave higher proliferation rates than those at 2,400 rph. or 300 rph. or less.

Further improvements have been made in techniques utilized in growth of mammalian cells in rapidly agitated fluid suspension cultures grown in shaker flasks by Messrs. Bryant and Schilling and Dr. Earle. By reduction of the size of the individual culture flasks, the entire agitation assembly can be placed within the carefully controlled temperature room, and manipulation of the cultures is greatly facilitated since the fluids can now be changed without transfer of the cells from the flasks. This new procedure now allows rapid proliferation of monkey kidney epithelial cells in a protein-free, chemically defined medium for the first time.

Dr. Evans is attempting to adapt *freshly* isolated cells of monkey kidney, mouse kidney and mouse fibroblasts to grow in a protein-free, chemically-defined medium NCTC 109. Maintenance of cells and some indication of growth has taken place, but it is too early to consider that cell lines have become established. Establishment of such lines in protein-free medium would be of importance to virology studies since the problems encountered with serum-containing media could be avoided. Furthermore, pleuropneumonia-like organisms would be excluded since the medium does not propagate these organisms.

In collaboration with members of the staff at the Navy Tissue Bank (National Naval Medical Center, Bethsda), Dr. Evans has also successfully cultured human skin epithelial cells in protein-free medium NCTC 109 in both static and agitated cultures.

Dr. Earle and Mr. W. T. McQuilkin are attempting to establish the cinemicrographic techniques to permit accurate characterization of cells in culture, including growth rates, cytokinetic periods, intermitotic intervals, and migration rates. Considerable variability of behavior among cells of pure line populations makes progress toward standardization of technique slow. Even with preliminary findings, however, it is evident that a cell line (NCTC 929-strain L) returned to serum-containing medium after an extended sojourn in a protein-free, chemically-defined medium (NCTC–109) reverts to a state exhibiting characteristics seen in the original strain, but not seen while grown in the protein-free medium. Thus, whatever the phenomena of adaptation to the chemically defined medium might be, the observed changes are not necessarily permanent. It may be recalled that last year's report indicated the changes seen when cells of this clone line were adapted to growth in a protein-free medium: the average generation time was prolonged; the migration rate on the glass surface was greatly increased; the cells became less compact, refractile and granular and rounded up only in prophase.

Transformation Studies. During the past few years Dr. Katherine K. Sanford and her associates in the Tissue Culture Section have studied many types of transformation—neoplastic, enzymatic, morphologic, and chromosomal—occurring within clones of mammalian tissue cells grown in long-term culture. With the identification of several biochemical markers, such as those determined by Dr. B. B. Westfall, and a number of nutritional variants, the tools may now be at hand to learn of the mechanisms involved in these transformations, to attempt to control and induce them, and to test for the transfer of genetic materials between cells. Recently Dr. Sanford and her associates have reported additional studies on the development *in vitro* of hereditary differences in morphology and further alterations in arginase and *beta*-glucuronidase activities within a clone of mouse tumor cells. By means of clonal analysis additional hereditary alterations in morphology, level of enzyme activity, tumor-producing capacity, and chromosomal constitution have been determined among clonal derivatives. Last year an initial attempt to identify "hybridization" of cells produced by transfer of genetic materials between cells was not successful, but encouragement for new attempts now comes from work by Barski, Sorieul and Cornefert, who, using clones obtained from Dr. Sanford, have demonstrated "hybridization" of cells as evidenced by chromosome markers (see also the report below in the section on Nucleic Acids by Drs. Law, Bradley, and Roosa).

Dr. Virginia J. Evans has studied three series of fresh C3H embryo explants cultured in medium NCTC 109 and 10 percent horse serum to determine the time and incidence of transformation to malignancy as determined by intraocular

injection following *in vitro* growth periods of different lengths. Tumors occurred after the cells were *in vitro* only 176 days, 147 days and 138 days, respectively, for the three series. Growth acceleration of the cultures occurred some 4 to 8 weeks prior to the time the transformation took place *in vitro*. The tumors produced in the eye were fibrosarcomas, with latent periods ranging from 17 to 125 days. Exploratory chromosome counts indicated that there were many heteroploid cells in the populations. Such information pinpoints rather sharply the time at which the investigator must look for leads in understanding the transformation and provides a fairly clear-cut calendar for other studies requiring knowledge regarding the state of the cells in culture.

Similar studies with medium NCTC 109 without added serum have been done with implantation intraocularly after 26 to 211 days *in vitro*. Resorption of all implants has occurred to date. It should be pointed out that under these conditions, the rate of growth *in vitro* has been found to be slow, with death occurring and waves of new cells growing out.

Fibroblasts obtained by implanting small pieces of epigastric fat pads in double millipore filter chambers (porosity HA) which were then placed in the peritoneal cavities of mice and grown through 12 successive chamber transplant generations have good growth *in vitro*. Implants into the eye survive, but do not proliferate.

Dr. Sanford also continues to investigate several variables of cells grown in culture beyond short term periods through study of effects of vitamins varied, singly or in combination, in their concentrations. Medium NCTC 109, a protein-free, chemically defined medium that supports long-term proliferation of cells grown in culture and was developed a few years ago, is extremely complex in composition. Although a simplified medium, NCTC 118, is an improvement, it nevertheless contains 18 vitamins. As reported last year, when each of the 18 vitamins was individually deleted from the medium, 5 vitamins (calcium pantothenate, choline chloride, niacinamide or niacin, thiamin and riboflavin) were identified as essential for cell survival. Vitamins not apparently required for cell survival, but which increased rates of cell proliferation were folic acid and pyridoxal (or pyrodoxine).

During the past year, two new media were evaluated, and the interrelationship of vitamin B_{12}, folic acid and thymidine were studied in their effects on cell proliferation and growth. Medium NCTC 119, containing only seven vitamins, failed to support cell survival for more than 2 or 3 weeks. Additional studies with five other vitamins showed an importance of biotin which could not be demonstrated in the presence of all 18 vitamins. Investigations with medium NCTC 120, which contains biotin, showed that the cells, though proliferating as rapidly as the controls in medium NCTC 118, were fragile and could not withstand the stirring used to set up quantitative experiments. When vitamin B_{12}, folic acid or thymidine were deleted individually from the medium, no detectable effect on cell survival or proliferation was noted. When all three were deleted, however, growth was poor, and enlarged cells with abnormal nuclei resulted. In the absence of folic acid and B_{12}, some reduction in growth was noted. A more marked reduction in growth occurred in the absence of thymidine and folic acid. In the latter medium containing only B_{12}, cells increased in nuclear mass and cell numbers decreased to a critical point. Although a few cells appeared to adapt to the medium, growth lasted only 4 to 6 weeks.

One nutritional variant which does not require pyridoxine or pyridoxal was isolated, but the cells are exceedingly fragile.

Immunological Aspects. Utilizing red cell agglutination techniques, Dr. M. A. Fink and Dr. Evans found that in guinea pigs antibodies were produced which were specifically reactive with only homologous erythrocytes when cultured monkey or human cells carried in culture for long periods of time were administered. Surprisingly, however, neither fresh monkey nor fresh human cells incited the production of antibody reactive with homologous erythrocytes. Tests for delayed hypersensitivity revealed that both the tissue cultured and fresh tissues contained common antigens. In a comparison of fresh *vs.* cultured human epithelium, it was noted that pigs injected with fresh tissue reacted to human serum, while those injected with cultured tissue did not. In tests for immediate hypersensitivity (Schultz-Dale technique) there was again evident a marked diminution or absence of reactivity in the uteri

from pigs injected with tissue cultured cells, but not in those from pigs injected with fresh tissue, when brought in contact with homologous serum.

These changes in antigenic characteristics seen in cells grown in culture suggest the possibility of loss of surface antigen with an uncovering or increase in availability or potency of the antigen responsible for hemagglutinating antibodies. Should histocompatibility antigens be involved, special culture techniques might permit growth of cells containing viruses from one species and subsequent transfer to experimental animals of a different species—another approach of importance to the study of human tumors. The above findings also confirm the usefulness of the Brand hemagglutination technique in determining the species of origin of cultured cells, a matter of considerable importance in establishing and operating tissue banks.

Enzymology. Dr. D. B. McN. Scott (University of Pennsylvania) and Dr. Sanford have evaluated certain oxidative enzymes involved in glucose metabolism in a cloned cell line that produces tumors rapidly upon implantation in mice and in three cloned cell lines that produce tumors slowly if at all upon implantation in mice. The former showed nearly three times the hexokinase activity, four times the glucose-6-phosphate dehydrogenase activity, and five times the phosphogluconate dehydrogenase activity compared with the latter.

Nine long-term mouse cell culture strains and two human ones were all found by Dr. Westfall to produce *alpha*-keto-glutaric and pyruvic acids, an indication that all of them posses active transaminating enzymes needed for the formation of these keto-acids, or, in the case of pyruvate, that it was formed from glucose. On the other hand, catalase activity in twelve long-term mammalian tissue cell strains, including three derived from mouse liver epithelial tissue, was found to be very low. These findings supply additional evidence to that previously obtained by Dr. Westfall which indicates no necessary parallelism among the various enzyme systems in cells grown for long periods in culture. Some enzyme systems not necessary for cell maintenance disappear; others remain active and may be necessary for cell survival growth.

Tumors arising from injection into mice of some of the mouse cells also had low catalase activity. Hepatomas arising from injection of the hepatoma strain of mouse cells likewise had low activity. Furthermore, the liver catalase of the hosts bearing these hepatomas was not depressed.

Studies on Proteins and Nucleic Acids

The great importance of proteins and nucleic acids in the control of functions of normal and abnormal cells and of whole organisms is attested by the vast amount of work, in many cases by leading scientists, on the subject. Like other members of the staff of the National Institutes of Health, investigators in the National Cancer Institute are making significant contributions to the knowledge of these important materials. The fundamental studies on proteins and nucleic acids provide a base upon which further understanding of disease processes, including cancer, are built. Contributions to the separation and characterization of complex protein mixtures made by Drs. H. A. Sober and E. A. Peterson have had far-reaching influence on research conducted on the proteins and nucleic acids.

Drs. Peterson and Sober have prepared two new cellulose ion exchangers (designated ECA-cellulose and ECTHAM-cellulose, respectively) for use in the chromatography of substances that are too tightly bound to the previously available adsorbents. Both possess basic groups that are weaker than those of DEAE-cellulose or ECTEOLA-cellulose. They have been used successfully in the chromatography of ribosomes and soluble ribonucleoprotein. New elution gradients and new buffer species have been evaluated. Of special interest is tris-succinate buffer since it gives much sharper peaks than phosphate buffer in the elution of serum albumin and, in studies on trace metals, is completely combustible in the carbon arc used for metal analyses.

Sephadex is becoming increasingly useful as a dialysis replacement; furthermore, the combination of Sephadex with the cellulose ion exchangers seems like a profitable avenue of approach since a multitude of stages of continuous salt displacement can be obtained by such a mixture.

Protein Characterization. Drs. Peterson and Sober, utilizing gradient and stepwise elution techniques in chromatographic fractionation on

cellulose ion exchangers, have determined additional properties of serum fractions.

Chromatography of crystalline bovine plasma albumin (BPA) on DEAE-cellulose with a suitable gradient yields a skewed peak, suggesting heterogeneity. Treatment of the BPA with cetyltrimethylammonium bromide (CTAB) before chromatography preferentially converts the earlier, major portion of the peak into complexes having little or no affinity for the adsorbent. As the major component is thus diminished, the residual, presumably unaltered, albumin can be seen to comprise at least 4 components. Unlike the CTAB complex of human serum mercaptalbumin, that formed with BPA is largely disassociated by dialysis.

Chromatography of an old sample of lyophilized Red Cross human Fraction V gave two approximately equal peaks. The first was pure monomer; the second was 70 percent dimer and trimer. Fresh Red Cross Fraction V, on the other hand, appeared as a single, highly skewed peak containing little or no dimer.

Esterase activity of highly purified albumin, which was first demonstrated by Kramer, may represent a true metabolic function of the albumin, or it may be a fortuitous consequence of the structure of the molecule. All of the chromatographic fractions of BPA showed equal esterase activity, indicating that the activity is not attributable to an enzyme impurity. The addition of CTAB caused a three-fold increase in esterase activity, although the same concentration of this compound had no effect on the substrate in the absence of albumin.

Progress on the isolation and characterization of the metal proteins of serum has remained slow because of difficulties in insuring the absence of contaminating metals in the solutions and equipment used. Use of plastic materials and the preparation of metal-free reagents should allow greater progress during the coming year.

Fractionation of the antihemophilic factor (Factor VIII, AHF) is progressing very satisfactorily through collaboration with Drs. Martinez-Canaveri and Casillas of Argentina. AHF has been purified with excellent recovery and the ensuing lability of the activity has been avoided in part by the addition of glucose. Although still in the preliminary stage, it also seems possible to adsorb the AHF directly on DEAE-cellulose from undialyzed and undiluted plasma from which interfering materials have been removed by prior aluminum hydroxide adsorption. This simple procedure shows considerable promise of easily providing AHF preparations that would be suitable for clinical use. Cooperative studies with the Protein Foundation are being instituted.

Dr. R. W. Hartley, Jr., working with Drs. Sober and Peterson, has studied crystalline bovine plasma albumin and a number of its derivatives by chromatographic fractionation. In the high degree of resolution obtained, the most striking result has been the separation of mercaptalbumin and nonmercaptalbumin. Subsequent experiments indicated that nonmercaptalbumin, after reduction with mercaptoethanol, behaved chromatographically as mercaptalbumin. This would indicate that some of the chromatographic differences observed are related to the presence or absence of a free SH group. Studies are under way to determine the position of this group in the albumin molecule. Albumin dimers have been found to be heterogeneous chromatographically as well as with respect to reduction by mercaptoethanol to the monomer. Dimers can be produced from the albumin monomer even in the absence of free SH groups and appear to be S–S dimers in that they are reducible by mercaptoethanol. Such studies are of importance in learning more of the physical and chemical forces involved in maintaining the native configuration and activity of many proteins.

Protein Synthesis Studies. Dr. E. L. Kuff continues his investigation of the cytochemical organization of normal and malignant cells, with particular reference to the problem of protein synthesis and replication of intracellular components. He has used as a particularly valuable source of cells the plasma cell tumors, which have been shown by Drs. M. Potter and J. Fahey to produce and secrete into the circulating blood, large quantities of myeloma proteins while simultaneously maintaining a high rate of mitosis. Electron microscopy by Dr. A. J. Dalton has revealed the presence of an extensive endoplasmic reticulum and highly developed Golgi systems within the cells. Ribonucleoprotein (RNP) particles were extremely numerous, both in association with the neoplasmic reticulum and as appar-

ently free entities in the cell sap. Fractionation of plasma cell tumor homogenates by standard methods of differential centrifugation gave two subfractions of the entire microsome fraction: one made up primarily of free RNP particles, and the other composed of fragments of the endoplasmic reticulum (membranes) with associated RNP particles (*vide supra* the collaborative work with Dr. M. Potter on the antigenic properties of these fractions).

Viruslike particles, regularly seen in neoplastic mouse plasma cells localized exclusively within the endoplasmic reticulum, have been reported by Dr. Dalton. Through the use of the nonionic detergent, Tritonx100, it has been possible for Dr. Kuff to disrupt the membrane component of the isolated tumor microsomes in such a way as to liberate the virus-like particles in morphologically unaltered form. Evaluation of the biological activity of these preparations is under way with Drs. Dalton and Law.

Through the use of a density gradient fractionation method, Dr. Kuff has been able to isolate Golgi membranes from a mouse plasma cell tumor. With Dr. R. F. Ziegel, it has been shown that the tumor Golgi fractions resemble those previously obtained from epididymus, both in their morphology and in their high content of lipids. The rate of incorporation of P^{32} into the phospholipids of isolated Golgi fractions of epididymus has also been compared with the corresponding rates for other intracellular fractions. For the relatively long incorporation periods thus far studied (1 to 3 hours), there was little difference between the Golgi and other cytoplasmic fractions.

These studies, particularly the immunochemical studies of plasma cell tumors with Dr. Potter mentioned above, should prove to be very valuable in investigating the relationships between the synthesis of specific secretory proteins and that of the actual cellular proteins themselves. Especially important will be the studies on the microsomal RNP particles, since they may represent the sites of synthesis of these proteins. If artificial redistribution of protein during the preparation of fractions can be ruled out or eliminated, the question can then be investigated whether the myeloma globulins are associated with all of the tumor RNP particles or only with those attached to the endoplasmic reticulum.

Drs. Peterson and Kuff have adsorbed rat liver microsomes isolated by differential centrifugation on a cellulose ion exchanger, allowing glycogen particles and residual protein to pass through unadsorbed. Because microsomes exhibit an unusual tendency to establish additional bonds with the adsorber, difficulties were encountered in the chromatography of these cytoplasmic fragments. However, with modification of techniques, clean preparations of particles having a sedimentation constant of 77S were obtained. Soluble ribonucleoprotein can also be obtained by chromatography of a clear, particle-free supernatant rat liver homogenate. Phenol extraction of the ribonucleoprotein yielded protein and an "S–RNA." The latter, upon re-chromatography, behaved identically with the original ribonucleoprotein. Further studies are under way to elucidate the reasons for the identical chromatographic behavior and to determine the completeness of the removal of protein. These techniques promise to permit separation of these important cell particulates under milder conditions than any yet reported.

Dr. M. Rabinovitz, as part of continuing attempts to introduce *in vitro* "biochemical lesions" within the protein synthetic pathway in normal and tumor tissues, has synthesized two antimetabolites, 4-thiamethione (antimetabolite of methionine) and S-carbamylcysteine (antimetabolite of glutamine), and has studied their inhibition of the incorporation of labeled amino acids into protein. The former inhibits incorporation of methionine, leucine, valine, lysine, and phenylalanine, and methionine does not prevent the inhibition. Glucose both prevented and reversed the inhibition, suggesting an interference with the oxidative energy supply supporting protein synthesis. The latter inhibited the incorporation of glutamine and other amino acids into protein. The inhibition of glutamine incorporation, which was not reversed by glutamine, was greater percentage-wise in the presence of higher concentrations of glutamine, and this inhibition was shown to be due to interference with the general stimulation of amino acid incorporation known to be produced by glutamine. Dr. Rabinovitz has also found that the antibiotic, puromycin, can inhibit the incorporation of amino acids into ribosomal protein, but not into soluble protein of cells.

Amino Acid and Peptide Synthesis. The preparation of a series of *alpha*-amino-beta hydroxy acids (in a single step reaction which involved the treatment of an alkaline solution of an amino acid with an aldehyde, in the presence of catalytic amounts of cupric ion) was reported last year by Drs. T. T. Otani, N. Izumiya, S. M. Birnbaum, and M. Winitz. Additional hydroxyamino acids have now been synthesized, as well as some acyl derivatives, and resolution of *alpha*-methyl-DL-serine and *alpha*-ethyl-DL-serine were attempted. Unexpectedly, the N-chloroacetyl derivative of the former compound could be resolved only partially by the usual enzymatic procedure since the hydrolysis continued on beyond the customary 50 percent point to reach 100 percent hydrolysis, indicating that the enzyme could not discriminate between the asymetry of both optical isomers of the molecule (i.e., the hydroxymethyl and methyl groups were not sufficiently dissimilar). Partial resolution could be demonstrated, however, if the reaction was stopped slightly beyond 50 percent hydrolysis. The resolution of *alpha*-ethyl-serine was accomplished in the usual manner.

Growth inhibition studies with *E. coli* were undertaken by Dr. Otani with several of the synthesized compounds. Inhibition was observed to be most effective with *beta*-hydroxy-DL-aspartic acid, *beta*-hydroxy-DL-leucine, and L–O-methyl-threonine.

Dr. Winitz has successfully synthesized and isolated the epimeric mixture of *delta*-hydroxy-lysine in about 50 percent over-all yield. Separation of the diastereomers was achieved by fractional crystallization of their *alpha*-choloro-acetyl-*epsilon*-carbobenzoxy derivatives. Resolution was achieved enzymatically with cobalt-activated acylase I.

Drs. N. Izumiya (Visiting Scientist) and Winitz have prepared the four steroisomers of valyllysine anhydride and of phenylalanyllysine anhydride to obtain additional information on the comparison of chemical structure and biological activity in a group of compounds represented by the polypeptide antibiotics, gramicidin S and tyrocidin A. These antibiotics have the same features in common: a) a basic character due to the presence of one or more diamino acid residues; b) a cyclic conformation; and c) at least one amino acid residue of the D-configuration. Although the synthesized compounds also possess these characteristics, no antibiotic activity was found against either Gram-positive or Gram-negative bacteria. Moreover, they were not susceptible to the hydrolytic action of trypsin or chymotrypsin.

Nucleic Acids. Drs. Sober and G. Rushizky (Visiting Scientist) are exploring modifications of chromatographic techniques that will permit separation of fractions containing large nucleotide sequences resulting from enzymatic digestion of nucleic acids. All of the mono-, di-, and trinucleotides resulting from ribonuclease digests of ribonucleic acids have previously been separated. Fractionation studies in collaboration with Drs. L. Heppel and M. Singer (NIAMD) of adenylic acid polymers from the hexa- to the deca-nucleotide and larger fragments, coupled with analytical paper chromatography, have indicated the presence of contaminating enzyme activities in the original enzyme preparation used to degrade the polyadenylic acid polymer. Homologous oligonucleotides are being used as model substances for nucleotide separations. Such studies are of considerable importance in the investigation of nucleic acids in that they serve to characterize the enzymatic action, to characterize the nucleic acid itself in terms of its nucleotide distribution and sequence, and to obtain a variety of pure oligonucleotides which can subsequently be used in physical-chemical and enzymatic studies.

Drs. Rushizky and Sober have prepared pancreatic ribonuclease digest of a high molecular weight ribonucleic acid from yeast and from tobacco mosiac virus (supplied by Dr. C. A. Knight, Virus Laboratory University of California, Berkeley). The digests were fractionated by a two-dimensional mapping procedure which combines paper electrophoresis and paper chromatography, and the fractions so isolated were further examined by physical-chemical and enzymatic methods. Comparisons were made with the complete digests. The following generalizations were obtained: a) oligonucleotides are released before the pyrimidine mononucleotides; b) with the pyrimidine mononucleotides, cytidylic acid was released before uridylic acid; and c) in each case, the cyclic intermediate of cytidylic acid or its oligonucleotides were converted to the terminal 3′-form before the corresponding uridylic

acid compounds. Pancreatic ribonuclease has been found to have a preference for the enzymatic hydrolysis of pyrimidine linkages adjacent to purine linkages, *i.e.*, these linkages are split before pyrimidine-pyrimidine bonds. This preferential specificity may be of considerable assistance in providing large and homogenous oligonucleotides in pure form which will aid in the determination of nucleotide sequences in nucleic acids.

As a further aid to work aimed at determining the structure of nucleic acids, Drs. Rushizky and Sober have purified two nucleases, "adenylase" and "guanylase," by means of conventional protein fractionation techniques, including precipitation and ion exchange chromatography. Both enzymes have been purified some 400-fold, and each is free of the other activity when tested at an enzyme-substrate ratio of 1:100. Since these two enzymes appear to hydrolyze adenylic and guanylic acid nucleotide linkages respectively, they will, especially when purified further, be particularly useful as tools for the specific cleavage of nucleotide significances of ribonucleic acid and provide therefore a complementary technique to that provided by the pyrimidine specificity of pancreatic ribonuclease.

Dr. Mary Maver, with Miss Antoinette Greco, has been purifying nuclease preparations derived from normal and neoplastic tissue, and has further characterized their activities. Utilizing beef spleen and liver, rat liver, two transplantable rat hepatomas and a rat lymphosarcoma, she has obtained ribonuclease preparations by means of chromatography whose activities are characterized by: (a) their pH optima on highly polymerized rat liver RNA; (b) the products isolated from their hydrolysates when cyclic purine and pyrimidine nucleotides are used as substrates; (c) the products of hydrolysis isolated in work with Dr. Rushizky from hydrolysates of yeast RNA by paper electrophoresis followed by paper chromatography; (d) the products of their hydrolyses of polyadenylic and polycytidylic acids, and (e) the effects of Mg ions and heat upon their RNAase activities. Two enzymatic activities which fractionate closely with the RNAase activities have been removed by repeated fractionation on carboxymethyl cellulose columns: (a) an acid phosphatase which preferentially dephosphorylates the 3′-adenylic acid and, (b) a phosphodiesterase, without RNAase activity, which hydrolyzes cyclic adenylic acid to yield the 2′-adenylic acid. These enzymes are much more active in calf spleen and liver and rat hepatoma 3683 than in normal rat liver.

An active "acid" RNAase with a pH optimum of 5.7 has been isolated which does not hydrolyze cyclic adenylic acid, but does hydrolyze cyclic cytidylic acid and polyadenylic and polycytidylic acids to yield the 3′-derivatives. With yeast or rat liver RNA as a substrate, both purine and pyrimidine linkages are broken and no core remains. The alkaline RNAase of spleen hydrolyzes cyclic adenylic and cytidylic acids to give the 3′-acids. Rat liver has been found to have a RNAase with a pH optimum of 6.5–6.7 besides the activities with optima of pH 5.7 and 7.3–7.8. The 6.5–6.7 RNAase is masked in crude preparations by an inhibitor which can be removed by treatment of the preparations with heat or *para*-chloromercuribenzoate. The RNAases of liver are considerably less active than those of other tissues studied.

Dr. Maver and Miss Greco have also been studying the sulfhydryl dependency of the RNAase activities of liver. As has been shown by others, *para*-chloromercuribenzoate (CMB) activates the alkaline RNAases by combining with an inhibitor. When this inhibitor has been removed by the chemical purification procedures used to obtain the more active nuclease preparations, often the alkaline as well as the acid RNAase activities can be activated (about 20 percent) by cysteine and inhibited by CMB. The acid RNAase is much more sensitive to CMB than the alkaline RNAases. The inhibitions of RNAase activities by CMB can be reversed by excess cysteine when the right quantitative conditions prevail. Spectrophotometric assays done according to Boyer show that the combination of the SH of the RNAase protein with the CMB is directly correlated in timed reactions with the inactivation of the enzyme. These studies also should not only aid in elucidating the nucleotide sequence of nucleic acids, but the sulfhydryl sensitivity noted above may have important implications in the regulation of RNAase activity which may control RNA production for protein synthesis and in the liberation of energy-yielding or accepting nucleotides.

Extensive and detailed studies on deoxyribonucleic acid (DNA) and neutral deoxyribonuclease

(DNAase I) have been made by Dr. J. Shack. Various conditions that affect enzyme activity (pH, substrate concentration, type and concentration of cations and of nonspecific salts) are not independent but rather are interdependent variables. Contrary to many earlier reports in the literature, it was found that proper control of these variables yields a substrate concentration-activity relation which corresponds to classical Michaelis-Menten kinetics. It was also found that the rate of hydrolysis of DNA falls off rapidly to a very low value when less than half of the susceptible bonds are broken. It appears that DNA contains linkages of widely different susceptibility to the enzyme and that the rate falls off as the more susceptible linkages are broken, rather than because of reduction of substrate concentration or inhibition by products of the reaction. Comparison of results with native and denatured DNA indicates that differences in susceptibility are not dependent on the specific secondary structure possessed by native but not by denatured DNA, but are more probably related to the sequence of nucleotides in the vicinity of the particular bond.

Dr. R. Kielley, utilizing the assay method of Dr. Kornberg and associates, has characterized some of the conditions influencing deoxynucleotide kinase activities in liver homogenates of mice and rats and has studied the extent to which these enzymes can catalyze the synthesis of deoxynucleoside di- and tri-phosphates (apparently the direct precursor substances of DNA) in normal mature cells. From studies of substrate concentration on other deoxynucleotide kinase activities, one can conclude that the enzyme activity levels of deoxycytidylic acid, deoxyguanylic acid, and deoxyadenylic acid kinases are comparatively high and would not therefore be limiting factors in DNA synthesis. The activity of thymidylic acid kinase is somewhat low and additional work is required to determine if it can be considered a limiting factor in DNA synthesis. Preliminary results with tumor tissues indicate that the course of deoxynucleotide tri-phosphate synthesis in tumors is radically different in some respects from that found in normal liver.

Deoxycytidine diphosphocholine (d-CDP-C) and deoxycytidine diphosphoethanolamine (d-CDP-E) were isolated from the Novikoff hepatoma by Dr. W. C. Schneider 2 years ago. Assay conditions developed and isotopically labeled substrates synthesized have permitted initiation of studies of the factors responsible for the concentrations of these compounds which are higher in hepatoma and regenerating liver than in normal liver. The enzymatic activities related to the synthesis of d-CDP-C are considerably higher in homogenates of Novikoff hepatoma and of regenerating liver than in those of normal liver. These compounds may prove to be of importance in DNA synthesis since they may lie on the pathway for conversion of ribonucleotides to deoxyribonucleotides.

With Drs. R. T. Bradley (last year an Anna Fuller Fund Fellow) and R. Roosa (now at the Wistar Institute), Dr. L. Law has provocative preliminary evidence obtained in three separate instances indicating that stable, DNA-induced transformations in mammalian cells may be demonstrable. P-88 sensitive cells incubated *in vitro* with DNA prepared from cells with a high level of 8-azaguanine resistance developed resistant colonies at a frequency 20 to 40 times that observed in sensitive control cultures.

Enzymatic Studies

Dr. S. M. Birnbaum and Mr. M. C. Otey have made comparisons of the rate of disappearance of various D-amino acids in rats (interpreted as *in vivo* oxidation) and the rate of oxidation *in vitro* of corresponding D-amino acids. Small amounts of the compounds were force fed to young rats in 6 divided doses over a 2-day period. The excreta were quantitatively collected and were assayed for the D-amino acid. The total excretion subtracted from the amount fed was assumed to give a measure of the *in vivo* oxidation. Although the fraction "oxidized" varied from 40 to almost 100 percent, depending on the nature of the amino acid administered, this percentage value was nearly constant for a given D-amino acid at whatever level it was fed. Study of the specific and total oxidative activity of rat kidney and liver homogenates against a series of D-amino acids revealed: a) the relative specificity of the two tissues is similar if not identical in this respect, and b) there is no apparent relation between the *in vitro* and *in vivo* oxidative rates for D-amino acids. This latter observation raises some question as to the physiological interpreta-

tion of the significance of *in vitro* enzyme measurements.

In previous studies on the isolation of cell particulates by Dr. R. E. Greenfield, Jr., it was noted that the addition of the high molecular weight polymer, polyvinylpyrrolidone (PVP), to solutions of sucrose resulted in a marked protection of cellular particulates. He has now made use of PVP-sucrose solutions and an interesting use of proteolytic enzymes administered intravenously to obtain suspensions of individual cells from different tissues. As suspension of single cells was obtained which contained 15–25 percent of the total liver nitrogen when the *in vivo* injection of trypsin and a fractional centrifugation procedure were used. This fraction was relatively free of cell debris and was made up predominantly of parenchymal cells. The staining properties of the cells would indicate that a majority of them are alive: *i.e.*, over 90 percent of the cells in most suspensions did not take up trypan blue or nigrosin and nearly all of the cells took up tetrazolium under anerobic conditions. The ease with which single cell fractions could be isolated from other organs varied markedly. Cells could be isolated in high yield from spleen, thymus, lymphosarcoma, and hepatoma. Only after multiple trypsin injections could the kidney be separated into cells, and even then the yield of cells was smaller than that found for the preceding organs. Following a single injection of trypsin the kidney separated into glomeruli and various lengths of tubules and blood vessels. Although muscle fibers which had been subjected to the *in vivo* trypsin injection could be easily teased apart, they had to be cut from the tendons. The brain and fibrous tumors separated into cells rather poorly, and the heart tissue did not separate at all.

Little change other than petechiae in the lungs could be seen on microscope examination of the organs following a single *in vivo* injection of trypsin. The animals did not appear in pain and remained healthy if permitted to survive.

When liver cell suspensions were kept at 0° in 20 percent PVP–10 percent sucrose solutions, the activity of aldolase, lactic acid dehydrogenase and catalase over an 8-hour period was nearly the same on a nitrogen basis as that found in the liver of origin. When the isolated liver cells were suspended in culture media at 38°, certain of these enzymes were lost. When 20 percent PVP–10 percent sucrose was added to NCTC 109, a protein-free, chemically defined medium, 80 percent of the lactic acid dehydrogenase was lost from the cells to the media in one hour while over 90 percent of the aldolase and the catalase could be recovered from the cells. Aldolase was rapidly lost when sucrose was removed from the media. The addition of methocel or serum did not prevent the rapid loss of enzymes.

Drs. M. Chirigos and A. Goldin have studied a transaminase system, which mediates the transfer of *alpha*-amino groups of several *alpha*-amino acids to *para*-hydroxyphenylpyruvate with the formation of tyrosine, in liver and in spontaneous and transplanted tumors. With the amino acids employed, a low degree of similarity in transaminating pattern was observed among the tumors when compared with the pattern for liver. Tumor extracts showed greater transaminating activity with glutamic acid than with glutamine, in contrast to liver preparations which exhibited similar activities with both compounds. The study also indicates enzyme systems not previously reported exist both in liver and in the tumors.

Dr. Helen Dyer has been studying glutamic-oxalacetic transaminase (GOT) activities of several tumors, including the Morris 5123 hepatoma, in a number of strains of rats and the activity in their sera. The level of serum GOT activity was not reduced during the first two months of feeding of several hepatocarcinogenic aromatic amines nor the chemically related nonhepatocarcinogenic compounds in the dosage used for the induction of tumors. GOT activity of livers of animals bearing tumor transplants did not differ significantly from those of normal animals. Although several transplanted tumors showed a lower GOT activity than normal liver, the Morris hepatoma 5123 had a higher activity. Furthermore, the GOT activity of sera of rats bearing hepatoma 5123 was considerably above that of normal rats, whereas the sera of rats bearing several other transplanted tumors was within the normal range. As the hepatoma 5123 grew in size, the serum transaminase activity increased accordingly. The serum level can also be raised by the intraperitoneal injection of the supernatent layer of a centrifuged homogenate of hepatoma 5123. Very little GOT activity was detected in the urine. The results suggest that the increased

serum GOT activity originates in the tumor tissue and that it accumulates in the blood because it can be excreted only slowly by the kidneys.

An intriguing experiment has been performed by Dr. D. Burk and Mr. J. Hunter in which thermophilic chlorella cells were introduced into the peritoneal cavity of mice. Severe and chronic hypoglycemia occurred that resulted in the death of the animal. Cells washed from the peritoneal cavity of the mouse, where they had remained for 17 hours, grew vigorously when placed in proper medium, atmosphere and light. The hypoglycemia could be corrected by administration of glucose. This experiment followed studies on the inhibition of anaerobic and aerobic glycolysis of Ehrlich Krebs-2 mouse ascites cells by the presence of thermophilic chlorella cells grown simultaneously with the tumor cells in Krebs-Ringer medium. Because the algae consumed glucose at a rate several times faster than the cancer cells, the inhibition appears to result from low availability of glucose for the cancer cells. The algae, like the tumor cells, can produce lactic acid from glucose, but D-lactic acid instead of L-lactic acid is formed.

Dr. H. Kahler, with Mrs. B. Moore, extended his earlier studies on the pH of tumor tissue. Following administration of glucose to rats, the pH fell rapidly and returned to normal in tumor tissue at sites in the tumor with good blood circulation. An estimate of the blood circulation was made by parenteral administration of Lissamine green dye; tumor tissue stains green if the tumor has a good blood supply, remains white if the blood circulation is poor, and remains red if there are pockets of blood in a static condition.

Drs. R. E. Madden and D. Burk have developed a procedure for comparing the metabolic behavior of tumors in the solid and ascites forms that may facilitate metabolic and chemotherapeutic studies of solid human tumors and may aid in establishing single-cell-cloned tissue cultures of a variety of normal and malignant cells from human and animal tumors. Single-cell preparations from several solid human and mouse tumors were prepared by stirring scissored tumor sections in media containing trypsin and deoxyribonuclease, centrifuging the strained supernatant fraction and taking the sedimented cells up in medium not containing the enzymes. With the animal tumors, comparisons were made with single-cell preparations from solid tumors and from the same tumor grown in the ascites form in terms of metabolic activity and viability (quantitative *in vivo* mouse inoculation studies). The behaviors of the two forms were essentially the same.

Chemotherapy Investigations

The search for chemical agents useful in the management of clinical cancer is under way on an unprecedented scale, and the stakes are high enough to warrant the tremendous efforts and large amounts of resources being expended. Two main approaches, not unrelated, have been employed, and, despite arguments to the contrary by proponents of one approach or the other, *both* need to be vigorously advanced. One, for the most part an empirical approach, involves screening of materials for their antitumor effects, the ultimate criterion being usefulness in the clinic. As yet, no satisfactory animal system screen has been developed that will predict the usefulness of drugs in the clinic, although some correlations exist between the responses of acute leukemia in children and leukemia L1210 in the mouse. Until much more data are available from studies on antitumor effects of various agents in both man and animal systems, it will be necessary to continue quantitative screening studies and to seek additional correlations. Investigation of many parameters must be continued, such as different agents of various classes, different tumor types (with more emphasis on nontransplanted tumors in animals), dosage schedules, routes of administration, combination therapy, immunological effects, and modifications of the host. The second approach, perhaps one in which the results might be of more far-reaching significance, but also perhaps slower in coming, is one aimed at understanding basic mechanisms of tumors, host-tumor relationships, and the mechanisms of action of chemical agents, both intrinsic and extrinsic, on the tumor and the host. Obviously, knowledge gained in one approach will often be helpful to the other, but until a satisfactory screen is attained, the second approach needs heavy support, perhaps comparatively more than it has received in the large-scale chemotherapy programs currently under way.

The Biochemical Pharmacology Section, under the leadership of Dr. A. Goldin, conducts exten-

sive programs in both approaches of cancer chemotherapy, in the former through effective use of the contract mechanism for a drug development and evaluation program. In this latter, which is under the direction of Dr. C. G. Zubrod, Clinical Director, NCI, and under the supervision of Dr. Goldin and Messrs. J. M. Venditti and G. A. Brandner, particular attention has been given to two factors that have limited the success of cancer chemotherapy: (a) the toxicity of drugs for the host; and (b) the capacity of the tumor to develop resistance to therapy.

Relative to the former problem, the drug development and evaluation program is devoted primarily to determining quantitatively the relative antitumor specificity of drugs of potential clinical interest. In particular, the program emphasizes structure-activity relationships among compounds which emerge as "active" from primary screens. Prolongation of the life-time of tumor-bearing animals is employed as the chief criterion for determining the relative therapeutic efficacy of drugs. Tumor size and body weight changes are, nevertheless, measured routinely. The program also investigates "active" compounds with respect to the optimal conditions for antitumor activity including the optimal schedule of treatment, route of administration, effect of combination therapy, etc. Such pharmacologic studies are needed to provide an accurate appraisal of the potential usefulness of a compound of interest.

Studies of the problem of the development of resistance to treatment have been facilitated by the emergence of active compounds of various chemical classes which have the capacity to inhibit growth at different biochemical sites and by the development of drug resistant sublines of tumors. The inclusion of studies of the effects of new compounds on drug resistant tumors is valuable from two standpoints. Such studies can uncover active compounds which may have clinical usefulness in cases which have become refractory to conventional treatment. In addition, drug resistant tumors are being utilized to explore possible mechanisms of drug action.

Tumor Assay Systems

In the past. three tumor assay systems have been studied most extensively:

The Advanced Leukemia L1210 Assay System. To date, 213 compounds (88 of which were tested in 1960) have now been evaluated for their ability to prolong the life of mice with systemic L1210. The most active compounds against this tumor have been the halogenated derivatives of amethopterin. For example, 3′,5′-dichloroamethopterin is about four times as active as amethopterin. Extensive structure-activity relationship studies among folic acid derivatives have shown that appreciable antileukemic activity is limited to the 4-amino derivatives of this metabolite. However, substitutions in various other positions (e.g. alkyl substitutions in the 10-position or halogenation in the benzene ring) can profoundly alter the degree of antileukemic effectiveness among the 4-amino derivatives. Cytoxan, a cyclophosphoramide nitrogen mustard, displayed activity equal to that of amethopterin. Uracil mustard and a number of ethyleneimines showed moderate antileukemic activity, but were only about 50 percent as effective as Cytoxan. Of 35 purines tested against advanced L1210, none appeared to be more effective than 6-mercaptopurine, which was 50 percent as active as amethopterin. Among the pyrimidines, 5-fluorouracil and its riboside displayed about equal effectiveness (about 40 percent of the amethopterin effect). 4-Amino-pyrazolo (3,4–d) pyrimidine was about 25 percent as effective as amethopterin. None of the various derivatives of this compound showed greater activity. Good activity was seen among the methylglyoxal-*bis*-guanylhydrazones. Three such compounds tested displayed activities of about 65 percent. Glyoxal-*bis*-guanylhydrazone, itself, was relatively inactive. Among the antibiotics, E–73 acetate, Streptovitacin A, and Actinomycin D were the most active, being about 30 percent as effective as amethopterin.

Special studies with the advanced L1210 assay system have been directed toward investigations of factors which may alter the host-tumor-drug relationship, including the influence of the route of administration, schedule of treatment, effect of therapy with drug combinations, etc. It has been shown, for example, that the efficacy of therapy with 3′,5′-dichloroamethopterin or 3′-bromo-5′-chloroamethopterin is substantially reduced when the drug is given orally. Consequently, the superiority of the halogenated derivatives over amethopterin, seen on subcuta-

neous administration, disappears when the drugs are given via the oral route. On the other hand, oral administration did not seriously reduce the effectiveness of 6-mercaptopurine. The methylglyoxal-*bis*-guanylhydrazones were only slightly less effective orally than subcutaneously. Studies of the influence of the treatment schedule on antileukemic activity showed that Cytoxan was more effective when given weekly than when given daily, twice daily, every 4 days, or every 11 days. In general, the activities of the antibiotics tested were not appreciably influenced by the treatment schedule. The methylglyoxal-*bis*-guanylhydrazones were more effective when given daily than when given every 4 days or as a single treatment.

Combination therapy with amethopterin and Cytoxan showed that, with appropriate use of the drugs, treatment with the combination was superior to treatment with either drug alone. Urethane, which was inactive in itself, did not potentiate the activity of amethopterin, nitrogen mustard, or Cytoxan. 6-Azauracil, also inactive when employed alone, did not potentiate the activity of 6-mercaptopurine.

Increasing emphasis has been placed on studies of the effectiveness of chemical compounds on drug resistance. To date, these have included antipurine- as well as antifolic-resistant variants of L1210. Drug-resistant tumors are being used to uncover active compounds which may have clinical usefulness in cases which have become refractory to conventional treatment and to explore possible mechanisms of drug action.

The Sarcoma 37 Assay System. A total of 70 compounds (26 tested in 1960) has been evaluated against early sarcoma 37 for ability to increase the life span of the mice. This tumor has been particularly insensitive to treatment and has therefore not been used routinely in the advanced form. Of the compounds tested against early S–37, alanine nitrogen mustard, Cytoxan, N-methylformamide and p–N, N-bis-(2-iodoethyl) aminobenzylphosphonic acid ethyl ester have provided appreciable increases in survival time. This tumor is of particular interest, because of the apparent lack of correlation between the degree of tumor inhibition and the increase in survival time attained as a result of therapy. For example, 6-mercaptopurine, Bayer 3231, and Actinomycin D were able to inhibit local tumor growth in mice with sarcoma 37 but provided little, if any, increase in survival time.

Special studies employing sarcoma 37 have been directed mainly to investigations of the influence of the treatment schedule on the effectiveness of compounds and to the influence of caloric restriction on local tumor growth and survival time.

The Adenocarcinoma 755 Assay System. Unlike sarcoma 37, Adenocarcinoma 755 (Ca–755) has been quite sensitive to certain compounds, particularly the purine antagonists. In earlier studies in this program, 45 compounds were tested against early Ca–755. Extensive increases in survival time as well as tumor-free survivors were observed as a result of therapy with 6-mercaptopurine and some of its derivatives. The high degree of sensitivity of this tumor to therapy indicated that it could be employed more advantageously in an advanced form for the quantitative evaluation of drugs. Within the past year, 40 compounds have been tested for their ability to increase the survival time of mice with advanced Ca–755. Many purine derivatives elicited substantial increases, but none appeared to be more effective than 6-mercaptopurine. Among nonpurines, Cytoxan was the only compound which showed activity in the same range as 6-mercaptopurine. Moderate activity was observed for Actinomycin D and 4-aminopyrazolo (3,4–d) pyrimidine.

Like sarcoma 37, Ca–755 displayed no correlation between local tumor inhibition and survival time extension. For example, three pyrazolo (3,4–d) pyrimidines tested produced approximately 50 percent inhibition of the local tumor of early Ca–755 without providing substantial increases in survival time.

Other tumor systems that might be suitable for quantitative assay are under investigation.

Ehrlich Ascites Tumor. Of eight compounds tested against the Ehrlich ascites tumor, only N-methylformamide provided an appreciable increase in survival time.

Hepatoma 129. Cytoxan and 6-mercaptopurine were effective in retarding tumor growth and increasing survival time of mice inoculated subcutaneously with hepatoma 129 (obtained from Dr.

Belkin), even when the treatment was withheld until the tumors reached about 1 cm. in size.

Moloney Virus Leukemia. The use of the Moloney virus for the production of indicator tumors in studies of the chemotherapy of leukemia in mice which is also being explored, has two advantages: (1) the induced neoplasm is a *host* tumor, and (2) the induced disease is similar to that which occurs spontaneously in nature. Drs. Goldin, J. Glynn and M. Chirigos and Messrs. J. M. Venditti and S. R. Humphreys, in collaboration with Dr. J. B. Moloney, have evaluated the therapeutic effectiveness of several drugs against this virus-host system, both with virus- and whole cell-induced leukemia. Drugs shown to be significantly effective in extending the latent period (time to death with leukemia) following inoculation of neoplastic cells are: Cytoxan, triethylenemelamine, and phenylalanine mustard (Sarcolysin); drugs of intermediate or no effectiveness are: Amethopterin, dichloroamethopterin, 6-mercaptopurine, meticortilone and 5-fluorouracil. With virus-induced indicator tumors, the following drugs were moderately effective: Cytoxan, methotrexate, and methylglyoxal-bis-guanlyhydrazone. The last-named compound produced marked reduction in spleen and thymus gland weights.

Spontaneous Tumors. Because of the poor correlation between results in animal transplantable tumor screens and in man and because of the growing body of information that indicates transplantable tumors are usually very different from spontaneous ones, screens involving spontaneous tumors need to be established in spite of the increase in variability among the experimental animals and the longer holding time and greater numbers of animals required. Drs. Goldin and Chirigos and Messrs. Venditti, Humphreys and N. Mantel have initiated studies testing the effectiveness of 3′,5′-dichloroamethopterin, 6-mercaptopurine and cytoxan on a spontaneous mammary tumor in strain C3H mice. As might be expected, the untreated control animals showed considerable variability from day of entry into the experiment to the day of death (median, 40 days; range, 5 to 117 days). Survival time was not increased although some retardation of tumor growth was observed. The median survival times observed suggested that drug toxicity was limiting with cytoxan and 6-mercaptopurine in the doses employed.

In spite of logistical difficulties, some data on therapeutic effectiveness of several agents against spontaneous tumors in noninbred experimental animals perhaps should be obtained as another comparative screen of importance to clinical cancer.

Biochemical Studies

3′-Fluoroamethopterin and 3′,5′-difluoroamethopterin were tested against leukemia L1210 by Drs. A. Schrecker, J. Mead and Goldin and Mr. Venditti. The former is comparable in effect to methotrexate and the latter to the dichloroanalog. These findings suggest that the greater effectiveness of 3′,5′-dichloroamethopterin over methotrexate in L1210 is not due to steric hindrance of the halogen atoms, but to the electronegative nature of the halogens. 3′,5′-dimethylamethopterin will be synthesized to test further the concept.

A simple fluorometric method has been developed by Drs. Chirigos and Goldin for the determination of small amounts of phenylalanine mustard (Sarcolysin), and time studies on the distribution of the drug following its administration to tumor-bearing animals have been done. Binding of the drug by cellular fractions of the blood was indicated.

In collaboration with Dr. N. O. Kaplan (Brandeis University), Dr. Goldin and Mr. Humphreys have studied several precursors, analogs and antagonists of diphosphopyridine nucleotide (DPN) and related compounds for their influence on DPN metabolism, their ability to reverse antileukemic activity of certain drugs and their antitumor effects. Dose response curves have been obtained on some 50 compounds, with structural differences producing a wide range of toxicity. Only 6-aminonicotinamide, *beta*-acetylpyridine and the thiadiazoles have evidenced any antileukemic effects. Nicotinamide has been shown to reverse the antileukemic action of 2-amino-1,3,4-thiadiazole and 2-ethylamino-1,3,4-thiadiazole. As a follow-up on the earlier finding that nicotinamide administration in the mouse led to more than a ten-fold increase in liver DPN, other

compounds have been shown also to result in rapid formation of liver DPN: 3-acetylpyridine, pyridyl-3-methyl carbinol, *beta*-picoline, monomethyl and monoethyl nicotinamide.

As an example of work representing a continuation of studies reported last year on the mechanism of action of antifolic drugs, investigations are summarized on biochemical studies of resistance to antifolics in two resistant cell lines (C82 and M66) derived from leukemia L1210. Drs. Schrecker, Mead and Goldin and Mr. Humphreys, in collaboration with Dr. M. Friedkin (Tufts University), have found that both the local tumor and the spleen of the M66 variant had the same amount of dihydrofolic reductase activity as the sensitive L1210 leukemia, while in the C82 subline the level of enzyme activity was increased thirty-fold. Formate–C^{14} incorporation into the acid-soluble adenine of spleen and local tumor was inhibited by antifolics to a much lesser extent in the C82 variant than in the sensitive L1210 line. With single treatment with methotrexate or 3′,5′-dichloroamethopterin, some inhibition was still obtained in the C82 line. With daily treatment, however, no inhibition at all was observed after several days of treatment. This would suggest that there are still some sensitive cells present in the C82 antifolic-resistant line and that they are eliminated early in treatment. It tends to support the hypothesis that, in the C82 subline, there is a mixed population of cells with varying amounts of dihydrofolic reductase, the larger proportion of cells having higher amounts of the enzyme. The cells that do not possess high activity may be the ones eliminated during the first few days of treatment. Whether in the original L1210 line some cells with high dihydrofolic reductase activity are already present has not yet been determined. In the M66 line, inhibition of formate incorporation by methotrexate or 3′,5′-dichloroamethopterin (single treatment) is of the same order as in the sensitive line. At present the effect of multiple treatment in the M66 line is under study. Preliminary results indicate that, here again, treatment induces drug tolerance. It would appear, so far, that the mechanism of resistance may be different in the M66 variant from that in the C82 variant. In the latter a high enzyme activity produces a high tolerance for the inhibitor.

Drug Resistance Studies

Dr. Law continues his studies on the mechanisms involved in inhibition of leukemic cell growth by drugs and in development of drug resistance. A subline of the lymphocytic neoplasm L1210 has been developed which is resistant to the effects of 6-mercaptopurine, 5-fluorouracil and amethopterin. The growth rate and strain specificity have become altered. Comparative studies with the parental cell line on the effects of several new agents are in progress.

With Dr. M. Lane (now at Baylor University), Dr. Law has found that the development of resistance to 5-fluoropyrimidines apparently has changed the sensitivity to cyclophosphoramide (Cytoxan); "resistant" lines (to three other compounds) were found to be more sensitive to cyclophosphoramide than the "sensitive" parental line. At optimal dosage levels, complete regressions of established tumors in both P–815 and L1210 lines have been observed. In collaboration with Dr. E. P. Anderson and Dr. W. Brockman (Southern Research Institute), strains of both these cell lines that are resistant to 5-fluorouracil were found to have decreased capacity (or decreased rate) of nucleotide formation. In contrast to findings reported by Reichart, phosphorylase and kinase activities, which probably constitute the major pathway for the biosynthesis of uridylic acid in mammalian tissues, are found in these resistant lines. Preliminary results indicate both enzyme activities are weaker in the resistant lines compared with the sensitive lines.

Previous studies with intact cells and cell-free extracts by Dr. Anderson on the biochemical effects of azaserine in azaserine-sensitive and azaserine-resistant cell lines of the plasma cell neoplasm 70429 indicated an "intact cell factor" was operative in the resistance mechanism, but to learn if this "factor" was related to greater impermeability of the resistant cells to the drug, it was necessary to determine specifically the entry of the compound into the cells. In collaboration with Dr. J. Jacquez (Sloan-Kettering Institute), Dr. Anderson has studied the capacities of the two cell lines for concentrative cell transport and intracellular breakdown of azaserine. No major differences were found in the capacity to concentrate azaserine within the cell. Quantitative differences, however, were found; the sensitive line showed a slightly higher initial velocity of up-

take, whereas, over a longer period, the resistant cells surpassed the sensitive ones in the final intracellular concentration which they could maintain. No appreciable differences were found in the extent of intracellular breakdown of the azaserine. Additional studies have been started on reversal of azaserine transport by normal amino acids in the two lines, since pool size of normal metabolites and their capacity to reverse transport of the antimetabolites are also important considerations in understanding the mechanism of development of resistance. Some difference in pool size of nucleotides has also been indicated by data already collected.

Glycolysis Studies

In previous Annual Reports, Drs. M. Woods and D. Burk reported that a high degree of malignancy is associated with greatly increased glycolytic capacity and lowered sensitivity to glycolytic inhibition of the anti-insulin type. In general, those experimental neoplasms in which the hexokinase reaction is under strong insulin-anti-insulin control respond to one or more chemotherapeutic agents better than do those cancers in which glycolysis is less readily inhibited by compounds of the anti-insulin type.

Human normal and leukemic leucocytes have been studied with special reference to their glycolytic responses to anti-insulin steroids, insulin and endotoxic polysaccharides by Drs. Woods and Burk and Mr. J. Hunter. Results obtained to date, though not conclusive, indicate that glycolysis in lymphocytic cells (normal and leukemic) is much more sensitive to inhibition by steroids than is the case with cells of the myelocytic series. The data indicate that insulin: anti-insulin regulation of glucose metabolism plays an important role in the metabolism of leukocytes (lymphocytic and myelocytic), and that endotoxic polysaccharides counteract such glycolytic inhibition in a manner resembling, although not identical with, insulin.

During the past year, studies on the action of fluorinated pyrimidines and related compounds on tumor glycolytic metabolism were extended. In collaboration with Dr. J. Seitz (Visiting Scientist from the Institute for Hematology and Blood Transfusion, Leningrad), the effects of fluorinated pyrimidines on "high energy phosphate" metabolism of tumor cells were studied. At low levels of inorganic phosphate, where *in vitro* glycolytic inhibition may reach 80 percent, no reduction in net acid-insoluble high energy phosphate was observed. All of this phosphate fraction, however, may not have consisted of simply ADP and ATP, but might have involved possible fluorinated derivatives.

While inhibitory action by a series of 5-fluorinated pyrimidines seems to depend on the presence of an active glucose metabolism during exposure to the drug, in a few instances, following *in vivo* exposure to 5-fluorouracil, the transplantability of ascites tumor cells was markedly inhibited without concomitant glycolytic inhibition. However, in all instances, there was a strong positive correlation between the *in vitro* glycolytic inhibitory activities of a series of substituted pyrimidines and their *in vivo* anti-tumor activities.

Evidence was obtained that the anti-glycolytic pyrimidines inhibit aerobic glycolysis in ascites tumor cells by competitively inhibiting the utilization of inorganic phosphate during initial stages of exposure to the drug. A secondary phase of inhibition is not reversed by increasing the concentration of inorganic phosphate in the medium. A recent report by Lemon is of interest in this connection: simultaneous intravenous administration of glucose with 5-fluorouracil in man is accompanied by reduction in host toxicity, with retention of anticancer effects.

Cytoxan, a cyclic phosphoramide derivative of nitrogen mustard, was found to inhibit tumor cell glycolysis. Comparison of the L1210 ascites strain resistant to cytoxan, developed by Dr. Lane with the L1210 ascites susceptible strain, showed a difference in the glycolytic and respiratory properties of the two strains with respect to cytoxan action. Cytoxan inhibited the glucolysis and respiration of the susceptible strain considerably more than in the resistant strain, the glycolysis and respiration of the tumor being determined after action of cytoxan *in vivo*. Nicotinamide and diphosphopyridine nucleotide reversed cytoxan action in the resistant more than in the susceptible strain. Further progress on the activation of cytoxan *in vitro* was made. In the presence of triphosphopyridine nucleotide (TPN) and magnesium, as well as adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN), cytoxan inhibition of glycolysis of liver

and mammary tumor homogenates and derived subcellular elements was obtained. Thus a connection of cytoxan action with magnesium and TPN was found. An inhibition of ATP-ase by 5-fluorouracil was demonstrated for the L1210 ascites tumor cells. Further study of the respiration and glycolysis of tumors and normal tissues was undertaken. Glucose usually produced a pronounced Crabtree effect (inhibition of respiration by glucose) in the case of ascites tumors, but this was not always true. TPN, with increased magnesium and nicotinamide, increased the glycolytic production of carbon dioxide three to four times above the amount produced in the presence of ATP and DPN only, in mouse mammary tumor homogenates, but not in liver homogenates. An increase in the amount of lactic acid produced was also found. This finding has been extended to whole cells for the thymoma ascites. The increased glycolysis in the presence of TPN is not so great with cells, but clearly demonstrable. There is no evidence to support the hypothesis that DPN is formed from TPN. Other nucleosides such as inosine, uridine, cytosine and guanosine triphosphates could be substituted for adenosine triphosphate partly or wholly in tumor glycolysis, in studies with thymoma ascites and mammary tumor homogenate.

Chemotherapy of Experimental Central Nervous System Leukemia

The clinical studies of Drs. E. Freireich and L. Thomas in the General Medicine Branch have revealed the importance of central nervous system involvement as a serious complicating factor in chemotherapy of clinical leukemia. Similar findings have been seen in mice treated sufficiently to give extensive increases in survival time. An experimental animal model for the study of central nervous system leukemia has now been developed by Drs. Goldin, Chirigos and Thomas and Mr. Humphreys. Treatment with amethopterin or 3′,5′-dichloroamethopterin increased the survival times of the mice inoculated intracerebrally with L1210 even when the therapy was initiated as late as a few days prior to the death of the control animals. The effectiveness suggests that these drugs may be capable of crossing the blood-brain barrier in sufficient quantity to exert direct antileukemic action in the brain. Cytoxan was found to have limited effectiveness against intracranial leukemia.

Service Functions

The many research contributions made by the investigative staff would not be possible without the very considerable assistance given by many others on the staff of the Institute. Messrs. W. Magruder and J. Murphy continue to provide invaluable assistance in meeting those many difficult day-to-day problems which must be handled expeditiously if research progress is to continue at a good pace. The skills shown by them are not only of tremendous importance to the research programs, but in a very real sense, often require a creativity just as unique as that needed for research.

The administrative clerical and secretarial assistance provided throughout the Institute, likewise, is in a very real sense a contribution to the programs of the Institute without which much of the work could not come to fruition.

The Pathologic Anatomy Branch continues to perform very superior work in pathologic anatomy, not only for studies in the NCI but for investigations throughout the NIH. Pathologic anatomy work-up of surgical specimens (2,521 specimens in 1960) and of autopsy material (276 autopsies during the year), including the extensive discussions held by the pathologists with other members of the staff, provide basic pathology information of the highest quality for the clinical studies under way at NIH. Nearly 50 scientists in the various laboratories at NIH have requested particular tissues from surgical and post-mortem services during the past year and, on many occasions, it was possible to furnish these investigators with fresh human material.

Six full-time residents in the Department of Pathologic Anatomy are receiving excellent advanced training in pathology.

The Cytodiagnostic Service, under the supervision of Drs. R. A. Malmgren and E. W. Chu, provides excellent exfoliative cytology service to NIH and constitutes a major element of the diagnostic facilities available to the clinical staff (10,161 slides, representing 3,055 accessions, were examined during 1960). The Service has par-

ticipated in quantitative studies on surgical wound washings, on fluids draining from such wounds, on body fluids such as spinal fluid, and on peripheral blood, utilizing both the Papanicolaou staining procedure and special filtration techniques for the collection of cells.

Mr. J. Albrecht and his staff of histopathology technicians continued to provide the excellent service that the research staff has learned to rely on from this superior group. They prepared 129,068 stained slides for the staff of NIH, of which 29,994 required special staining. The fine on-the-job training given to technicians, several of whom later work closely with members of the investigative staff, continues to pay dividends for the National Institutes of Health.

The analytical chemistry group in the Laboratory of Biochemistry under Mr. R. J. Koegel's supervision continues to provide high quality service. During 1960 the character of the requests changed significantly, with a reduction in requests for microchemical empirical elemental analyses, but with an equal workload of some 5,500 individual analyses.

NATIONAL HEART INSTITUTE

INTRODUCTION

This introduction to a review of the intramural research of the National Heart Institute will be brief. The material contained in the following pages, largely as reported by the leaders of its major research groups, speaks for the Heart Institute far more effectively than can any words of introduction. We believe the reports that follow combined with the published bibliography of the Heart Institute constitute a record of considerable achievement. That much of it is directly pertinent to the categorical responsibilities of this Institute will be apparent; we take considerable pride in the fact that this is accomplished without restriction of the freedom of the individual scientist to pursue, within the resources available, those problems which excite his intellectual curiosity. We believe the high level of scientific achievement is not separable from this freedom.

Several organizational changes have been effected in the Heart Institute. These are not reflected in the report which follows since the changes were accomplished toward the end of the year. The General Medicine and Experimental Therapeutics Branch has been for some two years an organizational designation without real operational meaning. Its three sections, Experimental Therapeutics, Clinical Endocrinology, and Cardiodynamics, have each operated quite autonomously. This independence has now been recognized and the Experimental Therapeutics and Clinical Endocrinology groups have been given branch status under the leadership of Drs. Albert Sjoerdsma and Frederic Bartter, respectively. The Cardiodynamics Section has been combined with the Cardiology Section from the Surgery Branch to establish the Cardiology Branch under Dr. Eugene Braunwald. That portion of the Cardiodynamics Section which has been the responsibility of Dr. Donald Fry has been established as the Section on Clinical Biophysics within the Cardiology Branch.

LABORATORY OF CELLULAR PHYSIOLOGY AND METABOLISM

Section on Cellular Physiology

The program of the Laboratory of Cellular Physiology and Metabolism, Section on Cellular Physiology, is aimed at the understanding of what might be called the "design" of protein molecules and with the factors concerned in their structure, biosynthesis and mechanism of action. This broad problem is being attacked from a number of directions involving biochemical, genetic and physical investigations. The major premise of the research is that the biological and physical properties of proteins are predictable from a knowledge of covalent structure alone and that much of the mysticism attached to proteins has grown out of lack of understanding of proteins as organic molecules. Thus, it is felt that such phenomena as irreversible denaturation and the loss of function resulting from treatment by such agents as heat, urea, etc., do not necessarily reflect irreversible changes in structure, but rather ineptitude and lack of information on the part of investigators. The following reports of specific projects summarize the experimental details and future plans of individual segments of the program.

(1) In previously reported studies it has been demonstrated that the four disulfide bridges in ribonuclease can be cleaved by reduction with mercaptoethanol and that the resulting inactive product can be converted to the original native molecule by simple exposure to atmospheric oxygen. This work has been extended in the direction of the elucidation of the factor concerned with the proper pairing of half-cystine residues. It is now clear from an examination of the effects of a variety of chemical and physical agents that the matching of proper half-cystine residues in disulfide bond formation is under the control of a large number of side chain interactions involving hydrogen bonding, electrostatic attractions

and repulsions, and Van der Waal's forces. For example, when fully reduced ribonuclease, containing eight sulfhydryl (SH) groups per mole, is allowed to reoxidize in the presence of 8 M urea, all of these SH groups disappear and a tightly coiled spherical molecule is produced which is completely inactive in spite of the fact that it has physical properties much like those of native ribonuclease. Upon re-reduction of this substance followed by reoxidation under optimal conditions of pH and protein concentration, a material which is indistinguishable from the native enzyme is obtained in yields up to 95 percent. Such an experiment demonstrates the importance of hydrogen bonds in the process since urea is known to be a strong hydrogen bond rupturing agent. The importance of the other interactions mentioned above may be demonstrated by the use of other substances and conditions.

Studies of this sort suggest strongly that all of the information required for the formation of a completely cross-linked and internally coiled globular protein is coded into the amino acid sequence of the molecule and that no further information is necessary to direct the formation of the complicated three-dimensional structure that characterizes this particular enzyme.

The studies outlined above have involved the development of new methods for handling reduced proteins which add considerably to the simplicity and reproducibility of the work. It is planned during the coming year to apply these methods to a variety of other protein molecules in an effort to test the generality of the hypotheses mentioned above. Preliminary findings show that another enzyme, lysozyme, behaves in the same manner as ribonuclease in relation to the reversibility of its reduction and reoxidation.

(2) Continuing efforts are being made to work out the structures, within biologically active proteins, that are essential for such functional processes as catalysis and hormonal stimulation. These studies involve specific degradation of ribonuclease and other proteins in an attempt to arrive at minimum structures that are still functionally competent. It has been possible to mask almost all of the positively charged side chain groups of ribonuclease by the chemical addition of strands of polyalanine to the amino groups of the lysine residues without loss in enzyme activity or of the ability to regenerate active material after preliminary reduction of disulfide bridges. It has also been possible to remove several amino acids from the carboxyl-terminal end of the enzyme without inactivation although more extensive degradation has led to complete loss of activity. The degradative and reductive experiments now make possible the preparation of fragments of the reduced form of ribonuclease which will serve as raw materials for preliminary studies on the organic synthesis of portions of the protein that might show enzymatic activity. Such work is of importance since it is necessary to show that those parts of the total structure responsible for activity, as indicated by degradative studies, are the same as those that will be required in the eventual synthesis of an active substance.

(3) Since only certain parts of enzymes appear to be essential for function, it seems likely that such parts of the structures would be less likely to undergo change during speciation and evolution in general. If this hypothesis is correct, it seems likely that a comparison of the same enzyme from different species and from mutants of the same species should indicate common denominators of structure associated with function. Along these lines, a number of different lysozymes are being investigated, both structurally and with relation to the genetic "information" of the organism in question. The structure of lysozyme from chicken egg white and from bacteriophage T4 are now under active investigation and structures of lysozymes from a variety of other species of birds are being screened in a qualitative way. During the past year a chromatographic technique has been perfected in this laboratory which permits the separation of the component peptides of egg white lysozyme following reduction and alkylation of sulfhydryl groups and trypsin digestion of the protein. This technique consists of a gradient elution, from phosphorylated cellulose ion exchange columns, using pH and salt gradients of a volatile buffer, ammonium acetate. The amino acid analyses of the peptides obtained by this method provide the background information and material necessary for further sequence work. Current attempts at specific blocking of selected sites during trypsin digestion are aimed at providing information which will make it possible to assemble the peptides in their proper order.

Among the mutants of bacteriophage T4 several have been isolated that produce lysozymes with physical properties differing from those of the enzyme in the original standard strain of phage. The lysozyme of one such mutant has been examined in detail and it has been found that its structure differs by a change in amino acid sequence from that of the standard enzyme. Similar examination of other mutants should provide information to test the hypothesis that the amino acid sequence of an enzyme is determined, in a direct fashion, by the sequence of genetic "information" coded into the DNA molecules that determine heredity. The selection for interesting enzyme mutants will be continued and the fine structure genetic map extended. Ultimately, it is by the determination of the nature of the changes produced in the enzyme as a result of mutation (particularly when induced by chemical mutagens with known mechanisms of action) that we hope to gain an insight into the nature of the "genetic code."

(4) Studies on the biochemistry of muscular contraction have continued with special emphasis on the chemistry of the myosin molecule. In earlier studies it was demonstrated that myosin is probably made up of three physically and chemically identical polypeptide chains, each coiled in the form of a helix, the three helices being wound together in the form of a rope. Present studies now suggest that these three strands may be completely dissocated from one another and under suitable conditions be made to reassociate to yield the original parent molecule. The results suggest that the subunit is so designed that its proper aggregation is predetermined by the location of interacting groups along the chain much in the way that such groups determine the re-formation of native ribonuclease from the fully reduced molecule as described above. The structure of the individual subunits is being studied by the preparation of enzymatic digests and the separation of the resulting peptide fragments for subsequent determination of sequence. The alignment of these fragments into the proper order should eventually yield information on the spacing of interacting groups along the chain and may make it possible to explain the aggregation process on a rational chemical basis. In the course of these studies, a careful determination of the molecular weight of myosin by light-scattering methods has indicated, in accordance with the chemical studies, that the native molecule is made up of three subunits, each with a molecular weight of approximately 200,000. These studies have elucidated some earlier experimental discrepancies which led to the postulation of a considerably lower molecular weight for this protein.

It was observed some time ago that the ATPase activity of myosin, the protein unit of the contractile mechanism, exhibits a biphasic response to titration of the SH groups, with a 3–4 fold stimulation of ATPase activity when approximately one-half of the SH groups are titrated, and complete inhibition on further titration. The initial phase of this process is also characterized by a marked alteration in the substrate and activation specificities of the protein. During the course of the titration, the ability of the molecule to react with another muscle protein, actin, is lost. This reaction is widely regarded as important in the mechanism of contraction. Identification of the amino acid composition of those portions of the molecule involved in these processes and their localization in the molecule should provide considerable insight into the biochemical process of muscular contraction.

Treatment of myosin with sufficient radioactive N-ethylmaleimide to react with one of its sixteen sulfhydryl groups leads to a marked acceleration of its calcium activated hydrolysis of adenosine triphosphate and a complete loss of its ability to hydrolyze inosine or guanosine triphosphate. Furthermore, the hydrolysis of adenosinetriphosphate is no longer accelerated by ethylenediamine tetra-acetate. This process is accompanied by the labeling of one of the cysteine containing peptides identified by the "fingerprinting" procedure. In addition, it appears that this peptide contains not one but two cysteine residues and the titration of this second group occurs during the inactivation phase of the biphasic response to SH titration.

(5) Previous studies have indicated that the processes of protein biosynthesis in hen's oviduct appear to involve the mediation of certain lipid-amino acid complexes which are in a very rapid state of metabolic flux. The chemical nature of these complexes is being studied following their partial separation on chromatographic columns. It was recently found that specific sites which

can distinguish L-valine from D-valine or L-serine exist in the lipids. Evidence that points to a covalent link between the carboxyl group of the amino acid and a lipid component was obtained. Preliminary findings suggest the existence of a lipo-peptide thought to be involved in the protein synthetic process of the oviduct.

The lipid-peptide complexes are also being studied in *E. coli* in collaboration with investigators at the Carnegie Institution of Washington. Such cells also contain these materials and the results suggest that they may be associated with the ribosomes which are known to be actively involved in protein biosynthesis.

In a collaborative project with investigators at the National Cancer Institute, chromatographic studies are being performed on the ergastoplasm of rat liver cells. The ergastoplasm consists of a lipid membrane, studded with ribonucleoprotein granules approximately 150 A in diameter. It is believed that "information" for making particular proteins is contained in the configuration of the nucleic acids of these granules. If this hypothesis is correct, one would expect the granules to be heterogeneous. The granules are being fractionated to determine if different granules lead to the synthesis of different cytoplasmic proteins.

(6) Studies on the structure and metabolism of heparin and related mucopolysaccharides, the relation of heparin to lipoprotein lipase and the role of lipoprotein lipase in fat metabolism are being continued. Olive oil, corn oil, cream and cocoa butter were fed to rats with cannulated thoracic ducts. The chyle was collected and chylomicrons prepared from it. These chylomicrons were then incubated with pancreatic lipase and lipoprotein lipase. The free fatty acids liberated by enzymic hydrolysis were isolated and the molar percentage composition determined by gas-liquid chromatography and compared to that of the substrate triglyceride. The relative amounts of free fatty acid and glycerol produced during hydrolysis were also determined. The results show clearly that, whereas pancreatic lipase hydrolyzes preferentially fatty acids esterified at the α-position of a triglyceride, lipoprotein lipase hydrolyzes α- and β-esters equally well. Lipoprotein lipase does not appear to have a specificity among the long chain fatty acids, saturated or unsaturated.

Section on Metabolism

The Metabolism Section of the National Heart Institute has a wide range of current interests which center primarily on the chemistry and metabolism of two classes of compounds, lipids and proteins. The two are in many respects interrelated but the study of lipids is perhaps paramount and can be considered as part of an overall program aimed at elucidating the causes of atherosclerotic heart disease.

Metabolic Activity of Adipose Tissue

One of the major projects in the laboratory involves the study of the metabolic functions of adipose tissue and their control. This has been approached experimentally in several ways. The mechanisms through which the rate of fatty acid release is regulated and altered by hormones is of primary interest and several kinds of experiments have been carried out to explore the hypothesis that alterations in the rate of fatty acid release may result from changes in the rate of fatty acid esterification.

Triglycerine Synthesis. Glycerides are synthesized from fatty acyl Coenzyme A and α-glycerophosphate by a mechanism apparently like that previously described in liver. The adipose tissue, however, cannot convert glycerol to α-glycerophosphate as can some other tissues. The synthesis of α-glycerophosphate from other metabolic interemediates was studied in the homogenate system. Synthesis from phosphorylated intermediates of the glycolytic cycle was obtained when DPNH was added to the system. Neither glucose nor glycogen was an effective precursor in this system. Synthesis of triglyceride from diglyceride and acyl Co-A precursors has been observed in chicken adipose tissue, but the excessive thiolase activity of rat adipose tissue has prevented successful studies in that species. One interesting aspect of the studies of incorporation of fatty acids into glycerides is that lipolysis continues, even though net esterification of fatty acids is occurring. Characteristics of the lipase active in this *in vitro* system suggest that it is different from lipoprotein lipase.

Influence of Hormones. It has been shown that epinephrine, ACTH, and glucagon (which increase fatty acid release) all decrease the in-

corporation of C^{14}-palmitate into glycerides by adipose tissue incubated *in vitro*. Serotonin, which does not increase fatty acid release, does not alter C^{14}-palmitate incorporation; while glucose, which stimulates palmitate esterification, decreases fatty acid release. The effects of these hormones and of a series of compounds related to epinephrine on the conversion of C^{14}-glucose to triglyceride have also been studied in an attempt to elucidate the interrelationships between carbohydrate and lipid metabolism in adipose tissue. Some similar studies have been carried out in tissues obtained from rats with altered thyroid function, comparing effects in these tissues to those obtained in tissues from normal animals in order to determine the role of thyroid state in the responses of adipose tissue.

Physiology of Adipose Tissue and the Mechanism of Free Fatty Acid Release

Fatty acids released by adipose tissue are carried through the plasma in association with serum albumin. The equilibrium relationships between fatty acids and albumin were studied in 1957 and 1958 by Dr. DeWitt Goodman. During 1960 an attempt was made to measure with a stop-flow apparatus the kinetics of the formation of the fatty acid albumin complex. The only conclusion possible was that the reaction is complete in so short a time that this apparatus is not satisfactory for a quantitative measurement. It may be concluded that the formation and dissociation of fatty acid albumin complex requires a time that is short in relation to the time required for blood plasma to pass through a capillary *in vivo*. Further preliminary experiments aimed at measurements of the diffusion of fatty acids through albumin containing media were undertaken. These indicated that fatty acids ions cannot diffuse appreciably faster than can the albumin to which they are attached.

Biosynthesis of Cholesterol

A major interest of one group in the laboratory has been the mechanism of action of MER–29. This compound has been found to affect experimental animals and man in the same way—namely, by inhibiting the reduction of the side chain of 24-dehydrocholesterol to yield cholesterol. As a result, the serum of patients treated with MER–29 contains a considerable quantity of desmosterol (24-dehydrocholesterol), which may be as much as ¼ of the total sterol present. It also accumulates in rat tissues and presumably in man as well. Desmosterol appears to participate in many of the metabolic reactions of cholesterol and is found to be esterified at much the same rate. Whether it may give rise to bile acids and steroid hormones in which the side chain is lost is the subject of a current investigation. Desmosterol yields less color in the standard Liebermann-Burchard color reaction than does cholesterol, with the result that the application of standard cholesterol methods to MER–29 treated patients results in a falsely low estimate of serum sterol levels. Colorimetric and chromatographic methods for the estimation of true cholesterol and desmosterol levels have been developed and publications currently in preparation or in press emphasize these data.

One of our investigators has had the privilege of working with Dr. George Popjak, both in London and during Dr. Popjak's tenure as a Visiting Scientist at NHI. Their collaborative work dealt with studies of the mechanisms of biosynthesis of some of the important intermediates in the pathway to cholesterol. It was found that both liver microsomes and a soluble enzyme were required for the cyclization of squalene. O_2 and TPNH were also essential. Studies with inhibitors indicated the essential participation of enzyme sulfhydryl groups in the reaction. The mechanism for the conversion of farnesyl pyrophosphate to squalene was also studied. The available evidence indicates that the mechanism of this condensation is asymmetric, one farnesyl pyrophosphate being converted to an intermediate which differs from that to which the other farnesyl pyrophosphate is changed. There have been important technical difficulties with this study which have made it impossible to define the details so far but the study is being actively pursued at this time.

Hypoproteinemia and Hyperlipemia of Nephrosis

Clinical studies of nephrosis which were pursued in previous years have been curtailed since the departure of Dr. Howard Goodman from this Laboratory but experimental animal work has continued. Plasmapheresis has been utilized to simulate the renal loss of proteins. This procedure results in consistent and significant increases

in serum lipids. The mechanism of this effect is under current investigation. The relation of carbohydrate feeding and infusion to serum triglyceride levels is also being scrutinized. Earlier experiments both in animals and in patients led to the finding that a high intake of carbohydrate, while decreasing the serum FFA level often led to an increase in serum triglycerides.

Relation of Serum FFA Levels to Lipid Deposition in Tissue

In a study that was completed during 1960 and has been prepared for publication, investigators of the Metabolism Section demonstrated that the sustained elevation of FFA levels following norepinephrine infusion was correlated with an increased deposition of triglyceride in the livers of dogs. Research in other institutions has indicated that the uptake of FFA by tissues (heart and liver) is related to arterial FFA levels. These various findings add further conviction to the conclusion that FFA transport is regulated largely by the adipose tissue that controls its release into the blood rather than by tissues that utilize it.

Clinical Studies of Hypoproteinemia

In this study, carried out in collaboration with the Metabolism Section of the National Cancer Institute, patients with various types of hypoproteinemia have been admitted and studied. Two patients with analbuminemia have been encountered. Each of these was given S^{35}-labeled amino acids and studied for evidence of incorporation into serum proteins. Surprisingly, the serum albumin fraction, however carefully purified, contained some radioactivity. This result suggests that each subject makes at least a small amount of endogenous serum albumin and agrees well with the report from Bennhold's clinic that analbuminemics, who have never been given albumin, have traces of this protein in their plasma.

A much larger group of hypoproteinemic patients has been shown to be suffering from "protein losing enteropathy." This condition is demonstrated by testing with I^{131} PVP and I^{131} albumin. Clinical and biopsy studies of this group of patients have led to the definition of a previously unrecognized disorder of the intestinal lymphatics, for which the term "intestinal lymphangectasia" is being proposed. The results of this study were presented at a meeting of the American Gastroenterological Association and are being readied for publication. I^{131} PVP has been prepared regularly with the help of the Clinical Center Pharmacy Department in such quantity that it has been possible to provide it to investigators in other parts of the country and of the world. This program has resulted in the discovery of a large number of similar cases in other centers and to the appearance of several additional publications, primarily in foreign journals.

Fibrinogen

An investigation into the basic structure of fibrinogen has been continued during 1960. The amino acid composition of this protein has been determined with exactness and the products of chemical and enzymatic hydrolysis are under study. The complexity of fibrinogen makes it unlikely that the complete amino acid sequence will be known soon but some important facts have emerged. Trypsin attacks fibrinogen and quickly degrades it into a few large fragments. The most important fragment has been isolated and appears to have a molecular weight of approximately 100,000. This may be equivalent to the spherical portions of the fibrinogen molecule seen in electron micrographs. The relation of fibrinogen structure to its role in the clotting mechanism is a basic interest in this work.

Fatty acid transport

Experiments have been designed this year to eliminate certain irrelevant biological variation when the metabolism of different fatty acids is being compared. This is accomplished by introducing simultaneously such acids as C^{14}-labeled linoleic acid and H^3-labeled palmitic acid. These have been fed or injected as free fatty acids (FFA) or chylomicron triglyceride fatty acids (TGFA). The results so far have revealed some hitherto unappreciated differences in the rates of removal from the plasma of these two acids as FFA. This is detected by an early drop in the ratio of labeled linoleic to palmitic in plasma. This, in turn, is explained in part by a very rapid preferential accumulation of linoleic acid by the lecithin in liver. A greater accumulation of palmitic by the liver triglycerides is not sufficient to overcome the effect on the apparent plasma

removal rates. This technique shows promise of permitting comparative measurements not otherwise available in events occurring as fast as the turnover of fatty acids.

Means of interpreting such experiments using tracer fatty acids have been improved by the programming of data obtained in animal and human experiments, such as the departure of radioactivity from the plasma or its appearance in the expired air, for the 650 computer. To this has recently been added electronic means of resolving sums of exponentials into components, a system developed in LTD–NHI. Combining these operations, it is possible with a few moments of calculation to obtain expressions for the probability of conversion of a fatty acid to carbon dioxide or of its recycling within the plasma lipid compartment. Presently under study is the question of how useful such operations are in interpreting biological data of this rather standard type, and whether unique information can be so obtained.

Regulation of Lipoprotein Synthesis

The regulation of plasma lipid and lipoprotein levels has been studied both from the standpoint of the lipid and the protein moieties and from differing experimental approaches.

Cholesterol Catabolism. The action of dietary fats on cholesterol metabolism was measured by using the turnover of bile acids as a sensitive indicator of cholesterol degradation. Preliminary data suggest that cholic acid turnover may be influenced by dietary fat, but final analyses are awaited for more certain interpretation. Studies of cholesterol degradation using measurement of the rate of appearance of end products of labeled cholesterol in feces have failed to demonstrate a significant correlation with the nature of the dietary fat.

Rate of Lipoprotein Synthesis In Vitro. It having been previously demonstrated in the laboratory that liver slices synthesized the protein moiety of serum lipoproteins, and that the rate of such synthesis was not influenced by factors increasing cholesterol synthesis, elaboration of other lipids in this system was studied. It has been observed that the presence of high concentrations of free fatty acids in the medium significantly increases the rate of triglyceride synthesis by the liver. Concomitant effects of glucose and of epinephrine were consistent with the overall hypothesis that increased mobilization of free fatty acids from adipose tissue to the liver could increase liver triglyceride synthesis and ultimately lead to higher levels of triglyceride and other lipids, and hence lipoproteins, in plasma.

Epinephrine-induced Hyperlipidemia. Epinephrine or norepinephrine were infused intravenously into anesthetized dogs. Rises in serum free fatty acids in all dogs were accompanied by large increases in liver triglyceride concentrations. Intraportal infusion of norepinephrine, which bypassed the effect of adipose tissue, did not change liver triglycerides. This, and the fact that the total content of lineoleic acid in the liver rose (despite the fact that this acid cannot be synthesized by the dog) indicate that the fatty liver must have arisen because more lipid was mobilized from the adipose tissue via the FFA pathway than could be oxidized. The above effects were associated with some rise in serum cholesterol, but not triglycerides. It is possible, thus, to relate influences, such as stress, to rises in serum lipoproteins, through the mobilization of FFA. This mechanism has considerable importance in understanding a number of currently vague influences upon plasma lipid levels.

Defective Lipid Metabolism

Plasma Post-Heparin Lipolytic Activity. There are presently no biochemical tests for segregating metabolic defects which may cause "essential hyperlipemia." Despite many studies of lipoprotein lipase activity, no attempt has been made to use a quantitative measurement of the activity of this enzyme in man after heparin administration as a means of such segregation. Such an enzyme assay has been developed, a set of optimal conditions determined, and rates of lipolysis established in a group of 25 normals, and these compared with subjects with hyperlipemia. The latter show great scatter, falling into three vaguely defined groups, equal to, lower, or higher than normal. More importantly, it was discovered for the first time, that the response to heparin in man varies with the previous diet.

Low fat intake for 7–10 days depresses enzyme activity to about half of normal. It is also interesting that normal individuals with such depressed activity do not have hyperlipemia even though their enzyme levels, as measured *in vitro*, are lower than those in some patients with severe familial hyperlipemia.

A New Lipidosis. An apparently new disorder of lipid metabolism has been discovered in two siblings of a family from the Island of Tangier in Chesapeake Bay. It is manifested by tonsillar enlargement and discoloration, lymphadenopathy, and enlargement of liver and spleen. Such changes in the reticuloendothelial tissues are associated with accumulation of foam cells containing lipid. Practically all the excess lipid appears to be cholesterol esters. The pedigree has been established and many relatives examined during a trip to Tangier Island. The paternal and maternal grandfathers five generations removed include brothers and the pedigree is *theoretically* typical for the manifestation of the disease as the homozygous phenotype of a rare recessive gene. Whether a heritable defect in lipid metabolism is responsible remains to be proved. Studies in process to elucidate the mechanisms include tonsillar incubations with cholesterol precursors and measurement of plasma cholesterol turnover in both children. One fortuitous finding which is being further explored is the potential value of the tonsil for study of lipid synthesis in the RE system. It is a relatively available tissue, and slices can be easily prepared which readily incorporates precursors into cholesterol.

Section on Enzymes

The activities of the Section on Enzymes are directed toward the elucidation of the detailed mechanisms by which various compounds are metabolized in living organisms. Attention is directed to the metabolism of those substances which is poorly understood and which offers a unique opportunity to study biochemical phenomena of fundamental interest and general significance. Specifically, the current research includes studies on various aspects of lipid metabolism, amino acid metabolism, the metabolism of heterocyclic compounds, of onium compounds, and of one carbon compounds. It also includes studies on complex biochemical phenomena such as the regulation of cellular metabolism, enzyme induction, and cellular differentiation.

Lipid Metabolism

Fatty Acid Synthesis in C. kluyveri. It was previously reported that cell-free extracts of *C. kluyveri* catalyze the incorporation of CO_2 into the unesterified carboxyl group of malonyl-CoA; this exchange reaction requires the presence of malonyl CoA and an acyl CoA derivative of a saturated fatty acid with 2–16 carbon atoms in addition to CO_2.

The cell-free extract of *C. kluyveri* has now been separated into two soluble partially purified protein fractions both of which are necessary for the exchange reaction. Although the mechanism has not been fully elucidated, evidence has been obtained suggesting that the exchange reaction involves the reversible condensation, with concomitant decarboxylation, of malonyl CoA and the acyl CoA derivative to form a β-keto thiolester derivative which is firmly bound to the enzyme:

$$COOHCH_2COSCoA + RCOSCoA + E \rightleftarrows E \cdot RCOCH_2COSCoA + CO_2 + CoASH$$

The likelihood that this reaction represents the first step in the biosynthesis of long chain fatty acids is supported by the observation that a completely soluble enzyme complex precipitating between 65–95 percent saturation with ammonium sulfate catalyses the synthesis of stearate, C–16, C–20 and C–22 fatty acids from the same reactants, namely, acetyl CoA, malonyl CoA and TPNH.

Fatty Acid Synthesis by Adipose Tissue. Submicrosomal particles derived from epidydimal fat pads of the rat have been found to contain an enzyme system capable of synthesizing palmitate and small amounts of stearate from acetyl CoA, malonyl CoA and TPNH. The stoichiometry of the synthesis of palmitate can be expressed as follows:

$$\text{Acetyl-SCoA} + 7\ \text{malonyl-SCoA} + 14\text{TPNH} + 14H^+ \rightarrow \text{palmitate} + 7CO_2 + 8\text{CoASH} + 14\text{TPN}.$$

The overall reaction proceeds most rapidly at pH 6.8 and is activated by mercaptans.

As with the bacterial enzyme preparation, the rat fat pad enzyme system was found to catalyze the interchange of CO_2 and the carboxyl group of malonyl CoA. The observation that the exchange reaction and the fatty acid synthesizing activity of the fat pad enzyme concentrated in parallel throughout a 60-fold purification provides additional support for the conclusion that the CO_2 exchange reaction is an integral part of the system required for fatty acid synthesis. A study of the specificity of the fatty acid synthesizing enzyme system for fatty acid acyl CoA derivatives indicated that odd numbered and branched chain derivatives could substitute for acetyl CoA. In such cases, odd numbered, iso, and anteiso long chain fatty acids were formed.

Incidental to this investigation has been the development of procedures for the non-enzymatic synthesis of β-keto acyl CoA derivatives and for the isolation, separation and quantitation of long chain fatty acids as their hydroxamate derivatives.

Propionate Metabolism. The roles of biotin and vitamin B_{12} coenzymes in the metabolism of propionate have now been established in collaboration with P. Overath, H. Eggerer and F. Lynen of the Max-Planck Institute, Munich, Germany. In studies with enzyme preparations of *Propionibacterium shermanii* it was shown that biotin which is enzyme bound (E· Biotin) serves as the CO_2 acceptor in a reaction equilibrating propionyl-CoA with methylmalonyl-CoA as follows:

$$\text{methylmalonyl-CoA} + \text{E·Biotin} \rightleftarrows \text{Co}_2\text{·E·Biotin} + \text{Propionyl-CoA}.$$

The CO_2·E· Biotin is presumed to be the carboxyl donor for the conversion of pyruvate to oxalacetate in this organism (Swick and Wood, 1959) and may react also with ADP and Pi to produce Co_2, ATP and regenerate E· Biotin (Ochoa, et al.). Vitamin B_{12} coenzyme was identified as a cocatalyst for the reversible isomerization of methylmalonyl-CoA to succinyl-CoA.

Investigations of the mechanism of enzymatic isomerization of methylmalonyl-CoA to succinyl-CoA have been made. Studies on this reaction have been hampered by lack of any model reaction in organic chemistry. Recently, however, the rearrangement of α-cinenic acid to geronic acid has been shown to involve a carboxyl-group transfer. The further demonstration that the rearrangement was actually a transcarbonylation, mediated by elimination of carbon monoxide and its recapture by a resonance-stabilized carbonium ion, has prompted a reinvestigation of the methylmalonyl-CoA isomerase mechanism, particularly since the B_{12} coenzyme for the enzymatic reaction could serve as a carbon monoxide carrier through its cobalt moiety. With lamb kidney enzyme preparations entirely dependent on the addition of B_{12} coenzyme a search was made for formation of $C^{14}O$ from succinyl-1, 4–C^{14}–CoA, or its incorporation into the latter in the presence of methylmalonyl-CoA. The results were negative.

In other studies with partially purified isomerase from *P. shermanii* it was demonstrated that the isomerization of 2–C^{14}-labeled methylmalonyl-CoA yields 3–C^{14}-labeled succinyl-CoA. Thus, it is established that the isomerization involves a migration of the thiolester carbon atom to the β-methyl carbon of methylmalonyl-CoA and eliminates from consideration the idea advanced by others that the isomerization involves intermolecular transcarboxylation reactions.

Metabolism of Onium Compounds

Anaerobic Fermentation of Choline. Acetaldehyde has been identified as an intermediate in the anaerobic conversion of choline to trimethylamine, acetate and ethanol by *Vibrio cholinicus*. The latter two substances are derived from the dismutation of acetaldehyde which is catalyzed by alcohol and acetaldehyde dehydrogenases present in the extract. This dismutation is associated with the esterification of inorganic phosphate. The alcohol dehydrogenase was purified 65-fold and was demonstrated to have an absolute requirement for TPN. The aldehyde dehydrogenase has been purified about 20-fold; it is TPN specific and is maximally activated by addition of CoASH. These observations, and the presence in cell-free extracts of phosphotransacetylase and acetokinase, suggest that phosphate esterification associated with acetaldehyde dismutation involves the intermediary formation of acetyl-CoA and acetyl-P, followed by a transfer of the phosphoryl group from the latter compound to ADP with the formation of ATP and acetate. This phosphate esterification is not inhibited by dinitrophenol; it

therefore probably accounts for the dinitrophenol insensitive portion of the total phosphate esterified during the fermentation of choline by cell-free extracts.

A new organism belonging to the genus *Clostridium* and capable of fermenting choline anaerobically has been isolated. Although considerable variation in the composition of the fermentation products is observed with different cultures, acetaldehyde, acetate, ethanol, trimethylamine and ammonia have been identified as major products. The formation of ammonia is particularly significant since it must reflect a considerable metabolism of the tertiary amino group under anaerobic conditions. The fate of the methyl groups is under investigation.

Sulfonium Compounds. In an effort to determine if the energy associated with the hydrolysis of the sulfonium bond (−21,000 cal.) can be used for cellular metabolism, an anaerobic organism capable of growing on dimethyl-β-propiothetin as the major source of carbon has been isolated in pure culture from soil by the enrichment culture technique. The fermentation balance for the degradation of propiothetin may be tentatively written as follows:

$$3(CH_3)_2\overset{+}{S}-CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3CH_2COOH + 3(CH_3)_2S + CO_2 + 3H^+ + CH_3COOH$$

The possibility that this fermentation involves a primary cleavage of propiothetin to dimethyl sulfide and acrylate which is followed by a secondary fermentation of the acrylate to propionate, acetate and CO_2 is indicated by the fact that cell suspensions are capable of fermenting acrylate to the latter products.

It has been established that cell-free extracts are capable of catalyzing the fermentation of propiothetin but the products have not been determined.

Incidental to this investigation has been the development of a new sensitive analytical method for the determination of dimethyl-propiothetin.

Metabolism of Heterocyclic Compounds

Riboflavin Degradation. It was previously shown that the oxidative dissimilation of riboflavin by an aerobic organism involves the intermediary formation of 1-ribityl-6,7-dimethyl-1,2,3,4-tetrahydro-2,3-diketoquinoxaline. This compound is subsequently converted to 6,7-dimethyl-2,3-quinoxalinediol by an oxygen dependent cleavage of the N-ribityl bond. In order to facilitate further studies on the nature of the cleavage reaction and to establish the fate of the ribityl moiety, 1-ribityl-6,7-dimethyl-1,2,3,4-tetrahydro-2,3-diketoquinoxaline-1•-C^{14} was prepared by a non-enzymatic synthetic procedure. The enzymatic degradation of this labeled intermediate is currently under investigation.

Plausible intermediates in the further enzymatic conversion of 6,7-dimethyl-2,3-quinoxalinediol to 3,4-dimethyl-α-pyrone-6-carboxylic acid and oxamide are 6,7-dimethyl-2,3,5-quinoxalinetriol and 3,4-dimethyl-α-pyrone-6-carboxylic oxamide. Attempts to synthesize the latter substances have been made, but as yet the desired compounds have not been obtained.

In order to simplify further studies on the organism responsible for riboflavin degradation, the factors present in yeast extract and peptone required for its growth have been determined. It has been established that methionine, lysine, threonine, valine, isoleucine and tyrosine are all needed for maximum growth. It was further determined that the degradation of riboflavin is an inducible metabolism in this organism and that ribose but not ribitol will serve as an energy source for growth.

Biosynthesis of Phenazine-1-Carboxylic Acid. The ability of various labeled metabolites to serve as a source of carbon for the biosynthesis of phenazine-1-carboxylic acid by washed cell-suspensions of *Pseudomonas aureofaciens* was investigated. The isotopic carbon from formate-C^{14}, acetate-1-C^{14}, lactate-1-C^{14}, alanine-2-C^{14}, glycerol-1,3-C^{14}, threonine-U-C^{14}, glucose-6-C^{14}, pyruvate-1-C^{14}, serine-3-C-^{14}, tyrosine-U-C^{14}, and tryptophan-3-C^{14} is readily incorporated into the phenazine pigment. On the other hand isotope from glucose-1-C^{14} was not incorporated. Efforts to demonstrate a common precursor role for some of these substances by means of the isotopic competition technique were unsuccessful. Thus, the extent of isotope incorporation with glycerol-1,3-C^{14} was uninfluenced by pools of unlabeled serine,

alanine, or glycine. Efforts to obtain mutant strains blocked in their capacity to synthesize the phenazine pigment were also unsuccessful. Inhibition of pigment formation has been observed with various aromatic compounds such as o-anisidine, aniline, o-phenylenediamine, anthranilic acid, etc.

Regulation of Biosynthetic Pathways

Aspartate metabolism. Regulation of diverse biosynthetic pathways by changes in concentrations of the ultimate products is achieved by two distinct mechanisms: (1) a repression of the synthesis of enzymes involved in the metabolic pathway, and (2) specific inhibiation of the activity of the first enzymatic step in the metabolic sequence. The first mechanism has been referred to as "repression" and the second is known as "feedback" control.

In collaboration with G. N. Cohen of the Pasteur Institute, Paris, France, it has been shown that in *E. coli* aspartokinase catalyzes the first step in a metabolic sequence leading ultimately to the formation of the three different amino acids threonine, lysine and methionine. Its control by either of the above regulatory processes therefore presents a unique situation in which excessive production of one of these amino acids could lead to decreased aspartokinase activity and consequently to a deficiency in the production of the other two amino acids.

Evidence has been obtained showing that in *E. coli* a partial solution to this dilemma is obtained through the synthesis of two different aspartokinases which are independently controlled by the concentrations of L-lysine and of L-threonine. One enzyme is specifically and noncompetitively inhibited by L-lysine and its formation is completely repressed when the organism is grown in the presence of 10^{-2} M L-lysine. The other aspartokinase is specifically and competitively inhibited by L-threonine but its concentration is not significantly influenced by growth in the presence of L-threonine. The biological implications of this discovery are supported by the further observation that in yeast, where the synthesis of lysine does not involve aspartate as a precursor, the lysine sensitive aspartokinase is virtually absent; here the threonine sensitive enzyme is the major if not the only enzyme present.

Metabolism of Amino Acids

Conversion of Phosphohomoserine to Threonine. The mechanism previously proposed for this unique transformation in which alcohol isomerization is coupled with phosphate elimination has now been confirmed. When this reaction is carried out in 100 percent D_2O, exactly 2 solvent hydrogen atoms appear in newly formed threonine. A complete hydrogen degradation of threonine has been developed, and has shown that in all cases solvent hydrogen is incorporated only into the α and β positions. The small incorporation of solvent H^3 previously reported reflects addition of protons to double bond 40 times faster than tritium ions. The study of the mechanism of this unique reaction is still at an early stage. While the coenzyme (pyridoxal-P) can be formulated as potentiating removal of phosphoric acid, shifting of double bonds, and oriented addition of water, neither it nor phosphate can as yet be invoked to account for the different products in the reactions: homoserine $\rightarrow$ α-ketobutyrate, phosphohomoserine $\rightarrow$ threonine.

Sulfur Transfer Between Homocysteine and Cysteine. In an effort to elucidate the detailed mechanism whereby the reversible transfer of sulfur from homocysteine to cysteine occurs in intact microorganisms, and in order to explain the nonreversibility of this process in mammals, the metabolism of cystathionine has been investigated at the enzyme level. An enzyme catalyzing the cleavage of cystathionine to α-ketobutyrate, cysteine and ammonia has been purified 800-fold from extracts of a *Neurospora* mutant believed to lack the ability to form cysteine and homocysteine. More slowly this enzyme catalyzes the conversion of homocystein to α-ketobutyrate and ammonia and of cysteine to pyruvate and ammonia. Qualitative differences between it and the homologous crystalline enzyme from liver are already apparent.

γ-Aminobutyric Acid Metabolism. It was previously established that extracts of *Clostridium aminobutyricum* catalyze the conversion of γ-aminobutyrate to γ-hydroxybutyrate and NH_3. This conversion involves the intermediary formation of succinic semialdehyde by a mechanism which is obligately linked with transamination to α-keto-

glutarate (needed in catalytic amounts) followed by DPN-dependent oxidative deamination of the glutamate formed. This is coupled with the reduction of succinic semialdehyde to γ-hydroxybutyrate. The further metabolism of γ-hydroxybutyrate to acetate and butyrate by dialyzed extracts requires orthophosphate, DPN, TPN and acetyl CoA: presumably, the acyl CoA derivatives of vinyl-acetate, crotonate, β-hydroxybutyrate and acetoacetate are involved as intermediates.

Elsden and his associates have shown that 10 μg of dry bacteria are produced for each μmole of ATP formed during the anaerobic fermentation of various substances by organisms of various kinds. In the present study it has been found that the fermentation of γ-hydroxybutyrate and of γ-aminobutyrate by *C. aminobutyricum* leads to the production of 8 to 9 μg of bacteria per μmole of substrate. It is therefore inferred that one ATP per mole is produced during these fermentations. This is greater by a factor of two than is anticipated from the established route of butyric acid-acetic acid fermentation and suggests that additional phosphate esterification may be available from anaerobic electron transfer reactions.

Reduction Deamination of Glycine. It has been found that exhaustive dialysis of partially purified enzyme preparations of *C. sticklandii* results in the loss of ability to catalyze the reduction of glycine to acetate and the concomitant synthesis of ATP. The activity of such preparations can be restored by the addition of a heat stable factor obtained from the partially purified enzyme. The properties of this material are under investigation.

One Carbon Metabolism

In studies with dried cells of *Methanobacterium omelianski* which catalyze the reduction of carbon dioxide to methane, it could be demonstrated, in collaboration with A. Lezius and F. Lynen, Max-Planck Institute, Munich, Germany, that carbon monoxide, formaldehyde, formate or methanol are not intermediates. CO_2 reduction in the presence of molecular hydrogen follows a long lag period during which time an extremely oxygen sensitive compound is produced. Both CO_2 and H_2 are required for the formation of this substance which, after reaching an optimal concentration, allows methane formation to proceed at a linear rate for hours or until the substrates are exhausted. This lag period is not associated with a deficiency in the hydrogenase system nor is it overcome by the addition of ATP or ATP-generating systems. Once the oxygen labile compound is formed, it can be kept for hours in an atmosphere of pure H_2.

The overall reduction of CO_2 to methane is inhibited by a number of metabolic poisons, notably by arsenate, iodoacetate, arsenite and low levels (10^{-4} to 10^{-5}M), 2,4-dinitrophenol. Inhibition by the latter compound suggests that coupled electron transport phosphorylation occurs during CO_2 reduction to methane. The fixation of $C^{14}O_2$ into the carboxyl groups of pyruvate and alanine by cell-free enzyme preparations was found also to be inhibited by dinitrophenol.

Coenzyme and vitamin analyses revealed that two different strains of methane producing bacteria contain exceptionally high concentrations of biotin and vitamin B_{12} coenzyme. The extraordinarily high level of these vitamins in organisms whose energy metabolism is restricted to the reduction of CO_2 to methane, is inferential evidence that they are catalysts in this biological process.

Lipoate in Metabolism of Lactate

Previous studies by Barker and coworkers have shown that in *Butyribacterium rettgeri* lipoate is required for the fermentation of lactate but not of pyruvate. It is thus suggested that in this organism lipoate is required for a process other than in the oxidation of an α-keto acid. The conclusion is supported by results of the present study showing that lipoate is apparently involved in the conversion of lactate to pyruvate but not in the subsequent dissimilation of pyruvate. The oxidation of lactate to acetate and CO_2 by washed cell suspensions of *B. rettgeri* is obligately coupled with the stoichiometric reduction of oxidized lipoate to the reduced form. On the other hand, lipoate cannot serve as an electron acceptor for the oxidation of pyruvate.

It has been further established that the fermentation of pyruvate by cells grown on lactate (in the presence of lipoate) are quite different. With glucose adapted cells, pyruvate is dismutated to a mixture of lactate, acetate and CO_2 whereas with lactate adapted cells, pyruvate is fermented to butyrate, acetate and CO_2.

Pyruvic Kinase Action

A continuing problem is represented by studies of the pyruvic kinase mechanism, particularly from the point of view of reconciling the abilities of the enzyme to phosphorylate alternatively an enolic oxygen, or inorganic fluoride in the presence of bicarbonate. Perhaps the narrowest way in which these dissimilar reactions could be reconciled would be to place F^- on the enzyme surface in place of C = C–C⁻, and $CO_3^=$ in place of $-CO_2^-$, and further to propose an intermediary acyl phosphate in both reactions. Accordingly experiments were carried out to test this hypothesis, by incubating either pyruvate or phosphoenolypyruvate with crystalline pyruvic kinase in $H^3{}_2O$, in absence of nucleotides. The enzyme was found to catalyze a slow exchange of solvent hydrogen into pyruvate, but none into phosphoenolpyruvate. This result constitutes evidence against the hypothesis.

Biochemistry of the Differentiating Slime Mold

Carbohydrate Metabolism. Apparent changes in the accumulation of glucose-6-phosphate dehydrogenase during differentiation of the slime mold *Dictyostelium discoideum* prompted an investigation, in collaboration with B. Bloom of NIAMD, to ascertain if the routes of carbohydrate metabolism undergo significant changes also. The relative yields of $C^{14}O_2$ from 6–C^{14}-glucose and 1–C^{14}-glucose were determined when these substances were incubated with the slime mold at 5 different stages of development. The ratio of CO_2 derived from the 6 carbon and the 1 carbon atom was 0.7 in the amoebae, 1.3 at pseudoplasmodium and preclumination stages and 0.3 at the fruit stages, thus indicating marked changes in the relative contribution of the hexose monophosphate-pentose-phosphate pathway and and glutamic-pyruvic transaminase were not atvelopment. Three possible pathways to account for the ratio of 1.3 observed at preculmination were dismissed as unlikely because of the inability to detect the required enzymatic steps in cell-free extracts.

Isocitrate and Glucose-6-p-Dehydrogenases. A heat-labile proteolytic-type agent(s) present only in extracts prepared at the amoebae stage of development was discovered to destroy specifically isocitric- and G6P-dehydrogenases. These enzymes could be fully protected by the presence of their substrates. Glutamic acid dehydrogenase and glutamicpyruvic transaminase were not attacked. Knowledge of this differential enzyme destruction as a function of the stage of differentiation has, in conjunction with related studies, suggested the possibility that enzyme activities *in vivo* may be controlled by sequential endogenous substrate utilization (and inhibition), thus accounting for certain metabolic changes occurring during differentiation.

Enzyme Induction

The accumulation of a specific enzyme upon induction could be due in whole or in part to a decreased rate of destruction, as well as to an increased rate of synthesis. In other words, a "basal level" inducible enzyme may be turning over more rapidly than other cellular proteins, and the inducer may act by combining with this enzyme and stabilizing it, thus allowing its accumulation. In an effort to detect possible differences in the turnover of inducible enzymes under conditions of high and low rates of synthesis, a 30 second pulse of highly tritiated leucine was given to exponentially growing *E. coli* cells in the presence, (a) of melibiose, a powerful inducer of β-galactosidase, and (b) of galactose, a poor inducer. The β-galactosidase formed in the presence of melibiose was ⅓ as radioactive as the average cellular protein whereas β-galactosidase formed in the presence of galactose was twice as radioactive as the average cellular protein. These results would be expected if increased enzyme accumulation on induction was in part due to a decreased turnover of β-galactosidase.

LABORATORY OF CHEMISTRY OF NATURAL PRODUCTS

The work of the Laboratory may be summarized in three areas. These are (1) the development and application of new methodology for the study of lipids and related substances, (2) investigations of the occurrence, structure and properties of plant alkaloids, and (3) studies of components of the kallikrein-kallidinogen-kallidin

system, and of the chemistry of human polypeptide vasodilators.

Lipid Methodology

Investigations of lipid metabolic problems important to atherosclerosis studies have been hampered for some time by the poor state of methodology in the field. Work on all aspects of lipids, from basic chemistry to nutrition, advanced very slowly in the past because of this situation. Some time ago this laboratory started work on the development of new laboratory techniques for studying naturally occurring lipids and related substances, with a view to developing new methodology. Significant basic discoveries in gas chromatography had been made earlier in England, and these were taken as the starting point. During 1959 methods were developed for the study of fatty acids and related long-chain compounds including alcohols, aldehydes and bases of the sphingosine type. This work required the development of new column materials for gas chromatographic work, and these column packings are now in rather general use in this country and abroad in both biological research work and manufacturing control laboratories.

The success of these methods in providing a new and important tool for studying long-chain compounds obscured the fact that gas chromatography was in fact more than a specialized tool or a new body of techniques of limited use. Early experiments suggested that steroids and other large molecules were not amenable to separation in this way but these experiments did not distinguish sufficiently between theoretical considerations and problems of technology. During 1960 the problem of extending gas chromatographic methods to the separation of steroids and other complex compounds of natural origin was undertaken. A series of studies was made with thermostable phases not previously investigated. The requirements for supports, for detection systems, for chromatographic columns and for vaporizing systems were investigated. Previous studies with long-chain compounds provided the necessary background of experience. It was found that with new techniques many classes of high molecular weight compounds could be separated. Steroids, alkaloids, vitamins A, D, E and K, many drugs and drug metabolites, and many metabolic products of varied structure can now be separated and studied by gas chromatography. The sensitivity of these procedures, the superb resolving power, and the ability to use complex mixtures of a few micrograms or less in total size (the ultimate sensitivity of current detection systems has never been used) are now well recognized.

Alkaloid Work

Studies of alkaloids of the Amaryllidaceae have been continued. Several biosynthetic studies were started in collaboration with Dr. A. R. Battersby. The biosynthetic work is being carried out primarily in England, in continuation of Dr. Battersby's present studies, and the chemical degradative work will be done here. Current experiments are designed to establish the origin of the ring systems of these alkaloids.

Structural studies were also continued. The structure of buphanamine was established, and additional degradative methods for studying the alkaloids were developed.

Additional supplies of galanthamine were isolated for physiological investigations. This Amaryllis alkaloid was introduced into therapy in Russia in 1958 in the treatment of myasthenia gravis. Several synthetic compounds, derived from the alkaloid, have been prepared for comparisons of activity.

Studies of *Cassia* and *Ormosia* alkaloids resulted in the determination of structures for jamaidine and jamaicensine. These compounds are found in seeds of *Ormosia jamaicensis;* material of Jamaican origin was used in the work. The relationship of the two alkaloids was demonstrated by the synthesis of jamaidine from jamaicensine. The *Cassia* alkaloid cassine is still under study.

The fungal antibiotic pleurotin was isolated from *Pleurotus griseus.* Structural studies are in progress.

Gas chromatographic techniques useful for steroid work were found to be immediately applicable to studies of complex mixtures of alkaloids. A preliminary survey indicated that many groups of alkaloids with widely different ring systems and different functional groups could be separated. An intensive study of two groups was undertaken. The gas chromatographic behavior

of the Amaryllis alkaloids was investigated, since many of these compounds were available from earlier studies. The separations on a non-polar phase (SE–30) were found to be excellent, and it was found that individual alkaloids of the Amaryllis group may be characterized in this way.

Morphine alkaloids were also investigated. It was found that the complex mixture derived from the plant (crude opium) could be extracted and the composition of the extract determined by gas chromatography. A "fingerprint" of the mixture was obtained. This work was carried out with a non-polar phase (SE–30). These results suggest that gas chromatography will become a valuable tool in alkaloid work, both in isolation and structure proof studies.

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.

A kallidin fraction was prepared by the action of human urinary kallikrein on human plasma kallidinogen. Two kallidins resulted when this material was purified. These compounds, kallidins I and II, are similar but not identical polypeptides, and both have vasodilator activity. Current work is directed to the preparation of an additional supply of the polypeptides. Preliminary comparisons have been made with bradykinin, and structural work on the composition of the kallidins has been started.

Studies of human plasma, pancreatic and urinary kallikrein were continued. Partially purified fractions were obtained, and additional work is projected to obtain material of greater purity. These kallikreins are not identical, but presumably each one yields the same kallidin.

Procedures for isolating human plasma kallidinogen are under development. This component of the system is needed to complete the proposed studies of kallidin formation.

LABORATORY OF CHEMICAL PHARMACOLOGY

Development of New Drugs

Reserpine Analogues

It is difficult to maintain a constant clinical effect with reserpine due to its cumulative effect and its instability in the gastrointestinal tract. In collaboration with Ciba Laboratories, reserpine analogues have been developed in which the unstable trimethoxy group is replaced by a methyl group. This ether exists in two epimeric forms (Su 8842 and Su 9064), both of which are stable in the gastrointestinal tract and are completely absorbed. Furthermore, they act reversibly, sedation and effects on brain 5HT lasting only about 8 hours in rabbits. In man the drugs act rapidly and the effects are easily controlled. Preliminary trials in treatment of psychoses look promising. Furthermore, since low doses of drugs cause a persistent depletion of peripheral norepinephrine (NE) without releasing brain 5-hydroxytryptamine (5HT) or evoking sedation, they will also be tested in the treatment of hypertension.

Benzoquinolizines With Reserpinelike Action

Benzoquinolizines, like tetrabenazine, act quickly, producing reserpinelike effects, yet they release only small amounts of brain 5HT. This suggests that they act directly, perhaps by mimicking 5HT in brain, rather than through release of 5HT. A number of sedative benzoquinolizines which do not release brain 5HT are under investigation to see whether they exert typical reserpinelike effects.

Imipramine Metabolite

A metabolite of imipramine, monomethyl norimipramine, has been found to be about 25 times as active as the parent drug in blocking reserpine action. This important lead may provide a highly active compound for treatment of mental depression.

Drugs for Arthritis and Gout

A few years ago, these studies were initiated when it was found that the two metabolites of phenylbutazone isolated in this laboratory were

active in man. Metabolite I exerted a pronounced antirheumatic effect; Metabolite II was strongly uricosuric. Now that Geigy Pharmaceuticals Co. has overcome certain synthetic difficulties, Metabolite I, oxyphenbutazone, has been introduced in Europe (Tenderil) as a potent, less toxic, antirheumatic agent and it will soon be introduced in the United States. Metabolite II led to development of an analogue, sulfinpyrazone (Anturan), now available as an extremely potent uricosuric agent for treatment of chronic gout.

Except for further trials with a methylsulfone analogue, which has an extraordinary half-life, this project is terminated.

Problems of Drug Administration in Long-term Therapy

Long-term therapy creates problems in clinical toxicity, not easy to foresee from usual animal toxicity experiments.

Biochemically Irreversible Drugs

Reserpine and monoamine oxidase (MAO) inhibitors are drugs with actions lasting long after cessation of dosage despite their rapid biotransformation. Since the drug effects are irreversible, they are usually given in small doses over a period of weeks to reach the required effect; large priming doses can produce profound toxic manifestations in susceptible individuals. This poses problems of dosage regulation since the effects of these inherently dangerous drugs are related not to plasma levels but to the extent to which they inhibit biochemical processes. Even with small doses drugs with cumulative effects can produce a delayed toxic action. For example, when Catron (JB 516) was given to dogs in 0.5 mg/kg doses daily it produced lesions in the inferior olivary nucleus or in the pyriform cortex after 100 days of treatment.

Effects of Drugs on Endocrines

Tranquilizing agents (reserpine, chlorpromazine) cause hypersecretion of ACTH and perhaps other pituitary hormones when given in sedative doses. If these compounds, as is likely, stimulate ACTH secretion in man, the biochemical and physiological consequences of this action after long-term treatment become important.

Drug Combinations

The use of drug combinations in long-term therapy has potential difficulties. A number of drugs, MAO inhibitors for example, may depress activity of the liver enzymes that inactivate other drugs. This potential hazard should always be tested especially if the other drug is a central nervous system stimulant or depressant.

One of the two drugs may induce an increased activity of the enzyme that metabolizes the other. As an example, in using the drug combination, phenylbutazone and aminopyrine, phenylbutazone induces an increase in the metabolism of aminopyrine so that the latter drug remains in the body for a very short time. Similarly, barbiturates may speed up the metabolism of anticoagulants and throw control of the coagulation mechanism out of balance.

If both drugs act centrally on amine receptor sites, a combination of drugs can exert effects that neither one causes alone. It has been shown that reserpine given to animals pretreated with a MAO inhibitor produces profound excitation rather than sedation. Perhaps only the fact that relatively small doses of reserpine are given to man has prevented catastrophic effects with this combination of drugs. It has also been shown that animals given an MAO inhibitor followed by imipramine (both in non-toxic doses) develop bizarre symptoms and hyperpyrexia. In patients this combination of drugs has caused peculiar motor signs, circulatory collapse and hyperpyrexia. Finally, the activity of chlorpromazine appears to be markedly increased after pretreatment with a MAO inhibitor. This has also been reported in man.

Biogenic Amines

Serotonin (5HT) and Norepinephrine (NE) in Brain

Using the compound α-methyl-m-tyrosine (MMT) it has been possible to show that reserpine action is associated with the effects on brain 5HT rather than on brain NE. In rats MMT reduces the NE of brain with almost no change in brain 5HT; the animals are not sedated. However, if reserpine is now given to these animals, 5HT is lowered and sedation ensues.

With the new tool, MMT, it has been possible

to show that the amines, released from binding sites and stabilized by a MAO inhibitor, elicit opposite effects in brain. Thus, if a rat is given a MAO inhibitor and then reserpine, excitation occurs as amines are liberated but stabilized. In contrast, if animals are pretreated with MMT, which depletes only the NE, and then given the combination of the MAO inhibitor and reserpine, only 5HT is stabilized in a free form and the animals now show sedation. Other experiments, which involve administration of MAO inhibitors to animals pretreated with reserpine, show that free NE at low levels in brain can elicit all the effects of large doses of amphetamine.

Brain Amines in the Newborn

Levels of brain amines were related to gross behavior in rats of various ages. At birth the amine levels are very low. The levels increase with age and are normal when behavioral patterns are developed. Newborn guinea pigs which have more fully developed behavioral patterns have a normal content of brain amines. The newborn rat does not lack ability to synthesize the amines but is unable to store them in brain.

Imipramine (Tofranil)

Imipramine is a clinically effective antidepressant drug which does not elicit excitation in normal animals or man. It is not an MAO inhibitor nor does it alter brain amines. The pattern of its pharmacologic effects is that of a weak chlorpromazine-like compound. Thus none of present screening procedures would show up this compound as an antidepressant. This action is dramatically evident in dogs and rats treated with reserpine. The following effects of reserpine are prevented by imipramine: depression, miosis, diarrhea, active closure of eyelids, hypothermia, potentiation of alcohol and barbiturate anesthesia, increased activity of central parasympathetic system. The activity of chlorpromazine is not affected by the drug. Preliminary data suggest that imipramine might act centrally by blocking the effects of free 5HT, but much more evidence for this is needed.

The latency in action of imipramine in animals and man has led to tests of one of its metabolites, monomethyl-norimipramine. This substance is about 25 times more active in blocking reserpine action than the parent compound. A dog given reserpine in sufficient dosage to produce coma and death is not depressed and lives if pretreated with 1 mg/kg of metabolite.

The discovery of the antidepressant effects of imipramine suggests that psychotherapeutic drugs with new kinds of activity might be discovered by screening them for the modification of the disturbances produced by other drugs.

Monoamine Oxidase Inhibitors

It had been previously shown that the antidepressant action of MAO inhibitors in animals was associated with the rise in brain NE and not in brain 5HT. It has now been established that this is also true for the new non-hydrazine inhibitors. As a result of this work, a rise in brain NE is regarded as a *sine qua non* for evidence that a compound is a MAO inhibitor antidepressant.

In studies of mechanism by which the inhibitors lower blood pressure, measurement of the postganglionic electrical responses induced by preganglionic stimulation of sympathetic ganglia indicates that the hypotensive effects are not due to inhibition of synaptic transmission.

Hypotensive Drugs, Guanethidine and Bretylium

Guanethidine has been shown to lower blood pressure by depleting NE from peripheral nerve endings. Its action is unusually specific for it does not release peripheral 5HT nor either amine from brain. Bretylium, a quaternary ammonium compound, lowers blood pressure by preventing the physiological release of NE from nerve endings.

Preliminary experiments on the mechanism of action of these compounds were based on the concept suggested by Burns and Rand that postganglionic adrenergic fibers may actually be cholinergic, with acetylcholine at nerve endings liberating NE, which in turn acts on muscle as a local hormone. Bretylium (at higher doses a ganglionic blocker) would then be a specialized cholinergic blocking agent with an affinity for sympathetic nerve endings and guanethidine (at higher doses a ganglionic stimulant) a specialized cholinergic stimulant which depletes NE by releasing acetylcholine at sympathetic nerve endings. Preliminary experiments favor this view since pretreatment of rats with bretylium antagonizes the NE-releasing action of guanethidine.

These drugs should prove useful tools in studies of the acetylcholine-NE relationship throughout the sympathetic nervous system.

NE in Sympathetic Synaptic Transmission

The function of the brain amines is difficult to study since functional units in the central nervous system are difficult to isolate. Since sympathetic ganglia contain considerable NE, the effects of depleting the amine were studied. After ganglionic NE has been depleted with reserpine, the postganglionic response to preganglionic electrical stimulation is greatly exaggerated. Similar results are obtained by the administration of adrenergic blocking agents and by bretylium. In contrast, if free NE is pooled in ganglia by the administration of an MAO inhibitor followed by reserpine, virtually complete ganglionic blockade results until the free amine diffuses away. These results suggest that the transmission across sympathetic synapses is regulated both by acetylcholine and NE and that during cholinergic ganglionic stimulation signals are put out to release NE, which in turn inhibits the effect of acetylcholine on synaptic transmission. This feedback mechanism would serve to prevent wide swings in ganglionic activity which otherwise might occur with changes in sympathetic output. Current investigations are aimed at determining whether NE and 5HT in brain also act by modulating synaptic transmission.

Reserpine Action

It is important to know the precise pharmacology of reserpine since it may serve to map out a neural organization in brain, modulated by 5HT. It has been proposed that such a neuronal system integrates parasympathetic with extrapyramidal and emotional functions (trophotropic system of Hess) and is stimulated by reserpine, whereas an opposing adrenergic system (ergotropic system of Hess) is inhibited by chlorpromazine. Pharmacologically, reserpine acts oppositely to chlorpromazine in that it stimulates central parasympathetic activity; chlorpromazine in contrast depresses central sympathetic activity. These stimulatory effects of reserpine are blocked by chloralose; those of chlorpromazine are not.

Electrophysiological measurements also show the difference between the two drugs. Chlorpromazine has its action on the reticular activating system by blocking stimulation from collateral input. Reserpine in therapeutic doses has its main action on the limbic system, producing a spontaneous electrical activity in the amygdala which spreads to the rest of the limbic system, but not to the neocortex. Chlorpromazine does not exert this action in therapeutic dosage.

Histamine

Although for many years histamine has been recognized as a normally occurring agent with marked pharmacologic effects, the question of its physiologic role remains unanswered. Projects in this laboratory designed to shed new light on this question have led to a sensitive and specific fluorometric method for the assay of histamine and for the enzymes responsible for its metabolism, diamine oxidase and imidazol-N-methyl transferase. It has been established that "histaminase" and diamine oxidase are the same enzyme, but that the diamine oxidases from various organs exhibit measurable differences. Experiments on the release of histamine from rabbit platelets have led to the demonstration that thrombin is an extremely potent histamine releasing agent, and that thrombin generation may be involved in the physiologic release of histamine from platelets.

Studies in Biochemical Behavior

Studies have been instituted to determine how body biochemical reactions are adapted to changes in environment through central nervous activity, and how these control mechanisms are affected by drugs through hypothalamic-pituitary-endocrine function and the autonomic system.

(1) In rats, cold, alcohol and depot ACTH elicit the same rise in plasma corticosterone, plasma free fatty acids, and liver tryptophan-peroxidase indicating that cold and alcohol release ACTH. Depot ACTH, however, produces a much greater rise in liver triglycerides, suggesting that this hormone can also exert an effect on lipid metabolism not mediated through the adrenals.

(2) Reserpine and allied compounds produce hypersecretion of ACTH. They act only in doses that deplete brain 5HT by 50 percent, a dose also required to evoke sedation. The response is not a "non-specific stress" since isoreserpine, which is

non-sedative, does not stimulate the pituitary even in lethal doses. Reserpine in doses of 2 mg/kg depletes pituitary ACTH by 80 percent over a period of 20 hours. Thus published reports that reserpine can prevent responses to stress are explained by the depleted pituitary. This was shown by experiments in which the pituitary ACTH of animals, cold-exposed for 20 hours, was also found to be drastically lowered, and the animals could not respond to a further stress.

(3) Chlorpromazine and other phenothiazines, but only in sedative doses, also cause the pituitary to release ACTH. This is not a "nonspecific" stress since non-sedative phenothiazines do not stimulate the pituitary even after huge doses. Central stimulants like amphetamine stimulate the pituitary only transiently. Thus the tranquilizing drugs produce an effect on the pituitary usually associated with stress.

(4) Studies suggest that the autonomic nervous system as well as the pituitary is involved in drug-induced deposition of triglycerides in liver. Thus ganglionic blocking agents prevent both the alcohol-induced triglyceride deposition in liver and the mobilization of fatty acids from adipose tissue without affecting ACTH hypersecretion.

(5) More than one mechanism is involved in aberrations of lipid metabolism induced by drugs. Carbon tetrachloride, but not alcohol, for example, produces deposition of triglycerides in the liver of hypophysectomized rats. Drug induced changes in lipid metabolism are effected by at least three mechanisms: (a) increased mobilization of free fatty acids from adipose tissue (alcohol, CCl_4 and ethionine), (b) activation of triglyceride synthesis in liver (alcohol), and (c) blocking of triglyceride removal from liver (CCl_4).

Passage of Substances Across Membranes

Membranes Within the CNS

It has been shown that if various sugars such as insulin, sucrose and mannitol are injected into the lateral ventricle they enter the brain through the ependymal lining slightly if at all. However, within 1 hour they have passed completely into the cisterna magna. Although these substances cannot enter the cerebrospinal fluid from plasma, they do pass from CSF into plasma rapidly and all at the same rate. These results indicate that they leave by fluid flow through large pores, probably in the arachnoid villi. It is possible that the rate at which the large molecules leave is related to rate of CSF turnover.

Penetration of Drugs into Cells

As reported previously, basic organic compounds cross the red cell at rates related to lipid-solubility. Organic acids also penetrate the red cell at rates roughly related to lipid solubility, but in addition completely ionized compounds like phenol red readily penetrate. Organic anions probably enter red cells by diffusing through large positively charged pores, the mechanism perhaps resembling that involved in the entry of chloride.

Active Transport Mechanisms

Data obtained in studies of the passage of pyrimidines across everted intestinal sacs (rat) suggest that there must be an OH group in the 6-position of the pyrimidine or purine ring for a compound to interact with the pyrimidine transport mechanism, either as participant or inhibitor. The nature of substituents in the 2 or 5 position seems unimportant. Two antitumor agents, 5-fluorouracil and 5-bromouracil, are actively transported across the intestinal epithelium by the pyrimidine mechanism. These are the first examples of drugs that are absorbed by active transport. Although purines (xanthine, uric acid, etc.) block pyrimidine (uracil) transport, they are not themselves transported.

The transport of pyrimidines is an adaptive process. Starvation of rats, for 4 days, increases the active transport of uracil by about 600 percent without the changing passive component. Hypophysectomy, but not adrenalectomy, also increases active transport by 250 percent. It is possible that lowered food intake is the common factor in starvation and hypophysectomy.

Transport of Catecholamines

The previously reported finding that 5HT is taken up in platelets by active transport has led to experiments designed to ascertain whether neurohormones in general and catecholamines in particular are also held in neurons by a "pump" mechanism. An important finding has been that labelled NE is also taken up by tissue slices by

a mechanism which obeys all the criteria of active transport. This process is blocked by cardiac glycosides and by reserpine. The process is present in brain, heart, kidney and pineal gland and absent from liver and muscle. Calculation of uptake of labeled amine indicates that the endogenous NE is present in tissue in two pools, only one of which is readily miscible with exogenous amine. This fits a picture of "free" amine in neurons maintained by the pump mechanism, and amine in granules bound to some cellular constituent.

A number of drugs inhibit uptake of NE *in vitro*, perhaps by competing for the transport process. These consist of sympathomimetic (cocaine and amphetamine) and sympatholytic (chlorpromazine) agents, depleters of tissue catecholamines (guanethidine) and thyroxine. Most of these compounds are known to enhance the action of administered catecholamines. This suggests that the uptake mechanism is an important means of terminating biological action of catecholamines.

Effect of Ouabain on Phosphatidic Acid

The synthesis of phosphatidic acid in rabbit brain slices is inhibited by ouabain in a concentration as low as 10^{-7} M and is 50 percent inhibited at 10^{-5} to 10^{-6} M. Higher concentrations of ouabain do not inhibit the synthesis of phosphatidic acid any further.

Drug Metabolism

Antimetabolites

6–Chloropurine (6–ClP) and its metabolite, 6-chlorouric acid, markedly inhibit the xanthine-oxidase uricase pathway. Both xanthine oxidase and uricase are blocked *in vitro* at concentrations corresponding to tissue levels *in vivo*. 6–ClP inhibits synthesis of RNA and DNA only slightly but markedly inhibits total lipid synthesis in liver slices.

6-Methylaminopyrine (methylated adenine or MAP) is a purine of unknown function occurring in trace amounts in cytoplasmic RNA. A methylating enzyme in rat liver homogenate transfers a methyl group from labeled methionine to form MAP mainly in cytoplasm.

Microsomal Drug Oxidation

This mechanism continues to command attention since it involves direct utilization of oxygen from air; it may share the same active oxygen donor as cholesterol; and a similar mechanism may hydroxylate steroids to form corticoids. In the last report it was proposed that TPNH + O_2 react with a constituent of microsomes to form a hydroxyl-donor which oxidizes substrate, and in absence of substrate forms H_2O_2. However, kinetic studies now show that the H_2O_2 cannot be formed from the hypothetical hydroxyl donor.

In view of the fact that cholesterol is formed in microsomes by a TPNH-requiring enzyme it may be of some importance with respect to mechanism that triparanol (MER 29) used clinically to block cholesterol synthesis also inhibits the metabolism of many drugs *in vitro*.

Induced Enzyme Formation

The mechanism by which many drugs increase the ability of rats to metabolize the same or closely related drugs is being actively pursued. The increased enzyme activity produced by pretreatment of rats with phenobarbital, phenylbutazone, chlorcylizine (an antihistaminic) and aminopyrine, is paralleled by accelerated drug metabolism in the intact animal and by a shorter duration of drug action. In man phenylbutazone also accelerates the metabolism of aminopyrine, and barbiturates seem to enhance the metabolism of certain anticoagulants. In rats the inductive effects are not prevented by starvation, by adrenergic blocking agents, or by lowered thyroid activity.

Studies with Ascorbic Acid

Many drugs not only induce the activity of drug enzymes in liver microsomes but stimulate synthesis of L-ascorbic acid by mechanisms that do not involve the pituitary-adrenal system. In studies of this mechanism of increased ascorbic acid synthesis it was found that UDPG dehydrogenase, a key enzyme in the series of reactions:

glucose ⟶ uridinediphosphoglucose ⟶ UDPG dehydrogenase ⟶ uridinediphosphoglucuronic acid ⟶ D-glucuronic acid–1–PO_4 ⟶ D-glucuronic acid ⟶ L-gulonic ⟶ L-ascorbic

is stimulated by methylcholanthrene.

In addition, this hydrocarbon increased activity of enzymes which metabolize ascorbic acid. The possibility has been mentioned in other reports that ascorbic acid may have a role in drug metabolism. In accord with this view the muscle relaxant action of Flexin is unusually prolonged in ascorbic deficient guinea pigs due to decreased activity of the Flexin-metabolizing enzyme in liver microsomes. However, other factors such as lowered protein intake might also explain this effect.

Of unusual biochemical interest is the finding that the 5-carbon analogue of ascorbic acid, L-erythroascorbate, can be formed from ascorbic acid in rat liver (decarboxylation of ascorbic to L-xylonic acid, followed by conversion to the ascorbic analog). The role and function of this substance might be of considerable interest.

Some of the symptoms of scurvy in guinea pigs are reversed by 3-methylcholanthrene by an unknown mechanism. These include a marked reduction in incidence of hemorrhage, reduction of bone resorption, preservation of dentinogenesis and prevention of death of odontoblasts.

Chemical Inhibition of Cholesterol Synthesis

Because of the possibility that compounds which inhibit conversion of mevalonic acid to cholesterol might not affect other metabolic processes, the search for antagonists has continued. Three analogues of mevalonic acid, which suppress the incorporation of mevalonic-2-C^{14} into cholesterol in rat liver homogenates, have the following order of potency: 3-hydroxy-3-methylvaleric acid, Δ^2-3-methylpentenoic acid, Δ^3-3-methylpentonoic acid. These antagonists are not particularly potent.

Development of Methods of Analysis

(1) A method for estimation of imipramine and its metabolite monomethyl-norimipramine has been developed. A method which separates these two substances is important since the action of imipramine is probably mediated through the highly potent metabolite.

(2) A method for chlorpromazine which involves oxidation to the highly fluorescent sulfoxide. This should be applicable to humans in therapeutic dosage and may answer some of the vexing problems with this drug.

(3) A method for direct determination of triglycerides in liver.

(4) A new and much more rapid method for determination of ascorbic acid in tissues.

(5) A method has been developed for measurement of cotinine in tissues. Since this is a metabolite of nicotine, and is fairly stable in animals, it might provide a good measure of the amount of nicotine inhaled from cigarettes.

(6) A method for the tissue estimation of Librium, a widely used tranquilizer, has been developed.

LABORATORY OF TECHNICAL DEVELOPMENT

The further development and application of gas chromatographic methods to problems of biochemical and medical research are continuing. Additional information has been acquired on the range and dependability of combined mass and radioactivity detection systems. This has been applied to the solution of specific problems in collaborative research projects with scientists from other laboratories and institutes.

Efforts have been continued to develop the techniques for measuring the quantity and radioactivity of labeled materials utilizing the anthracene column scintillation method previously devised in this laboratory combined with various mass detectors. Its utility in the study of sterols, a situation in which high temperatures reduced the efficiency of the original method, has been extended; the modified method provides a modified collector that can be cooled by drawing through it cool air in addition to the column effluent.

Leakage of radioactivity into the gas stream and penetrating radiation were shown to make the argon ionization detector unsuitable for low level radioactivity assay. A non-radioactive, nondestructive, high-sensitivity detector was required. Special electronic circuits were developed for this purpose.

Application of gas chromatographic methods to a wide variety of biological problems has shown that no single detector is universally satisfactory. Requirements for sample recovery or

for trapping radioactivity in an anthracene column preclude the use of highly destructive electric discharges or radioactive ion sources. Radioactivity detection on anthracene columns must be checked for the presence of quenching materials in the effluent. Some ionization systems require a very low level of organic vapors to operate efficiently and in the case of fluorinated esters of the amino acids, the halogen quenched the ionization so that the RF detector proved more suitable.

These applications have indicated that the most effective use of gas chromatography requires attention to a great many details of physical conditions, as well as consideration of chemical properties. A particular example of simplification by control of conditions was the idea of adding ammonia to the carrier gas to make possible the chromatography of the amino acid esters without N-substitution.

In the course of exploring methods for using an ion chamber as a radioactivity detector, it was noted that unlabeled organic vapor flowing through an unpolarized ion chamber produced currents the sign and magnitude of which were different for different vapors and easily measured by the vibrating reed electrometer. This may be a serious obstacle to application of the ion chamber, but the effect may be applicable as another type of detector for gas chromatography or other applications.

The study of detectors has been extended to the examination of the applicability of the hydrogen flame, as well as other detectors, to liquid-liquid chromatography effluents. Preliminary experiments have shown that it is possible to volatilize the solvent and convey the less volatile solute into the hydrogen flame to utilize the high sensitivity of this device for indicating the presence and quantity of a component coming off a column.

The collaborative research projects carried on with other scientists have made possible the analysis of all but a few amino acids, tryptophan being especially resistant and with arginine and tyrosine yielding multiple derivatives.

A study on the relationship of the composition of serum lipoproteins to the lipids of the dietary intake has been carried out in collaboration with Dr. Kayden at New York University. The results indicate that lipoprotein composition reflects the dietary fat composition for at least a day or two, and indicate the need for rigid dietary control in studies of the circulating lipids.

Several other collaborative projects were carried on as joint efforts and members of this laboratory have participated in the design of the experiments as well as in the adaptation of gas chromatographic methods to the specific problems.

The ultrasonic velocity gas chromatography detector developed in this laboratory has been improved by redesign of the cell. Its performance has been shown to conform with theory. It is unique in that, without destroying the sample, it provides high sensitivity, and very rapid, predictable response.

The application of the electronic frequency response correcting circuit for use with intravascular catheters has been improved by simplification of the procedure by circuit stabilization and development of a square wave test procedure for matching the circuit to the specific catheter.

Analogue devices for analysis of biological problems have been set up in response to specific problems presented by scientists from other laboratories. A method of simulating regurgitation curves partially accomplished last year is now complete. Fourier series, sums of exponential functions and distribution curves are being analyzed with equipment developed in this laboratory.

The development of a general theory of transport phenomena in linear biological systems has been continued and applied to the analysis of experimental data on fatty acid metabolism. The theory is being developed to provide a formal language and method for analysis of a large number of biological problems.

The catheter tip ascorbic acid detector has been perfected. The method now has practical utility in observation of the form of indicator dilution curves for the detection of cardiac shunts and determination of the extent of heart valve regurgitation. Special waveforms, electrode designs and techniques of application are under continued study to eliminate remaining artifacts which stand in the way of quantitative determinations of flow by indicator dilution curves.

In a cooperative project with the Surgery Branch, a study was undertaken to determine the physico-chemical properties of plastics responsible for initiation of clotting or unfavorable tissue response. The contract services of Battelle Me-

morial Institute were engaged to provide pure specimens for *in vivo* testing at NIH; physical-chemical characteristics were determined at Battelle. While there were several encouraging materials in the group tested, no clear pattern of properties has yet emerged. This project was an exploratory program to initiate the application of industrial research technology, through contract, to a pressing biological problem. A university group which has shown considerable interest and competence in the field was encouraged to continue the work.

A practical method for the rapid analysis of micro-milliliter samples of renal tubular fluid is still being pursued. Sample handling and alkali metal recoveries continue to be inadequate. Sensitivity and the possibility of a relatively simple method encourage continued effort to utilize the helium glow discharge to produce the characteristic alkali metal emission.

Phosphorescence excitation, emission spectra, and emission lifetimes have been determined for a series of substituted benzoic acids in acid and alkaline media to show the relationship between structure and phosphorescence. In general, the experimental observations have shown that phosphorescence lifetimes and intensities fall into series that relate to the pi electron distribution. It would appear, therefore, that phosphorescence intensity and lifetime data can be used for characterization. On the other hand the data obtained could be used to predict phosphorescent behavior.

The application of nuclear magnetic resonance to the development of a practical flowmeter has progressed in the direction of improved electronic methods for the reproducible high sensitivity detection of the proton signal. Improvement in sensitivity makes it possible to show flow effects in a 2 mm tube and the detection of trace quantities of paramagnetic salts.

The electronic refinements developed in the course of the flowmeter application are equally applicable to detection of paramagnetic indicators and susceptible nuclei for application to, as yet, undefined problems.

LABORATORY OF CARDIOVASCULAR PHYSIOLOGY

The activities of the Laboratory of Cardiovascular Physiology during 1960 included the initiation of several new and interesting lines of investigation, but it was predominantly a year in which previously initiated, long range projects were brought to fruition. The information gathered has proven to be meaningful and has permitted a considerable degree of systematization and generalization.

Homeometric Autoregulation in the Heart

One type of intrinsic response exhibited by the isolated heart is the well known Frank-Starling mechanism. This endows the ventricles with performance characteristics such that the heart ejects whatever volume is put into it. If inflow is augmented and end diastolic pressure and fiber length are thus increased, the ventricle contracts more forcefully and expels an augmented stroke volume. This occurs on a beat-to-beat basis and may be designated as heterometric autoregulation. This year an intensive study of a second type of autoregulation in the isolated heart, one which apparently does not utilize the Frank-Starling mechanism, was initiated. Homeometric autoregulation, as we have called it, requires at least a few beats to develop fully after an increase in activity (the tension developed per unit of time). The ventricle then exhibits performance characteristics of such a character that its end diastolic pressure and fiber length tend to be maintained at or near the initial level because of an increase in myocardial contractility. The intrinsic ability of the ventricle to rearrange its contractility in this fashion is construed to be a matter of substantial importance in the understanding of cardiac function. Current studies are designed to determine whether, when homeometric autoregulation occurs, a net loss of potassium from the heart takes place and whether this can be reasonably construed as being related to the increased contractility.

The Atrium

Mitral Valve Closure

Further evidence was acquired to indicate that mitral valve closure can be made to occur solely as the result of atrial activity. Data were also obtained indicating that the contractility of the atrium, rather than the level of blood flow, is

the primary determinant when atrial activity produces mitral valve closure.

Left Atrial and Left Ventricular End Diastolic Pressure

Since, at a constant level of sympathetic activity, the stroke work of the ventricle is a function of its end diastolic pressure (fiber length), and since the mean atrial pressure constitutes the central pressure which must be exceeded by returning venous blood, the level of mean atrial pressure necessary to produce any given ventricular end diastolic pressure was submitted to examination. It was found that sympathetic stimulation lowers and parasympathetic stimulation substantially elevates mean left atrial pressure relative to the left ventricular end diastolic pressure. The change with parasympathetic stimulation occurs without any observable effect on ventricular contractility and is the result of alteration in atrial function. These studies have been extended to man by the Cardiology Section.

Catechol Amine Metabolism of the Heart

The observation that cardiac sympathetic nerve stimulation regularly produces measurable increases in the concentration of catechol amine in coronary venous blood was substantiated. With the use of the trihydroxy indole method, the coronary venous output of catechol amine was shown to be a function of the stimulation frequency used.

It was determined that di-isochloroproteranol, which renders the heart unresponsive to cardiac sympathetic nerve stimulation, does not do so by blocking the release of norepinephrine for the latter was readily recovered in large amounts in coronary venous blood under such circumstances. By implication, therefore, the block must occur further down the line.

It was determined that the initial pressor response to bretylium tosylate, a recently introduced antihypertensive agent, can be attributed at least in part to an initial release of catechol amine from the heart, since in the minutes after the administration of this agent, increased amounts of catechol amine were found in coronary venous blood. This finding coincided in time with an observed positive inotropic and chronotropic myocardial effect.

Peripheral Circulation

Carotid Sinus Activity and Oxygen Consumption

Whereas an extremity at rest exhibits a slight decrease in its oxygen consumption when carotid pressure is lowered, the same intervention will substantially increase oxygen consumption in that extremity if it is exercising. This important finding is in line with the view that a major physiologic responsibility of the baroreceptor is to safeguard the biochemical status of peripheral tissues as their activity varies. The pathways (effect on atrial and ventricular function) by means of which this is at least partially accomplished is outlined in last year's annual report and current publications.

The effects of direct sympathetic nerve stimulation indicate that the increased flow in the exercising limb results from a functional sympatholysis, which is a graded curvilinear function of the local oxygen consumption.

Experiments employing the cannulation of lymphatics and the collection of lymph from the lower extremities of dogs at rest and during electrically induced exercise have been begun with a view to ascertaining whether an increase in kallikrein activity occurs in the perivascular space during exercise to account at least partially for the observed functional sympatholysis. This study is still in its early stages.

The Kallikrein System

Further investigation of the pharmacologically active hypotensive proteinases called kallikreins have revealed that the kallikreins derived from a variety of sources hydrolyze those synthetic substrates which contain arginine as the specific amino acid residue. The major portion of the esterase activity in the urine of normal man, as measured by its ability to digest p-toluene-sulfonyl-L-arginine methyl ester, is due to kallikrein. Attempts to differentiate plasma kallikrein from the permeability factor of Miles and Wilhelm or the glass-activated proteinase have been unsuccessful. On the other hand data have been obtained which would suggest that the plasma of patients deficient in Hageman factor are not deficient in kallikreinogen, but rather lacking in kallikreinogenase or some prior enzymatic step required for the activation of plasma

kallikrein. Partially purified human urinary kallikrein has been shown to be antigenic in rabbits and the antibody is capable of inhibiting the vasodilator activity of several of the kallikreins. Two active polypeptides, called Kallidin I and II, have been isolated from human plasma following the action of human urinary kallikrein. Methods have been developed during the year which have made it possible to purify human urinary kallikrein an additional 20-fold; human pancreatic kallikrein, 10-fold; and human plasma kallikrein, 30-fold.

Reflex Factors in Renal Vascular Resistance

The diuresis observed during stimulation of the stellate ganglion led to a series of studies concerning the effect of carotid sinus stimulation on renal vascular resistance. Although this problem has been approached by numerous investigators, the reported data have seemed to be more confusing than enlightening.

Experiments were designed to determine not only whether an increased renal vascular resistance occurs during carotid hypotension but also whether such a resistance increase is exclusively intrinsic (autoregulatory) or is, to an appreciable extent, attributable to sympathetic stimulation. In lightly pentothalized dogs, occlusion of both common carotid arteries produced an increase in calculated renal vascular resistance as measured by clearance techniques. A method was then designed by means of which the blood flow through one kidney could be directly metered without producing any ostensible disturbance of the renal innervation. This was accomplished by hooking an angled occlusive cannula into the lumen of one renal artery after introducing it from below through an iliac artery and then perfusing that kidney at a metered, constant flow before and during carotid artery occlusion. During carotid hypotension renal arterial pressure rose markedly, the increment sometimes exceeding that in the aorta. The responses in this type of experiment have been consistent; sympathetic impulses, reflexly engendered by the carotid sinus, produce substantial changes in renal vascular resistance and blood flow. This study, among other things, suggests that the consequences of a change in carotid sinus pressure itself can influence blood volume.

Pulsus Alternans

Alternating End Diastolic Fiber Length as a Causative Factor

The variety of techniques and preparations now available makes possible a comprehensive and sophisticated analysis of myocardial phenomena. One example in point is the definitive analysis of *pulsus alternans*. Observations on this phenomenon have been made in the dog and cat papillary strip, the isovolumetric, innervated *in situ* dog heart and the isolated, supported dog heart. The data obtained are not consonant with the most popularly held explanations, namely (a) a primary alternating impairment of contractility from beat to beat; (b) an alternating deletion of fractionate contractions, and (c) an alternation of the excitation mechanism. The data do indicate that, when *pulsus alternans* occurs, the weaker beat (WB) can be accounted for on the basis of a shorter initial fiber length from which the WB initiates. The shorter fiber length prior to the WB is due to an inadequate period for diastole; this does not allow time for complete relaxation and/or a comparable period for filling. This limited diastole is due to the large percentage of the total cycle time (larger stroke volume) required for systole in the previous, stronger beat (SB). The WB has a shorter systolic period due to its smaller stroke volume. This allows a longer period for relaxation and filling and thus a longer initial fiber length to be acquired prior to the SB. Thus *pulsus alternans* occurs when myocardial contractility is continuously depressed relative to the existing heart rate and stroke volume. When, under these circumstances, contractility is increased (shortening of systole), as by norepinephrine, stellate stimulation or homeometric autoregulation, *pulsus alternans* abates.

Chemoreceptors

The Carotid Body

This laboratory has made a major research investment in the investigation of baroreceptors as sense organs which reflexly influence the circulation. More recently attention has been turned to the chemoreceptors. Investigations of the reflex effects upon the heart and systemic circulation of stimulation of the carotid bodies with hypoxic and hypercapnic blood were made in

the open-chest dog. The carotid sinus-body area was independently perfused using a double disc oxygenator system which permitted rapid change from blood of one gas composition and pH to that of a different composition and pH without changing perfusion pressure. Continuous measurements of the pO_2 (polarographic) and pH of the perfusing blood were made. Caudal and cephalic blood flow were separately measured utilizing a Potter electroturbinometer and a Shipley-Wilson rotameter respectively. Left ventricular end diastolic pressure, aortic pressure and heart rate were simultaneously recorded. Puzzlingly, bradycardia is the primary heart rate response to carotid body stimulation confirming the findings of deBurgh Daly and Scott. Preliminary data suggests that this negative chronotropic effect is due principally to parasympathetic activation but that, in addition to the pathways found by deBurgh Daly and Scott, sympathetic withdrawal may also be present. These data permitted construction of ventricular function curves relating left ventricular end diastolic pressure and stroke work at constant heart rate (electrically paced). The relative effects of increased peripheral vascular resistance and changes in ventricular contractility, and the roles of neural and humoral factors in these responses are being investigated.

Rise in Arterial Pressure Following Occlusion

The rise of arterial pressure following occlusion of the celiac and mesenteric arteries in the cat under chloralose has previously been ascribed, on the basis of previous work in this laboratory, to a pressoreceptor reflex. It now appears that, at least for the time being, this position must be revised. The results of a variety of more recently conducted experiments which included completely isolating the splanchnic vascular bed, both with and without cross perfusion, and the recording of action potentials, indicate that both mechanical and reflex factors are operative. The occlusion of vessels *per se* increases the peripheral resistance. Also, blood runs off from vessels distal to the occlusion into the active circulation and so tends to increase heart output by acting as an infusion. Pressor reflex effects due to local irritation as ligatures were tightened on arteries were regularly found, but when these were excluded in cross perfusion experiments, lesser reflex pressure changes could still be elicited by arresting the flow in splanchnic vessels. Whether pressoreceptors, chemoreceptors or ischemic pain receptors are involved remains to be established.

Mammalian Myocardium

Some progress has been made in the study of isolated strips of heart muscle. The less complicated geometry and the element of control (shortening vs. tension) possible has afforded the opportunity to examine more precisely the kinetics of contracting myocardium with a view to a better understanding of changes in contractility.

Simultaneous length-tension measurements were made in dog and cat papillary muscle. It was found that in myocardium the intensity of active state can increase, the force-velocity curve can be changed, and thus the intrinsic rate of contraction changed in accord with varying conditions, allowing increased force of contraction without systolic prolongation. Force velocity and maximal work curves shifted to the right with increased initial length, increased Ca^{++} and norepinephrine; to the left with increased K^+ or acetylcholine. Using a modified quick release method (Ritchie), three phases of active state were determined: 1) *Intensity*, by redeveloped dp/dt. 2) Duration of *Full Intensity*, by departure of redeveloped dp/dt from initial dp/dt. 3) *Decay*, by decline of redeveloped dp/dt. Increased initial length, increased Ca^{++} and decreased K^+ increased intensity without essential change in duration of active state. Increased rate and norepinephrine increased intensity while shortening duration. Decreased temperature (30°–15° C.) prolonged duration and decay with little change in intensity. Dichloroisoproterenol blocked norepinephrine effects, but not rate staircase or Ca^{++} effects. These data demonstrate that in myocardium, unlike skeletal muscle, variations in the intensity and duration of active state are experimentally dissociable.

Shortening and Tension Development in Heart Muscle

It is hoped that the less complicated geometry of the isolated heart muscle strip will make it possible to gather data on the relation of myocardial oxygen consumption to tension in a situation in which the undetermined variable due to

radius of curvature (La Place) in the intact ventricle would not be present. Progress has been blocked by the lack of a sufficiently stable and sensitive pO_2 electrode. It now appears that this instrumentation problem has been overcome.

LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM

The mechanism of bicarbonate reabsorption in Necturus is being reinvestigated. It is now generally assumed that bicarbonate reabsorption in both the proximal and distal segments of the kidney is effected by an exchange of hydrogen ions derived from the tubular cells for intraluminal sodium ions with the resultant formation of carbonic acid in the tubule lumen. The carbonic acid is thought to be converted to CO_2 and water, the CO_2 escaping by diffusion. It is implicit in the theory that the rate of formation of CO_2 is equal to the rate of bicarbonate reabsorption. It has been pointed out that the intraluminal concentration of H_2CO_3 necessary to provide the driving force for the noncatalyzed dehydration of H_2CO_3 to CO_2 and H_2O at the required rate would result in an intraluminal pH approximately 1 unit less than that when equilibrium is achieved. Alternatively were the reaction catalyzed by carbonic anhydrase residing in the luminal membrane the *in situ* pH would not necessarily be depressed but administration of carbonic anhydrase inhibitors should lower rather than elevate it.

It is not possible to evaluate these alternative possibilities by determining the pH of fluid removed from the tubule lumen for measurement, since subsequent equilibration of CO_2 and H_2CO_3 in the measuring receptacle by noncatalytic means would raise the final pH and mask any *in situ* differences. For these reasons much of the effort in the past year has been directed towards the development of an appropriate micro pH electrode which would permit the *in situ* determination of pH in the proximal tubule fluid of Necturus. Thus far this has proved to be difficult and only a few pH-sensitive micro electrodes have been developed. In preliminary studies, no clearcut evidence of acidification in the proximal tubule of Necturus has been observed, suggesting that the dynamic pH (*in situ*) does not appreciably differ from the equilibrium pH. If confirmed, this observation would lend support to the view that the dehydration of carbonic acid within the tubule lumen is catalyzed by carbonic anhydrase residing in the tubule cell membrane.

Certain aspects of phosphate transport in the dog kidney, a process intimately related to urinary acidification, are being reinvestigated. Although net phosphate secretion in the chicken has been previously reported from this laboratory, it had generally been agreed that phosphate is not secreted in either dog or man. In recent years, however, several studies have appeared in the literature in which net secretion of phosphate was reported to have occurred in the dog. Since in none of these studies was the filtered phosphate load maintained at a reasonably constant level, the evidence is inconclusive. In order to establish the presence or absence of net phosphate secretion in the mammal, the renal excretion of phosphate is being studied under steady state conditions in a variety of experimental situations which include most of those previously reported to have been associated with the observation of net phosphate secretion. Neither prior administration of parathormone for a variable period of time, prior administration of parathormone and superimposed mannitol diuresis, prior loading with sodium phosphate for three days before study, or acute lowering of the glomerular filtration rate, has resulted in the excretion of phosphate in excess of that filtered.

An investigation of the effects of the infusion of ammonium salts on electrolyte excretion in the kidney has been completed. It had been observed that the administration of ammonium chloride into the renal portal venous circulation of the chicken resulted in a predominately unilateral increase in sodium chloride and water excretion on the injected side, as well as a rise in the excretion of ammonia. This effect has been shown to be due to the ammonium ion itself, since it occurs with the ammonium salts of sulfate, acetate, nitrate, as well as chloride. Equivalent acidosis produced by hydrochloric acid does not result in similar changes in urinary composition. The natriuresis is also independent of the rate of ammonia excretion. It occurs when the ammonia excretion is minimized by prior alkalinization of the urine. Although in all

studies net potassium secretion diminished in association with the fall in sodium reabsorption, no change in net hydrogen ion transport was observed. The mechanism of the response to ammonium salts is unclear. It is possible that the effect is secondary to interference with potassium uptake by the renal tubule cells. NH_4^+ ion may substitute for K^+ on the contraluminal Na-K exchange pump. It should be noted that substitution of ammonium ion for potassium on the sodium-potassium exchanger has been suggested on the basis of analogous studies in the red cell in other laboratories.

A number of studies of the mechanism of urinary dilution and concentration are in progress. The effect of one of the benzothiadiazine diuretics, chlorothiazide, on urinary dilution and concentration in the dog has been investigated. These agents diminish the rate of free water excretion during water diuresis in both man and dog. It has been assumed that this is a consequence of interference with the reabsorption of sodium and chloride in a water-impermeable segment of the renal tubule. No analogous studies during the elaboration of hypertonic urine have been reported. The present studies have also indicated that the decrement in solute-free water excretion when chlorothiazide is administered can be quantitatively accounted for by the increment in NaCl excretion suggesting that the major site of action of this drug is at the site within the renal tubule at which removal of sodium and chloride leads to dilution of the urine.

The relationship between negative free water and solute excretion over a wide range of solute excretion has been examined in hydropenic animals pretreated with vasopressin. Results obtained during the administration of chlorothiazide have been compared with those during the injection of nonabsorbable solute such as mannitol. At low rates of solute excretion, negative free water clearance is unaltered by chlorothiazide. In contrast, at high rates of solute excretion, a uniform increase in negative free water clearance occurs when chlorothiazide is administered. Since massive solute diuresis induced by nonpharmacologic agents is associated with a progressive fall in negative free water clearance, the rise observed with chlorothiazide under these circumstances may be indicative of the delivery of a more concentrated solution from the distal convolution to the terminal concentrating site. In this view the drug does not exert a primary effect on the terminal concentrating mechanism.

The effects of this agent in patients with nephrogenic diabetes insipidus have also been examined. It had previously been reported that the benzothiadiazine diuretics not only increased urine osmolality in patients with diabetes insipidus but also diminished urine flow. Although the increase in urine osmolality is clearly the result of a diminution in sodium transport in the distal convolution (see above) this single action can not account for the associated antidiuretic response. Oral administration of either chlorothiazide or hydrochlorothiazide to four patients with vasopressin resistant diabetes insipidus resulted in a uniform increase in urine osmolality though never to a concentration exceeding that of plasma, and a 30–40 percent diminution in urine flow. The effect was considerably greater when the patients were maintained on a low sodium intake than when maintained on a high sodium intake. Furthermore, the antidiuretic response could be maintained despite withdrawal of the drug if the restricted sodium intake was continued throughout the course of the study. A reversal to the polyuric phase was uniformly observed when sodium was readministered in the diet. On the basis of these observations, it has been suggested that antidiuresis produced by chlorothiazide is a consequence of a reduction in body sodium content. The fall in sodium content reduces volume flow to the distal portion of the nephron either by a reduction in filtration rate or by an increase in proximal reabsorption. If this interpretation is correct, salt depletion produced by other agents should also result in an antidiuretic response in these subjects. This possibility is being examined at present.

In association with the above studies, the effect of chlorothiazide has been examined in adrenalectomized rats. It has previously been suggested by others that the antidiuretic and chloruretic effect in diabetes insipidus is secondary to interference with the renal action of aldosterone. The conclusion is based largely on the report that chlorothiazide is ineffective in adrenalectomized rats. In contrast, the present studies indicate that the diuretic response does not differ in normal animals and in adrenalectomized animals maintained on an adequate salt intake.

Further experiments relating to the concentrating mechanism are being pursued in an attempt to develop a method for the estimation of renal medullary blood flow in the intact animal. It has been established that medullary and papillary tissue of the kidney is hypertonic to plasma. The maintenance of this concentration gradient within the medullary and papillary tissue is dependent upon the unique anatomical arrangement of the vascular tree within the deeper portions of the kidney, sequestered solute being effected by countercurrent exchange of water and solute between the opposing limbs of the vessel. In order to measure one of the variables affecting the efficiency of the countercurrent exchanger and thus the concentration of the urine, the possibility of applying a modified Fick principle for the estimation of medullary flow is being investigated utilizing hydrogen as an indicator.

Net water movement and the unidirectional fluxes of tritiated water through a mesityl oxide membrane have been measured. In contrast to prediction, the flux ratio of water through mesityl oxide, a presumably nonporous organic membrane, deviates significantly from the activity ratios. The possibility of droplet movement through the mesityl oxide has been eliminated by comparing the flux of sodium ^{24} with that of water. Since it is apparent that neither active transport of water nor bulk flow occurs through this membrane, the discrepancy between the flux ratio and activity ratio is inexplicable though it points up the difficulty of accepting the usual criteria for bulk movement at face value. Preliminary measurements of the water content of the mesityl oxide equilibrated with water of various activities have indicated a significant departure from linearity between the water activity and the water content of the equilibrated mesityl oxide. If this nonlinearity is assumed to persist at the two interfaces of the model membrane this may explain the unexpectedly high flux ratio observed.

Studies of water and solute movement through toad bladder have been initiated using isolated halves of toad bladder suspended as closed sacs in test solutions. In the absence of vasopressin net water movement in response to an osmotic gradient established with sodium chloride, mannitol or urea, is approximately equal. In the presence of vasopressin, however, net transfer of water from a dilute urea solution within the bladder is greater than from comparable solutions of mannitol or sodium chloride. Furthermore, net water gain into hypertonic solutions of urea is less than into equivalent concentrations of mannitol or sodium chloride. These differences are dependent upon the associated rapid penetration of urea through the bladder wall presumably via aqueous pores in contrast with the minimal penetration of either mannitol or sodium chloride.

Studies concerning the mechanism of active cation transport in human red blood cell ghosts have been continued, correlating the activity of the sodium pump with the activity of a specific membrane, "ATPase." The latter is presumed to be involved in some fashion in the active transport of cations. The effect of various substrates and other agents on the ATPase activity (as measured with isolated membranes permeable to ATP) has been compared with active sodium transport (measured in reconstituted ghosts). It has been observed that both sodium extrusion, an estimate of pump activity, and ATPase activity, specifically require ATP. Other high energy phosphate compounds were ineffective in both systems. Both sodium transport and ATPase activity were inhibited by sodium fluoride and copper chloride, but neither was affected by iodoacetate, sodium azide or sodium arsenate. The action of strophanthidin was similar in both systems. Both systems require magnesium and calcium inhibits both the ATPase activity and sodium extrusion. These comparative studies provide further evidence in support of the view that an ATPaselike enzyme residing within the cellular membrane is involved in the active transport of sodium and potassium across the red cell wall.

The characteristics of an ATPase isolated from the renal cortical tissue of dog, guinea pig and rat are being studied. Thus far the studies are in a preliminary form and the precise conditions for maximal activation of the enzyme system have not yet been developed.

A reliable automatic coulometric-amperometric titration method for chloride analysis has been developed; in the past year the method has been

further improved. The analysis of chloride in a large variety of biological fluids has been undertaken in order to demonstrate its validity as a useful biochemical method. It has been applied in a project designed to characterize the distribution of chloride across tissue cells in nephrectomized rats. According to the simplified Conway hypothesis, appreciable amounts of chloride should shift into the muscle cell in the presence of hyperkalemia and acidosis. To test this hypothesis the distribution of chloride and of isotopically labeled inulin has been compared in nephrectomized rats with severe acidosis and hyperkalemia. In the two studies completed the ratio of inulin to chloride spaces was 0.95 in the hyperkalemic animal, a value not significantly different from that observed in muscle tissue of normal rats. According to the Conway hypothesis the ratio should have fallen to about 0.75 in the presence of the observed degree of hyperkalemia.

An analysis of the myocardial chloride content in experimental cardiac failure in the dog has been completed. The increment in water and chloride content in heart muscle of dogs with congestive heart failure can be accounted for solely on the basis of an expansion of the extracellular space of this tissue. It is apparent that these alterations in water and electrolyte content do not constitute the primary defect responsible for the heart failure though they may in all probability contribute to a further depression in myocardial function.

An infinite thickness liquid counting method utilizing the gas flow detection chamber has been developed in order to facilitate the estimation of C^{14} labeled inulin. Data obtained using this method have confirmed results obtained in this laboratory a number of years ago which provided evidence that inulin clearance does not vary with changes in plasma inulin concentration; the use of radioactive inulin has extended the range of plasma concentration an additional order of magnitude lower.

Investigation of the role of a cardioglobulin system in mammalian plasma has been continued. It had previously been reported that cardioglobulin exerts a positive inotropic effect on the isolated frog heart. Using this as an assay it has been shown that the cardioglobulin content of human plasma is elevated in hypertensive disease, markedly depressed in some patients with idiopathic myocarditis, and may be acutely lowered during the course of extracorporeal perfusion of blood. The present efforts are directed at determining the role of this protein system in the mammal. For this purpose the cardiovascular effect attendant upon depletion of cardioglobulin in the rat has been assessed. Chronic cardioglobulin depletion by exchange transfusion has proved virtually impossible, as a result of the extreme rapidity of the regeneration of the protein. Although it has been possible to demonstrate an acute diminution in cardioglobulin concentration in rat plasma, a return to normal values occurs with 24 hours. The acute cardiovascular effects of administration of cardioglobulin-free plasma to the rat have thus far proved to be negligible, in part due to methodological difficulties. In acute studies administration of cardioglobulin rich plasma to the failing rat heart-lung preparation does not affect cardiac performance as estimated by alterations in heart rate, blood pressure, blood flow through a shunt and venous pressure. Further experiments are planned in order to verify this conclusion.

The mechanisms regulating the secretion and metabolism of aldosterone in dogs with secondary hyperaldosteronism have been studied. A reexamination of the role of the arterial baroreceptor in the control of aldosterone secretion has been completed. On the basis of these studies, it may be concluded that the baroreceptors in the aortic arch and the carotid arterial tree are not essential to either the hypersecretion of aldosterone or the almost complete sodium retention observed in dogs with inferior vena caval constriction, and further, that marked reductions in pulse pressure in the carotid arterial system and in the mesenteric arterial tree fail to increase the rate of aldosterone secretion. More recently, in collaboration with Drs. Anderson, Haymaker and Spense of NIAMD, a role of the midbrain in the regulation of aldosterone excretion in the same experimental preparation has been excluded. Thus, following midbrain transaction, acute hemorrhage results in a striking increase in adrenal vein aldosterone secretion in dogs with vena caval constriction.

The response does not differ appreciably from that observed in vena caval dogs with intact midbrains. It has also been shown that the adrenal and kidney transplanted to the cervical region, though devoid of nervous connections, respond in the "normal" fashion to acute hemorrhage. Aldosterone secretion increases and sodium retention supervenes in the cervically transplanted organ, despite an absence of both nervous connections and an increase in venous pressure in these organs.

The most interesting observation made in the past year in relation to this problem has been the demonstration that the kidney releases an aldosterone stimulating hormone which signals the release of mineralocorticoids from the adrenal. In the absence of ACTH, enhanced secretion of aldosterone from the adrenal in dogs following acute hemorrhage is considered presumptive evidence for secretion of aldosterone secreting hormones. In a series of successive ablation experiments, removal of the anterior pituitary, removal of the head, of the liver, etc. was generally followed by an increase in aldosterone secretion in response to acute hemorrhage. In contrast, removal of the kidneys prevented the increase in aldosterone secretion generally attendant upon acute blood loss. Furthermore the intravenous infusion of saline extracts of kidney resulted in a marked increase in aldosterone production. These data provide conclusive evidence for the renal origin of an aldosterone stimulating hormone.

The aldosterone stimulating hormone derived from kidney does not appear to be renin. Experimental hypertension in the dog is not associated with increased rates of aldosterone secretion. Renin administration does not augment aldosterone secretion although injection of hypertensin II may in some instances stimulate its release. An associated increase in corticosterone secretion due to hypertensin II, not generally seen following hemorrhage in vena caval dogs, suggests that the response to hypertensin II is not analogous to that due to aldosterone stimulating hormone of renal origin. More recently it has been shown that there is a marked fall in aldosterone secretion following nephrectomy in hypophysectomized dogs with constrictions of the inferior vena cava. This is additional support for the role of the kidney in the elaboration of a tropic hormone.

LABORATORY OF CLINICAL BIOCHEMISTRY

Amine Biogenesis and Metabolism

It is now certain that there is one enzyme, designated as aromatic L-amino acid decarboxylase, that is responsible for decarboxylation of all the normally occurring aromatic L-amino acids including 3,4-dihydroxyphenylalanine, 5-hydroxytryptophan, tryptophan, tyrosine, phenylalanine, histidine and kynurenine. Substrate-enzyme studies have been carried out for all these amino acids. In addition to this general amino acid decarboxylase there is also found a specific L-histidine decarboxylase. This means that there are at least two different catalysts for converting the dietary amino acid histidine to the potent pharmacologic agent histamine. In comparing the two mechanisms for histidine decarboxylation, it was found that the specific enzyme is present in tissues that are rich in mast cells (mast cell tumors) ; it acts only on L-histidine, has the higher affinity for histidine (K_m 10^{-4}M) and has a pH optimum near 6. It is not inhibited by α-methyl DOPA. By contrast histidine decarboxylation by L-aromatic amino acid decarboxylase occurs in organs containing few mast cells, the K_m is nearer 10^{-2}M, pH optimum is about 8.5 and the activity is inhibited by low concentrations of α-methyl DOPA.

The significance of these two separate mechanisms for histidine decarboxylation is not apparent. However, it has been suggested by Dr. Richard Schayer, that there is a marked increase in histidine decarboxylase in some tissues (up to 10 fold) during conditions of stress. Studies on the mechanism of this apparent enzyme induction are now in progress.

Inhibitors of aromatic L-amino acid decarboxylase were studied further and it has been found that the two most potent ones, α-methyl DOPA and α-methyl metatyrosine, have two actions in animal tissues *in vivo*. The first action apparently results from its known enzyme-inhibiting activity and leads to a fall in the serotonin and dopamine concentration of brain. However, 24 hours after a single dose of about 100 mg/kg the concentration of these two amines has returned to normal. On the other hand, the concentration of noradrenaline in heart and brain falls to values

less than 10 percent of normal and remains low for periods of several days. Various experimental procedures indicate that the α-methyl amino acids prevent the binding of noradrenaline by tissues. It would appear, therefore, that they act in a manner similar to reserpine with the important difference that their effects are limited to noradrenaline. This property makes them extremely valuable as tools in studying the functions of brain amines since it is possible to prepare animals which have normal amounts of brain serotonin and dopamine but little noradrenaline. This is essentially the situation 24–30 hours after a single dose of α-methyl metatyrosine in mice, rats, guinea pigs and rabbits (100–200 mg/kg). It is of interest that after an initial short period of apparent sedation the animals appear perfectly normal. Studies on the mechanism of the antihypertensive action of these agents in man by the Section on Experimental Therapeutics are complementary to those in this laboratory. In addition, members of the Laboratory of Chemical Pharmacology have used the α-methyl amino acids in their studies on the physiology of the amines.

One of the consequences of the existence of a nonspecific L-amino acid decarboxylase is that amine derivatives of all of the normally occurring amino acids should be formed. This appears to be the case and phenylethylamine, tyramine and tryptamine have been shown to be excreted in the urine. Ortho and meta tyramimne are also found although it is not yet known how they are formed. Other interesting amines which have been found in urine are synephrine and norsynephrine (p-hydroxyphenylethanolamine). The former, which may be considered as adrenaline with one less ring hydroxyl group, is found in urine in amounts which are comparable, and frequently greater, than that of the normetanephrines. Since synephrine is a fairly active pharmacologic agent, its presence in such amounts may have physiologic significance. However, of more biochemical interest was the indication that the side chain oxidation, such as occurs in the conversion of dopamine to noradrenaline, can occur with other compounds. It was subsequently found that purified preparations of the enzyme, dopamine-β-oxidase, oxidize tyramine to norsynephrine and that the enzyme is therefore not specific for dopamine. The finding of the N-methylated compound in urine indicates that the enzyme noradrenaline N-methylpherase is also nonspecific. It appears, therefore, that most of the enzymes involved in aromatic amine biogenesis are nonspecific. This nonspecificity explains the presence of the numerous aromatic amines and their metabolites in urine.

An important route of metabolism of serotonin leads to the pineal gland hormone, melatonin (N-acetyl-5-methoxytryptamine). In conjunction with members of NIMH it has been shown that in the pineal gland serotonin is first acetylated and then methylated to yield melatonin. The two enzymes have been partially purified and studied. The methylpherase is specific and is found only in the pineal gland. Melatonin which leaves the pineal gland is metabolized in the liver by the microsomal hydroxylating system to yield 6-hydroxymelatonin.

Amine metabolism is also carried out by nonspecific enzymes and many products are to be expected in urine. Methods for the assay of the o-methylated metabolites of the epinephrines and of their acid end product, 3-methoxy-4-hydroxymandelic acid, have been developed. These are specific and precise. Assays of a similar type are applicable to norsynephrine and p-hydroxymandelic acid. All these assays are now being used in collaboration with the Section on Experimental Therapeutics to study these amine pathways in man, normally and in various disease states. In the case of noradrenaline two major routes of metabolism are possible, oxidative deamination and o-methylation. Studies on animals *in vivo* indicate that only in the liver is o-methylation the major route of noradrenaline metabolism. In brain, heart, and other organs the major route is oxidative deamination. This means that noradrenaline synthesized and released within an organ is metabolized by monoamine oxidase, only that fraction which leaks out into the blood is methylated on passage through the liver.

The enzyme, monoamine oxidase (MAO) has now been obtained in soluble form from mitochondria and has been purified at least 10 fold from this source. This represents a considerable advance in purification. It has been found that the purified enzyme splits N-dimethyl amines into the corresponding aldehyde and dimethylamine and that N-dimethylamine oxides are deaminated in the absence of oxygen. It may be

that MAO catalyzes a direct oxidative attack on the nitrogen and that the N-oxides are intermediates. While a few N-oxides have been isolated from natural sources no enzymatic studies of this type have ever been carried out. Other oxidative deaminases such as L-amino acid oxidase, D-amino-acid oxidase and diamine oxidase are really dehydrogenases, the oxygen coming from hydrolysis of an intermediate imino compound. In the case of MAO, oxidation must then occur through atmospheric oxygen. Experiments with O^{18} are now in progress to verify this point.

Choline Biogenesis

The biogenesis of choline has been studied in animals. Although serine is the ultimate precursor it was not possible to demonstrate direct decarboxylation of this amino acid to yield free ethanolamine. These studies indicated, rather, that serine is incorporated into phospholipid and in this form is decarboxylated to phospholipid-ethanolamine (cephalin) and, still in lipid form, is methylated through intermediate mono and dimethyl forms to phospholipid choline (lecithin). All the methyl groups arise from the methyl groups of methionine. Utilization of one carbon sources such as formate or formaldehyde occurs only by prior conversion to methionine methyl groups. Overall synthesis of choline from serine, in phospholipid form, also follows from the work of Dr. David Greenberg and collaborators at the University of California. Such a pathway may have great physiologic significance. Conversion of a dimethylamine to a quarternary amine in a phospholipoprotein structure would be a most effective way to alter the charge on a membrane and thereby regulate transport mechanisms. In liver the entire series of reactions occurs in the microsomal portion of the cell. It may be that they are involved in determining the lamellar structure of the endoplasmic reticulum.

Aminobutyric Acid Metabolism

Further studies on γ-aminobutyric acid (GABA) metabolism were carried out. The major work has been on the identification of the GABA-histidine containing peptide in brain as γ-aminobutyryl histidine (homocarnosine). It represents the GABA analogue of carnosine. The latter is found mainly in muscle and other peripheral organs and does not occur in brain. Homocarnosine is found only in the central nervous system and urine. Methods for assay of the several carnosines have been developed and brain levels have been measured in many animal species. In man, monkey and cattle, brain contains as much as 30 μg/gram. Although this is considerable it represents only 5 percent of the total GABA content. The unique localization would appear to be indicative of the availability of β-alanine or GABA in the various tissues rather than of enzyme specificity. When significant GABA levels were maintained in peripheral organs (by including GABA in the diet) appreciable amounts of homocarnosine appeared in muscle. Normally it is not found there. These findings corroborate experiments of Meister concerning the lack of specificity of the enzyme, carnosine synthetase. The significance of homocarnosine in brain remains to be determined. Its presence in urine may provide another measure of central nervous system chemistry since it is normally formed only in brain.

Amino Acid Transport

Studies on the uptake of aromatic amino acids by various tissues *in vivo* and *in vitro* have been continued. L-Tyrosine uptake by brain *in vivo* unquestionably involves a mechanism of facilitated transport. It has now been found that it has a most rigid stereospecificity. In previous studies it was found that following administration of D-tyrosine some tyrosine appeared in brain, though much less than with L-isomer. It has now been found that the tyrosine entering brain after administration of D-tyrosine is all in the L-form indicating that racemization had taken place before penetration into brain. The D-form does not pass the "blood brain barrier." Little or no distinction between uptake of D and L-tyrosine occurs in other tissues. These and other findings suggest that the same anatomical area which is considered as the "blood brain barrier" also possesses mechanisms to facilitate the transport of essential metabolites of the central nervous system. Such findings make it necessary to point out what should have been obvious before this, that the "blood brain barrier" theory

requires the existence of mechanisms of active transport *at the same site*, to explain the uptake of metabolites with physical properties comparable to those which are usually prevented from penetrating. Although much of the mechanism for facilitated transport of L-tyrosine resides in the "blood brain barrier" and can be demonstrated only in the intact animal, brain slice uptake of tyrosine is also unique. Brain slices concentrate tyrosine as much as 3 fold over the incubation medium. To do this they require an energy source which can be supplied by glucose, mannose and related compounds. Metabolic poisons inhibit this uptake. Muscle, liver, spleen and kidney slices do not concentrate tyrosince. In rat diaphragm muscle all the uptake of L-tyrosine can be explained on a diffusion mechanism. These differences between brain and other tissues in uptake of L-tyrosine no doubt extend to many other amino acids and metabolites and it will be of interest to examine other metabolites in the same way.

Mechanisms of Aromatic Hydroxylation

Some additional studies have been carried out on the mechanism of aromatic hydroxylation. Using tritium labelled phenylalanine (ring) and T_2O it was found that the hydrogen in the para position of phenylalanine does not become labile in the presence of phenylalanine hydroxylase. An intermediate substrate activation should have yielded exchange between the para hydrogen of phenylalanine and water. This did not occur. Similar studies are being started with acetanilide labelled with tritium in the para position. In this case microsomal aromatic hydroxylase will be used.

Collagen and Hydroxyproline

Studies on collagen biosynthesis and hydroxyproline formation have been extended. The presence of rapidly turning over forms of collagen, suggested by studies on urinary hydroxyproline have been confirmed by direct studies on tissue collagen. This confirms our previous conclusion that although body collagen as a whole is metabolically inert there are small pools of collagen which are in the dynamic state as are other tissue constituents. Some of these findings are being explored in man by the Section on Experimental Therapeutics. Studies on chick embryos have shown that collagen (hydroxyproline peptide formation) occurs in microsomes and its requirements are similar to those of other protein syntheses. In preliminary studies it has been possible to demonstrate "collagen" formation *in vitro* in microsomal material isolated from chick embryos.

Studies have been initiated to investigate the metabolic relationship between proline, hydroxyproline and ketoproline found in the various actinomycin molecules. Micro-procedures for the isolation of the different actinomycins were devised. In an initial study with C^{14} labeled L-proline it was found that the actinomycins synthesized by washed suspension of *S. antibioticus* were isotopically labeled. Moreover, hydroxyproline was found to contain appreciable radioactivity. Formation of the actinomycin chromophore, 2-amino-4,6-dimethyl-3-phenoxazinone-1,9-dicarboxylic acid, has been achieved using cell-free extracts of *S. antibioticus* and 3-hydroxy-4-methylanthranilic acid as substrate. In addition, the cell-free system will condense a number of other ortho-aminophenols for example, 3-hydroxykynurenine, 3-hydroxy-4-methylanthranilic acid methylester and 3-hydroxyanthranilic acid to form phenoxazones. The purification and properties of this enzyme system are now under investigation. It may be possible to achieve an overall enzymatic synthesis of the proline and hydroxyproline actinomycin chromopeptides.

GENERAL MEDICINE AND EXPERIMENTAL THERAPEUTICS BRANCH

Section on Clinical Endocrinology

The research program of the Section on Clinical Endocrinology has included studies on (1) aldosterone metabolism in edematous states, (2) calcium metabolism in metabolic bone disease, (3) renal function, with special reference to free water clearance, and (4) experimental atherosclerosis.

(1) Studies on aldosterone metabolism included clinical studies on the role of aldosterone in fasting, in primary and secondary aldosteronism, in idiopathic edema, and in potassium depletion,

both spontaneous and experimentally induced. The role of the kidney and of various pressor agents in the regulation of aldosterone secretion has been explored in dogs.

It was confirmed that the sodium loss with fasting is greater than that with sodium deprivation alone; in some patients this was associated with failure of aldosterone secretion to rise with salt loss, but these patients lost potassium with fasting, and the relative hypoaldosteronism may have been a result of this. Eight patients with primary aldosteronism were studied before and after surgery. The use of a low sodium diet preoperatively allowed restoration of potassium deficits and an increase in aldosterone secretion. The same result was seen with aldosterone antagonists, which thus might increase aldosterone secretion in primary aldosteronism (by promoting potassium retention) as well as in secondary aldosteronism (by inducing sodium loss).

The expansion of intravascular volume with albumin, on the other hand, did not lower aldosterone secretion in primary aldosteronism as it does in secondary aldosteronism. Direct measurements of blood volume in patients with edema showed an inverse relationship of intravascular volume and aldosterone secretion; a similar inverse relationship related arterial pulse pressure and aldosterone secretion. Methodology for determination of aldosterone was improved by introduction of C_{14} aldosterone biosynthetically prepared, and of a "visible" aldosterone marker. Studies on gas chromatography of aldosterone were initiated.

A study of the possible role of diurnal rhythms in the cause of edema was instituted by extensive "mapping" of diurnal rhythms in normal female subjects. Aldosteronism was found associated with hyperplasia of juxtaglomerular apparatus in a normotensive boy, and the role of the kidney in control of aldosterone secretion was studied in extenso in dogs. Nephrectomy was found to reduce or abolish the response of aldosterone secretion to caval constriction in the hypophysectomized dog. Contrary to reported findings, this response was found to occur in hypophysectomized dogs both with and without suprapontine brain removal. Hypertensin (Angiotensin II) and renin were shown to induce aldosterone secretion in the hypophysectomized, nephrectomized animal, and did so in some preparations.

(2) Studies on calcium metabolism and metabolic bone disease included clinical balance studies in osteoporosis, in renal osteitis, in sarcoidosis, and in hyperparathyroidism, studies on renal phosphate clearance with special reference to parathyroid function, studies of labile calcium in the parathyroidectomized dog, and studies in the metabolism of vitamin D.

Patients with idiopathic osteoporosis (defined as "idiopathic" by an absence of the response of calcium balance to estrogens or androgens) were studied to ascertain the mode of action of albumin in inducing positive calcium balance and to test the effect of strontium. The possibility of a purely oncotic action of albumin could not be ruled out; strontium appeared to be without effect. Patients with renal osteitis were found to absorb little or no calcium from the gastrointestinal tract, as judged from balance studies and the fecal excretion of orally fed calcium[47]. Vitamin D markedly improved calcium absorption, but aluminum hydroxide did not.

Patients with sarcoidosis were found to absorb abnormal amounts of calcium from the gastrointestinal tract, to increase this absorption further with vitamin D, and to lower it markedly with carbohydrate-active corticosteroids even in the presence of vitamin D. Blood levels of vitamin D were never elevated, suggesting hypersensitivity to, rather than hyperabsorption of, vitamin D.

Renal phosphate and calcium excretion were studied in patients with hyperparathyroidism and in normal control subjects. Normal subjects could not be distinguished from those with hyperparathyroidism on the basis of either maximal reabsorption or clearance of phosphorus, but phosphate excretion in response to a calcium load was abnormal in hyperparathyroidism, as was renal calcium excretion on very low phosphorus intake. Both phenomena could be reproduced in normal subjects with chronic administration of parathyroid extract.

The role of the parathyroids in the renal excretion of phosphate was studied with clearance techniques in dog and man. No evidence for tubular secretion of phosphate could be found, despite phosphorus loading, acidosis, and use of parathyroid extract. Clearcut tubular maxima were found, and considered to be evidence against the possibility of secretion.

Parathyroidectomized dogs were found to have decreased amounts of readily labile bone calcium, as judged from dynamic response of extracellular fluid calcium to versene-induced hypocalcemia. Parathyroid extract and vitamin D could restore labile calcium to normal and, with higher doses, to above normal. H_3 vitamin D was prepared for us by a commercial firm, and C_{14} vitamin D will be prepared in the near future. Chromatographic systems for isolation of vitamin D have been developed, and *in vivo* and *in vitro* studies of its metabolism and fate have been begun.

(3) Studies on renal function included measures of free water clearance in patients with edema and in patients with hyponatremia, and studies with Amphotericin B.

Free water excretion was studied in patients with cirrhosis and compared with that in patients with cardiac failure and in normal subjects depleted of sodium. Results indicate that in all these conditions free water excretion is limited, not because there is persistent or excessive secretion of antidiuretic hormone, but because there is excessive reabsorption of salt and water in the proximal tubules. Whereas this appears to be aldosterone-dependent (as judged from the response to aldosterone antagonists) in the patients with cirrhosis as in the normal subjects, it appears not to be aldosterone-dependent in some patients with cardiac failure: in these patients, the filtration fraction appeared to be above normal: catechol amine antagonists had no effect in decreasing proximal sodium reabsorption.

Further studies were carried out in the syndrome of hyponatremia resulting from "inappropriate" secretion of antidiuretic hormone. The syndrome was uncovered in two patients with intermittent acute porphyria. Some fifteen cases with pulmonary or intracranial diseases have been uncovered in various clinics and are the subject of a review in preparation. The syndrome could be reproduced with pitressin in normal subjects, either by deliberate choice of fluid intake or by voluntary *ad libitum* drinking.

Amphotericin B was found to induce reversible decrease of glomerular filtration rate and renal plasma flow in patients receiving the drug for therapeutic purposes.

(4) Studies in experimental atherosclerosis included measures of the rate of penetration of labeled cholesterol and of labeled albumin into the aorta of the dog. It was found that both substances entered more rapidly in the proximal and progressively less rapidly down the distal aorta. The gradient for albumin does not depend upon pulsatile pressure or upon absolute circumferential tension, but the rate does appear to be pressure-dependent. Further studies, including measurement of lipoprotein in the aortic wall, are in progress.

Section on Experimental Therapeutics

The investigative approach of this laboratory to clinical cardiovascular problems is a combined biochemical-pharmacologic one. For convenience the studies are described under four headings: (1) biogenic amines, (2) amino acid metabolism, (3) action and metabolism of drugs, and (4) miscellaneous.

Biogenic Amines

Using improved methods, the urinary excretion of catecholamines (norepinephrine plus epinephrine), their methoxy-amine metabolites (normetanephrine and metanephrine) and 3-methoxy-4-hydroxymandelic (MOMA) was measured in 23 patients with pheochromocytoma and in a large group of patients with essential hypertension. For purposes of separating patients with pheochromocytoma from the rest of the hypertensive population, a suitable upper limit of normal for each assay was found to be 0.1 mg/day, 1.3 mg/day and 6.0 mg/day respectively. It was determined that the production of catecholamines as indicated by urinary products is not elevated above normal in essential hypertension.

The fact that the excreted methoxy-amines repsents a much smaller fraction of the metabolites in normals and hypertensives than does MOMA supports the idea that oxidative deamination is the initial mode of metabolic degradation of that norepinephrine formed at nerve endings. Studies in the rat tend to support this concept. Monoamine oxidase activity of brain and heart was found to be several times that of catechol-0-methyltransferase. Endogenous norepinephrine accumulated when monoamine oxidase was inhibited by drugs; exogenously administered norepinephrine accumulated in the heart under the same conditions. Neither of these effects could

be produced by inhibition of catechol-0-methyl transferase.

The vascular response to various sympathomimetic amines is being compared with the effects of these agents on blood unesterified fatty acid (UFA). While norepinephrine alters both variables, equipressor doses of dopamine in patients were found not to provoke an UFA response.

In collaboration with Dr. J. Pisano (LCB), N-methyl - p - hydroxy - phenylethanolamine (synephrine) was isolated and identified in human urine. That tyramine may be the precursor of synephrine of norsynephrine is indicated by the finding of elevated amounts of p-hydroxy-mandelic acid in the urine after infusion of tyramine in man. The overall metabolism of tyramine and the physiologic significance of the synephrines are under investigation.

A sensitive and specific chemical assay for histamine in human urine has been developed. Normal values are in the range of 20 to 100 μg/day. In a unique case of the carcinoid syndrome arising from a primary lesion in the stomach, characterized chemically by the excretion of large amounts of 5-hydroxytryptophan, serotonin and 5-hydroxyindoleacetic acid in the urine, urinary histamine was 545–580 μg/day.

Amino Acid Metabolism

It has been established that urinary hydroxyproline (HPr) arises primarily from the breakdown of body collagen, and that the amount excreted is a useful index of collagen synthesis and degradation. There is a progressive decrease in the amount of urinary HPr in successive decades of life, presumably reflecting the "metabolic age" of an individual's collagen. The possible application of this finding to gerontologic problems as well as to selected disorders of connective tissue is apparent.

Studies of the inhibition of aromatic amino acid decarboxylation by α-methhyl-3,4-dihydroxy-DL-phenylalanine (α-methyl-dopa) have been extended. Inhibition by this agent of the formation of dopamine, tryptamine, tyramine, serotonin and phenylethylamine from their corresponding amino acids in man has been shown. That the decarboxylase step of serotonin synthesis is specifically attacked was demonstrated in two carcinoid patients by the observation of an elevated excretion of 5-hydroxytryptophan (5HTP) coincident with treatment. Administration of α-methyl-dopa to two patients with pheochromocytoma resulted in a slight (20 percent) but probably significant reduction in the excretion of catecholamine metabolites. Decarboxylase inhibition with α-methyl-dopa was shown pharmacologically in the dog by blockade of the inotropic response to L-Dopa and in man by decreased intestinal motility response to 5–HTP. Several additional inhibitors have been synthesized in the Merck, Sharpe and Dohme laboratories and are in various stages of animal and clinical evaluation.

Action and Metabolism of Drugs

In studies with eight different monoamine oxidase (MAO) inhibitors, we have observed an orthostatic hypotensive effect rather uniformly in hypertensive subjects when evidence of effective MAO inhibition was demonstrable. The latter is indicated by increases in the urinary excretion of amines such as tryptamine and tyramine. Inhibitors containing the hydrazine moiety have frequently produced toxic effects in man at the doses required to lower blood pressure. Thus the preliminary finding that N-benzyl-N-methyl-2-propynyl-amine HCl (MO–911) is an effective antihypertensive agent and potent MAO inhibitor in man is of special interest. Also, since the structure of this agent is different from that of previous inhibitors, the evidence of a causal relationship between MAO inhibition and blood pressure lowering seems fairly conclusive. Nonetheless, the exact mechanism is undetermined. Neuropharmacologic experiments in dogs and cats have shown a sympathetic ganglion blocking action of several inhibitors; however, this effect occurs only with large doses and is rather evanescent. Possibly only a slight modification of ganglionic function is sufficient to produce postural hypotension in man. Among other possibilities, peripheral and/or central accumulation of an amine such as dopamine could result in competitive block of norepinephrine action.

It is claimed that MAO inhibitors benefit patients with angina pectoris. A rational basis for this might be the observations in two patients receiving the inhibitor, isocarboxazid, of obtunded rises in pulse rate and blood pressure during exercise. This implies diminished cardiac response during exertion.

In 40 hypertensive patients treated for six weeks to fifteen months with α-methyl-dopa, initial sedation followed by subtle tranquilization and lowering of blood pressure which is predominantly orthostatic have been observed rather uniformly. The potency of the compound is enhanced in patients with impaired renal function and it is uniquely effective in emergency control of severe hypertension particularly if used intravenously. Recent availability of the L isomer (Aldomet) has largely solved the problem of high daily intake of capsules for maintenance therapy. Broad evaluation of this agent as an antihypertensive is currently in progress in 16 clinics. Only three of eight carcinoid patients have shown symptomatic relief with α-methyl-dopa. Development of hypotension has often prevented attainment of effective dose levels. However, decarboxylase inhibition appears to be a sound approach to treatment of carcinoid, in part because it now seems doubtful that it is a basis for development of hypotensive drugs. Biochemical studies in rats and guinea pigs (LCB), pharmacologic studies in dogs, and combined studies of drug metabolism and blood pressure responses in patients all suggest that α-methyl-dopa has an activity above and beyond decarboxylase inhibition. For the moment, it is assumed that a selective depletion of tissue stores of norepinephrine accounts for the lowering of blood pressure. Clinical studies of other decarboxylase inhibitors, some with and others without norepinephrine-depleting properties, will be initiated shortly.

Section on Cardiodynamics

This Section has as its ultimate objective the study of the biophysical and physiologic behavior of the cardiopulmonary system of normal and diseased human subjects as they go about their usual daily activities and are subjected to various physiologic, psychic, pharmacologic, and other stressful interventions. The measurement of a large number of physiologic variables under conditions most nearly simulating normal activity has made the development of new methods of instrumentation essential. Progress in this broad area has and will continue to depend on a vigorous program in instrumentation development as well as a comprehensive study of the biomathematical models of the vascular and pulmonary systems. Thus, the major efforts of this Section have been directed along three lines: (1) instrumentation, (2) biophysics of the vascular system, (3) biophysics of the pulmonary system.

Instrumentation. The needs for improved instrumentation have made necessary a continuous instrument evaluation program. Most of the efforts in this area have been toward the development of a data processing and computing unit. Commercially available equipment has been modified to meet the biological and biophysical requirements of the Section. Many technical problems have been solved so that there is now available an efficient, smooth working, physiological recording system which permits the acquisition of multiple channels of physiological information from animal or man. These data may be recorded on conventional direct writing recorders, and at the same time stored by an electromagnetic FM tape system. Uniquue methods of programming the electromagnetic tape system to work either with an analogue to digital converter or with an analogue computer have been worked out. For example, a given time increment of information, such as one heart beat, may be searched out and programmed to play repetitively for study and computation.

Pressure measurement has remained one of the most important unsolved problems, and therefore, new techniques are being sought. Several miniaturized pressure transducers have been investigated, one of which has shown considerable promise. However, none has been found sufficiently accurate for the more precise biophysical measurements. An extravascular pressure sensing device is being developed in conjunction with the Astra Corporation. This device can be surgically implanted in animals, and, it is hoped, will open the avenue to accurately telemetered blood pressure from living, intact, active animals. This information has not been available previously, and if successful, the attempt to measure it will be a major step toward the accomplishment of the primary objective.

The measurement of instantaneous blood flow in the living intact organism has remained one of the primary objectives. Studies in this area have been carried out in mechanical models and in the living animal. Experimental studies with a mechanical flow generating device have revealed

that the relationship between the instantaneous pressure gradient in a cylindrical tube and the instantaneous pulsatile flow is such that it may be predicted mathematically with reasonable accuracy. The velocity profile was examined by the introduction of blue dye strands into the pulsatile flow and indicated that in the range of physiologic frequencies the velocity profile across the tube is blunt, with most of the fluid shear occurring at the boundaries of flow. The more peripheral sleeves of flow appear to be in phase with the pressure gradient, while the more central portions of the flow appear to be in quadrature with the pressure gradient. This finding is qualitatively in agreement with mathematical prediction.

In the animal, blood velocity has been compared from the simultaneous recordings from a Kolin electromagnetic flowmeter and the computed pressure gradient technique. Agreement of peak blood velocities by the two methods is quite good.

Cardiovascular Biophysics. The viscoelastic properties of the living vascular tissue have been studied. The results indicate that the pressure-diameter relationships of almost all blood vessels, in the systemic as well as pulmonary system appear to be described to a first approximation as a simple elastic system, particularly for the lower frequencies of pulsation. It would appear also that the longitudinal motion of blood vessels may be neglected as a boundary condition assumption in the solution of the hydrodynamic equations of flow.

The instantaneous blood velocity, pressure, pressure gradient and vessel radius are being measured and recorded on an electromagnetic tape system so that computation of the various terms of the Navier-Stokes equations may be accomplished. Preliminary evidence indicates that a large majority of these terms may be neglected. Therefore, it is reasonable that a relatively simple, yet realistic equation describing the pressure, flow, diameter relationships in the vascular system can be established.

In order to study the theoretical aspects of heart function, it is necessary to determine the nature of the load against which the heart must work. To this end, studies are being carried out to examine the input impedance of the pulmonary artery and the aorta. This is done by measuring simultaneously the instantaneous blood flow and pressure at the input of these two major vessels. These curves may be broken in to their respective Fourier series. Each term in the series may be represented by a complex number. The ratios of the corresponding harmonics of pressure and flow permit the computation of the complex impedance. The results to date indicate that the input impedance to the pulmonary artery consists of a very large real term and a relatively small imaginary term. Although the interpretation of this finding is somewhat involved, the major impedance to blood flow into the pulmonary artery seems to be related to vascular resistance. This is in contrast to the systemic side in which large reactive components of impedance appear.

Pulmonary Biophysics. Theoretical considerations have been developed which indicate that abnormal stress distribution within the structure of the lung may be the major factor in the production of the disruptive lesions of emphysema. The abnormal stress distribution could be the result of either congenital or acquired abnormalities of the smaller terminal air passages. Therefore, studies have been undertaken to examine the relationship of stress to strain in three dimensions in excised lungs. By using an improved intraesophageal pressure measuring system, the intrathoracic pressure is being measured simultaneously at three different sites along the esophagus. The difference between oral pressure and the intraesophageal pressures has been studied under static conditions in normal subjects. It has been possible to describe this relationship as a function of balloon volume, balloon position, and lung volume. The relationship seems to be independent of the rate of change of pressure or of the amplitude of pressure changes. This initial part of the project, which is of interest in itself, is preliminary to studying the pressure differences between the three balloons and the conditions of air flow, varying rates of respiration, and rapid respiratory maneuvers such as cough. If it can be demonstrated that there are significant differences in the stress distribution in the lung of normal individuals, as compared to those with chronic bronchitis, evidence is gained for support of the theory that the disruptive lesions of pul-

monary emphysema may be the result of abnormal physical stress.

New methods of studying the mechanical behavior of the lung have been developed. A unified three dimensional presentation of the three major variables controlling the lung behavior in health and disease has been developed in this laboratory and used to examine normal, cardiac, and emphysematous subjects. From these considerations a unique relationship between the maximum achievable expiratory flow and the degree of lung inflation has been discovered. Theoretical considerations of this unique flow-volume relationship have far reaching physical and physiological implications. Therefore, this relationship is being extensively studied in normal human beings. Theory would indicate that this relationship is controlled by the density and viscosity of the gas as well as the dimensions and physical properties of the intrathoracic airways. If it can be shown, experimentally, that the effect of either density or viscosity on this relationship was of minor importance, it would greatly simplify the mathematical description of this curve. Preliminary findings indicate that the viscosity of the gas breathed has very little effect on the maximum achievable expiratory flow at any given degree of lung inflation. This finding is in sharp contrast to the effect of gas density. If these preliminary findings are verified, it may be possible to establish a realistic and useful mathematical model of the lung from which the flow volume curve may be analyzed mathematically.

SURGERY BRANCH

The investigative projects of the Section on Clinical and Experimental Surgery of the Surgery Branch have, as in past years, centered largely around methods for improving the surgical treatment of patients with congenital or acquired heart disease and in elucidating the physiologic factors which apply before, during and following the operative correction of such lesions. An important group of studies has continued to center around the clinical application of the artificial heart and lung machine. The artificial heart and lung machine itself has not been modified in the past year since it had been found to provide adequate support of the circulation for periods of up to two hours. It may be of interest that the machine has been accepted for commercial production.

Perhaps the most pressing clinical problem in the field of cardiovascular surgery is the surgical correction of mitral and aortic regurgitation. For the past two years, efforts in both the experimental laboratory and in the operating room have been directed at the perfection of a prosthesis suitable for total replacement of the mitral valve. Valves have been constructed with a dacron fiber base covered with a thin layer of polyurethane foam. They have been implanted into five patients with irretrievably damaged valves. Although no patient is presently living with a total valve replacement, the valve was in place in one patient for more than four months. Autopsy examination revealed that this valve was free of clot, covered with a thin layer of fibrin and had remained mobile. This result is sufficiently encouraging that further refinements in the design of the valve are being made in the hope that it will ultimately prove useful for routine clinical practice. An important part of the problem of prosthetic valve replacement, whether for the aortic or the mitral valve, is a choice of material. It is particularly difficult to evaluate materials when valves are constructed and placed in experimental animals since the technical aspects of the operation itself are so formidable. Accordingly, various fabrics such as dacron, silk, silastic, pericardium and nylon have been implanted into the wall of the right atrium in dogs. A study of the behavior of these materials in this location will undoubtedly indicate that certain fabrics are more desirable than others in valve construction.

In the prosthetic mitral valve which has been employed clinically, artificial chordae tendinae are necessary to prevent eversion of the valve leaflets from the ventricle into the atrium. An experimental study is underway to evaluate the ideal material for such artificial tendinae. Strands of various materials, such as stainless steel, silk, silk covered with plastic, etc., have been passed through the substance of the heart so that the fibers traverse the cavity of each ventricle. In addition, various methods have been employed to fix the strands to each ventricular wall. Preliminary observations indicate that clotting is primarily related to the point at which the

strand pierces the endocardium and that the substance of the strand may be of lesser importance. Fixation of the strands by mechanical means has been found inferior to direct suture.

In the clinical treatment of patients with various types of congenital and acquired heart disease principal efforts in the past year have been directed at surgery of the mitral and aortic valves. Continuing experience with the open surgical correction of calcific aortic stenosis, with followup catheterization information has revealed that in the majority of patients simple commissurotomy is not effective in relieving left ventricular outflow obstruction unless aortic regurgitation is produced. Accordingly, a portion of the aortic valve has been replaced with a prosthesis, ordinarily of woven teflon cloth, in an increasing number of patients. It soon became apparent that the prolonged period of aortotomy necessary in these operations could not be provided by coronary perfusion alone. For this reason selective hypothermia of the heart, induced by perfusion of the coronary vessels with refrigerated blood and a slush of ice and water around the left ventricle has been adopted. Utilizing this technique, safe periods of cardiac arrest up to two hours have been achieved. A study comparing the effects of cardiac hypothermia and coronary perfusion on ventricular function has been initiated.

One year ago two patients with functional hypertrophic subaortic stenosis were subjected to operation. It is impossible to resect the obstructing muscle mass and in these patients the lesion was treated by a vertical incision or myotomy across the contraction ring within the left ventricle. Although the initial hemodynamic results seemed encouraging, the procedure was not again used until these patients were studied one year later. At this time each was found to have virtually complete relief of the obstruction. Since increasing numbers of patients with this form of aortic stenosis are being seen, it is likely that this new operation will prove of increasing value.

Previous studies have indicated that it is advisable for every patient undergoing open heart surgery to be digitalized beforehand. There has been much conjecture as to whether or not extracorporeal circulation removes digitalis from the heart muscle and whether patients after open operations should receive additional digitalis. An experimental study was undertaken in which animals were digitalized and their digitalis level titrated before and after cardiopulmonary bypass. Preliminary work indicates that digitalis is fixed in heart muscle and no decrease in its effective level could be demonstrated after bypass. The work will be continued, more precisely, with the use of radioactive digitalis preparations.

It has been occasionally noted in patients undergoing prolonged cardiopulmonary bypass that oliguria or anuria occurs. Although a high level of free plasma hemoglobin is frequently present following long perfusions there has been no correlation between the occurrence of a high plasma hemoglobin level and the occurrence of renal complications. In an experimental study, p-aminohippurate and creatinine are being utilized to measure renal plasma flow and glomerular filtration rate in dogs in which the kidney is supported by an extracorporeal circulation. By means of this preparation the effects of pure hemoglobin and red cells debris on renal function may be evaluated.

In many clinics extracorporeal circulation is presently being used concurrently with deep hypothermia, heat exchange being accomplished in the extracorporeal circuit. It has been generally assumed that as the body temperature falls blood flow to muscle masses is decreased while that to the "vital organs" remains high or is increased. Experimental studies have been undertaken to measure the total blood flow and the vascular resistance of various circulatory beds during profound hypothermia. It has been a surprise to learn that at 10°C. the flow to the lower extremity is increased while that to the brain and splanchnic bed is lower than normal. Continuing investigations concerning the changes in hepatic blood flow under these circumstances are underway. When profound hypothermia is used, ventricular fibrillation is an almost invariable complication of the technique and in clinical practice large doses of quinidine and sometimes procaine amide have been given to prevent this arrhythmia. The direct myocardial effects of these agents were not known and, accordingly, left ventricular function curves were constructed in dogs in which quinidine had or had not been given. It was found that quinidine produced a marked depression of left ventricular function and a profound fall in mean arterial pressure

indicative of peripheral vasodilation. It would seem that the protective effects of quinidine are outweighed by these undesirable side effects.

Fifteen patients have now been studied in whom left ventricular outflow obstruction was found to be due to massive hypertrophy in the outflow tract of the left ventricle. An attempt to produce this lesion experimentally was made by constriction of the ascending aorta in puppies. These animals have now been followed for one year and all have been found to have massive hypertrophy of the outflow tract of the ventricle and in several a pressure gradient within the left ventricular cavity has been found at retrograde arterial catheterization. Although this experimentally produced lesion is probably not akin to the asymmetric hypertrophy most common in patients, it does provide an experimental tool by which operative treatment of this unusual form of aortic stenosis may be evaluated.

The complete anatomic correction of certain congenital lesions such as severe tetralogy of Fallot and true truncus arteriosus will necessitate replacement of the pulmonary artery. In a previous study, single pulmonary arteries were replaced with grafts of plastic material and this work has been extended to permit total replacement of the main pulmonary artery and its major branches. It has been found that the length of the rigid graft is of critical importance but that when this parameter is controlled, that dogs can survive for prolonged periods with such a prosthesis not containing a valve.

While the heart and peripheral circulation are physiologically separated during cardiopulmonary bypass, a unique opportunity is afforded for studying the various direct myocardial and peripheral actions of many pharmacologic agents. The effects of various pressor amines and digitalis have been described and these studies suggested that a detailed investigation of certain anesthetic agents would be of value. In a study carried out in conjunction with the Department of Anesthesiology the anesthetic agent Halothane was introduced into the oxygenator for a period of five minutes. In six patients it was shown that there was immediate and progressive depression of myocardial contractility and a concomitant fall in systemic blood pressure indicating peripheral vasodilation. The effects of carbon dioxide and other gaseous agents on the heart and peripheral circulation can also be studied by this method and such projects have been initiated. The clinical observation was made that, following operations necessitating occlusion of the thoracic aorta, paradoxical hypertension following restoration of the circulation was far more frequent when Halothane anesthesia was employed. In an experimental preparation the aorta was occluded in dogs under either nitrous oxide-oxygen or Halothane anesthesia. Postoperative hypertension was uniformly noted when Halothane was employed. This action of Halothane may originate in an alteration of renal blood flow and this aspect of the problem is under investigation.

During the past year the Surgery Branch has supported a Resident in Orthopedic Surgery. Two investigative projects have been carried out in collaboration with the National Institute of Arthritis and Metabolic Diseases. There is no detailed knowledge concerning the pathogenesis of the bone changes secondary to cyanotic heart diseases. Various experimental procedures have been employed to reduce arterial oxygen saturation and a series of animals in which cyanosis has been produced are being X-rayed at intervals to determine the progression of their pulmonary osteoarthropathy. Also a new lesion of the small blood vessels in joint tissue has been observed in a series of patients who had no symptoms of joint disease. The lesion can be characterized as an occlusive vascular one whose significance or etiology is entirely unknown. A detailed investigation of the incidence of this vascular lesion has been undertaken in an effort to determine its possible relationship to the aging process.

Clinical studies in the Section of Cardiology during the past year have again focused on the applicability of Starling's law of the heart to man. A method was developed by which it is possible to determine directly the interrelationships between ventricular end-diastolic fiber length, diastolic tension, systolic tension and the rate of development of tension. These measurements may all be carried out by means of a specifically modified Walton-Brodie strain gauge arch sewn to the surface of the human heart. These studies have shown that as human myocardial fibers are progressively stretched. a progressive increase in diastolic tension, in the ten-

sion developed during systole, and in the rate of development of tension takes place. These observations are direct evidence of the operation of Starling's law of the heart in man. In other studies, on patients with mitral stenosis and atrial fibrillation, further evidence of the applicability of Starling's law was obtained. Continuous alterations of the length of a segment of left ventricular muscle were recorded at operation. On a beat-to-beat basis the length of a segment of left ventricular muscle at the end of diastole was found to correlate extremely well with the characteristics of ventricular contraction; the latter was assessed by a detailed analysis of individual left ventricular pressure pulses. Similarly, the left ventricular end-diastolic pressure appeared intimately related to the subsequent ventricular contraction in several patients with a closed chest studied at the time of left heart catheterization. Ventricular function or "modified Starling" curves were obtained in seven subjects with normal cardiovascular systems studied while under complete ganglionic blockade. "Effective" left ventricular end-diastolic pressures, cardiac output, and arterial pressure before and after the transfusion of 1,500 ml. of blood were measured. In each instance ventricular filling pressure correlated well with the left ventricular stroke volume and stroke work, further supporting the concept that Starling's law of the heart applies to man.

Hemodynamic observations on the function of the left atrium were carried out on 50 patients with various forms of heart disease. It was found that in patients with left ventricular disease (arteriosclerosis, aortic valve disease, etc.) left atrial contraction elevated left ventricular end-diastolic pressure while maintaining the left atrial (and therefore the pulmonary capillary) pressure at a substantially lower level. This dissociation between left ventricular end-diastolic and mean left atrial pressures provides an "index" of left atrial function and emphasizes the clinical importance of left atrial contraction in patients with left ventricular hypertrophy. It was shown that, just as in the left ventricle, the pressure in the left atrium just prior to the onset of atrial contraction is an important determinant of the characteristics of atrial contraction.

Currently, measurements of two other basic parameters are being carried out. The left ventricular end-diastolic volume is being correlated with the left ventricular stroke volume and stroke work. Similarly, the rate of development of left ventricular pressure, i.e. the slope of the isometric pressure gradient, is being correlated with the end-diastolic segment length. These studies are designed to provide more detailed information about the hemodynamic determinants of the characteristics of ventricular contraction in man.

In a continuing investigation of the pharmacology of drugs which act primarily on the cardiovascular system it was shown that digitalis glycosides augment the contractile force of nonfailing human hearts. In these studies, carried out on patients at the time of "open heart" operations it was also observed that digitalis exerts a direct arteriolar constrictor effect. These studies may serve to change current concepts of "prophylactic digitalization." In other observations on digitalis glycosides it was demonstrated that the production of mild hyperthyroidism greatly augments digitalis requirements. It was shown that large doses of rauwolfia alkaloids abolish these increased digitalis requirements. Guanethidine, a drug which apparently releases tissue stores of catecholamines, was found to abolish many of the manifestations of triiodothyronine-induced hyperthyroidism. In other studies the effect of large doses of syrosingopine, a rauwolfia derivative, on the cardiac response to acute hypoxemia and exercise was evaluated. It was found that the tissue catecholamine depletion produced by this drug failed to alter the cardiovascular response to these two stimuli.

The development and evaluation of newer diagnostic technics was continued during the past year. In a critical appraisal of the Kr^{85} inhalation test for the detection of left-to-right circulatory shunts, in over 300 patients in whom the presence or absence of such a shunt was subsequently proved, it was shown that the Kr^{85} inhalation test probably represents the most accurate means for the clinical detection of circulatory shunts. A simplified technique for the detection of patent ductus arteriosus was developed. This consists of the injection of Kr^{85} into the thoracic aorta and measuring the appearance time in the expired air. This technique has been found particularly valuable in excluding a patent ductus arteriosus in patients prior to open heart opera-

tions. Both ascorbic acid and iced saline have also been employed as indicators in the study of patients with congenital heart disease. Ascorbic acid is detected by means of a platinum electrode, while temperature is sensed with a small thermistor. Both of these elements may be introduced directly into the arterial blood or into the heart. By these techniques indicator-dilution curves may be recorded without sampling blood and therefore they constitute an important advance in the use of indicator-dilution curves. Solutions of Kr^{85} have also been employed as the indicator for the measurement of cardiac output by the indicator-dilution technic. Since Kr^{85} does not recirculate, inscription of the dilution curve is obviated, thus greatly simplifying the technic of cardiac output measurement. Transseptal left heart catheterization, developed in this laboratory 2 years ago, has been modified so that: (1) surgical exposure of the saphenous vein is avoided, (2) a smaller needle is employed for atrial puncture, (3) a larger catheter can be introduced into the left side of the heart, and (4) left heart angiography may be performed conveniently. This has greatly improved the clinical value of this technic.

Observations on 15 patients with idiopathic hypertrophic subaortic stenosis have been continued. This is the largest number of patients studied at any single hospital and detailed clinical, phonocardiographic, hemodynamic and angiographic observations have permitted a comprehensive description of this disease entity. In particular, a simple hemodynamic technic for the detection of this condition was described. This technic is based on an analysis of the response of the arterial pressure pulse to a premature ventricular contraction.

In studies performed in the experimental laboratory of the Section of Cardiology the effects of a variety of stimuli on the arteriolar and venous tone of the dog were examined. These investigations were carried out employing a preparation on total cardiopulmonary bypass in which the heart and lungs were excluded from the circulation. Thus, it was shown that veno-constriction accompanies arterial constriction when the pressure in the carotid sinuses and in the left side of the heart are lowered. Similarly, hypoxia and hypercapnia produce veno-constriction, but the increased arterial pressure which usually occurs with these stimuli results from an accompanying increase in the cardiac output. Hypothermia was shown to produce arterial and venous dilatation while hyperthermia has the opposite effect.

The mechanism of the inotropic, chronotropic and pressor effects of guanethidine was studied in normal dogs and in dogs following chronic cardiac denervation. The results of these experiments support the contention that guanethidine produces a positive inotropic and chronotropic effect by suddenly releasing the myocardial stores of catecholamines.

GERONTOLOGY BRANCH

The resarch program of the Gerontology Branch is directed toward (1) describing the biochemical, physiological and psychological changes that take place with increasing age in man and (2) investigating the basic biology of aging in order to understand age-dependent alterations in performance in man.

Physiological Studies

Longitudinal Studies

Age differences in biochemical, physiological and psychological characteristics of normal people still living successfully in the community are being evaluated. These subjects, ranging in age from 18 to 100 years, have agreed to return to the laboratory every 18 months for the remainder of their lives so that age changes can be recorded in individual subjects. Almost 200 subjects have received the first series of tests and 50 have been tested a second time. The program has been well received as evidenced by the fact that we are now making appointments with new recruits for December 1961. Due to limitations in space and staff, we can accept only two new subjects per week.

Because of the long term nature of this project, results on successive measurements cannot be analyzed as yet. However, members of this longitudinal sample have served as experimental subjects for many of the other studies on humans.

In collaboration with Dr. Bernice Cohen of the Johns Hopkins School of Hygiene and Public

Health, the testing schedule has been expanded to include information on genetic background which should shed light on the question of the relation of assortive mating to longevity. Dietary habits of the subjects will also be surveyed in collaboration with the Maryland State Health Department and the Heart Disease Control Program of the USPHS (Dr. McGandy).

Renal Studies

Experiments have been conducted to determine whether the hemoglobin-haptoglobin complex is cleared from the plasma by the reticulo-endothelial system. Preliminary experiments indicate that Kupffer cells may be isolated from liver homogenates. If this method is successful, experiments will be performed using hemoglobin tagged with Fe^{59} to determine the presence of the hemoglobin-haptoglobin complex in isolated Kupffer cells in the dog.

No age differences in glomerular permeability were found using infusions of dextran of different molecular weights.

Studies of glomerular filtration of free hemoglobin (corrected for binding to haptoglobin) relative to inulin clearance have been carried out in 47 subjects aged 20–88 years. The ratio C_{Hb}/C_{In} averaged 0.055 (S.D. 0.019) and showed no significant correlation with age. Thus there is no evidence of a change in glomerular permeability with age. Tubular reabsorption of hemoglobin, calculated as the difference between filtered load and urinary excretion averaged 1.43 mg./min (S.D. 0.96).

Measurements of creatinine clearance made over a period of 12 hours indicate similar rates of decline with age in both hospitalized and community residing groups.

Body Composition Studies

Estimates of lean body mass and body fat have been made utilizing the equations proposed by Keys and Siri which are based on body density and total body water determinations. In addition, a new equation was developed which introduces corrections for bone density (estimated from X-ray measurements of the phalanx) and for muscle mass (estimated from creatinine excretion). When fat content of the body was calculated from the Keys equation and expressed as a percentage of body weight an increase of 0.06 percent per year was found. However, when the new equation was used there was a decrease of 0.12 percent per year. The new equation gives a better estimate of body fat by introducing corrections for bone density and muscle mass, both of which show significant age decrements.

Energetics of Arm Motion

In cooperation with Dr. R. L. Ramsey, Medical College of Virginia, a description of the mechanical relationships of the human arm swinging maximally in alternating back and forth movements has been developed. During this maximum wagging exercise linear relationships were found between (1) period of swing and angular displacement (amplitude), (2) the impulse of the force applied to the limb and the log of the angular displacement and (3) action and angular displacement. The hypothesis that the slopes of these relationships were related to mechanical efficiency was not supported for any of them. Mechanical efficiency was calculated from the excess oxygen consumption associated with the work done. A family of curves was developed which related mechanical efficiency to age at different amplitudes of swing. Young subjects (age 30) showed an increase in efficiency with increasing amplitude of swing, whereas in old subjects (age 70) efficiency did not increase with increasing amplitudes of swing. In both old and young subjects, the angular proportion of the swing during which force was exerted by the muscle decreased (93 percent to 57 percent), with increase in amplitude (0.36 Rad to 1.85 Rad).

The delineation of the mechanics of arm movement provides a basis for understanding age changes in the ability of an individual to perform simple tasks efficiency. These measurements may provide a simple objective test of muscular efficiency that can serve as one factor in an index of aging.

Psychological Studies

Investigators in the Psychology Section provide information on the psychological performance and personality characteristics of subjects included in the longitudinal study of aging. In addition to standard tests of intellectual performance, standard questionnaires to evaluate personality characteristics are being administered.

Estimates of reaction time, α-frequency of the EEG, spinal reflex amplitude, heart rate variability and motor time in these subjects are also being made.

A substantial correlation (0.81 with $p < 0.01$) between mean reaction time and mean alpha wave frequency of the electroencephalogram has been observed in a group of 13 subjects. Correlation between these variables within individuals varied over a wide range of values (+0.03 to +0.65). When the effects of several other variables related to reaction time were partialed out by means of multiple regression analyses, the low individual correlation of 0.03 between reaction time and alpha wave frequency was increased to 0.29. The variables partialed out were amplitude of spinal reflex, heart rate and motor time (the time which elapses between the appearance of a muscle action potential and the mechanical response of the muscle). These variables are regarded as indices of the activity level of the brain stem reticular system. These measures also account for ⅓ of the total variance of the speed of response. Hence, it may be inferred that speed of response is influenced by general excitatory states of activity in the brain and spinal cord which may fluctuate from moment to moment.

The effect of interpolated activities on short term memory was tested in subjects with a high level of educational attainment. In contrast to the previous study which showed greater interference among old than young subjects with a low level of education, age differences in this group were not statistically significant. However, when the task was shifted to the judgment of a time interval (length of time a stimulus light was on), the older subjects showed a significantly greater interference effect. In this experiment the young subjects were more effective than the old in learning the original stimulus series so that a covariance analysis was required to test the significance of the age difference in re-learning scores. This analysis showed that the age difference was statistically significant. It is inferred that external cues were available in the experiment requiring judgments of size which were not available in the experiment involving time judgments and that the group of more capable subjects used these cues to a greater extent than did the subjects of lower capabilities.

Basic Biology

Cellular and Comparative Physiology

The general research objectives and activities of the Cellular and Comparative Physiology Section continue in a twofold direction: (1) the description of cellular and organismic changes in humans and appropriate experimental animals during aging and (2) the measurement of the effects of alterations in the environment on the performance and mortality of experimental animals.

Histochemical methods for the localization of reductases in tissue cultures of developing embryonic muscle tissue have made possible the demonstration that succinic dehydrogenase, DPN · H-cytochrome *c* reductase and TPN · H-cytochrome *c* reductase increase dramatically concomitantly with multinuclearity. This finding indicates an increase in mitochondria following cell fusion.

In order to determine whether the multinuclear cell becomes dependent on oxidative pathways exclusively, cultures were grown in the presence of antimycin A. The results indicate that although the mononucleated cell can maintain itself and proliferate in the presence of the blocking agent, the multinucleated cells are selectively killed. Thus muscle differentiation may depend upon an obligatory aerobic metabolic process. These studies lend support to the hypothesis that growth (cell proliferation) and differentiation are mutually exclusive processes.

Histochemical localization of reduced nucleotide reductases has also been useful in demonstrating cross-striations in developing muscle tissue in tissue cultures. These striations can be detected since the mitochondria are oriented along the A bands.

The recording spectrophotometer has been modified to permit continuous scanning of the absorption spectrum of individual cellular elements. By using appropriate corrections, the standard error of replicate readings has been reduced to 1–3 percent of the mean value.

Using this method, measurements of ploidy variability in developing chick muscle cultures have been made to compare the distributions in mononuclear and multinuclear cells. Preliminary results show a unimodal distribution of DNA values for the muscle cell nuclei which corre-

sponds to the lower peak (diploid) of the bimodal distribution found in mononuclear cells. If this result is confirmed, it will offer evidence that muscle is a true syncytium. This technique will make it possible to determine whether the nuclei of the multinuclear muscle cells are, in fact, nonproliferative. Measurements of DNA per cell will be extended to comparisons of cells from young and old animals to test the hypothesis that the frequency of mitotic "accidents" increases with age.

Muscle cells obtained from biopsy material taken from adult and aged humans can be maintained in tissue culture. Cells originating from mature skeletal muscle tissue are capable of neoformation of multinuclear muscle cells. After 2–3 weeks of culture, long multinuclear ribbons form in the outgrowth area of the original explants. These resemble the early multinuclear cells of chick embryonic muscle and have a highly refractile cytoplasm which, after fixation and staining, shows many longitudinal fibrils. Removal of the original explants prior to multinuclear cell formation did not affect the process. This indicates that the multinuclear cells do not arise simply by the "budding" of injured mature muscle cells. Efforts are being made to isolate the various cell types found in mature muscle in order to determine the source of the myogenic elements.

Further studies have been made of the age pigments which accumulate in the human myocardium. The particles have the following average dimensions: major axis, 1.39 ± 0.06 micra; minor axis, 0.97 ± 0.04 micra. Specific gravity of the particle is 1.18 ± 0.03. Measurements of reduced vs. oxidized difference spectra indicate that isolated age pigment particles contains very little flavoprotein, cytochrome or cytochrome *c* reductase which are present in mitochondria. Tests for some of the Krebs cycle oxidases have also been negative. Cathepsin and acid phosphatase activities were pronounced and there were traces of acid deoxyribonuclease and ribonuclease in the particles. Thus the enzymatic behavior of the age pigment particles resembles that of lysozymes more than mitochondria.

Alkaline hydrolysis of lipids extracted from age pigment does not greatly alter the fluorescence. Gas chromatography of fluorescent fractions separated on silicic acid columns and subjected to methanolysis has revealed a preponderance of unsaturated over saturated fatty acid esters. The yellow fluorescent material also shows an infrared spectrum very similar to that of old oxidized samples of animal cephalin.

Data have been obtained that indicate that the photoreduction of TPN (in spinach chloroplasts) does not require the utilization of high energy phosphate bond energy (ATP). The firefly assay system was adapted for the continuous measurement of ATP levels in illuminated chloroplast suspensions.

Studies of the effects of environmental factors on the longevity of *Drosophila melanogaster* have been continued. Flies reared in environments free from micro-organisms live slightly longer than flies reared in a nonsterile environment. Exposure to 50,000 R of radiation increases (by 50 percent) the longevity of flies in nonsterile environments, but reduces longevity by about 20 percent in flies reared under sterile conditions. When flies are reared in an environment of 100 percent oxygen, the rate of aging is increased by about 50 percent, whereas, at lowered oxygen tensions (1–2 percent O_2), the rate of aging is reduced by about 35 percent.

Extensive time lapse records of the development, function, senescence and death of a number of species of coelenterates have been made. The individual species of this phylum have widely different longevities, ranging from a few days to many years. Stolonic fusion, hydranth regression, digestion, respiratory movements, growth, cleavage, and other functions have been recorded. Senescence and death of individual hydranths of Campanularia begins with a gradual contraction of the tentacles and is followed shortly by lysis and contraction of the hydranth with the return of its contents to the colony.

Nutritional Biochemistry

Three general areas have been investigated during the past year within the Section on Nutritional Biochemistry. These included studies on (1) age differences in tissue metabolism of the rat, (2) biochemical and physiological differences between normal rats and those whose life span may be increased through dietary restriction and (3) the effect of diets with respect to their potential to increase life span.

Among these studies has been an investigation on the effect of age on protein catabolism. Previous studies have indicated a high catheptic activity in the livers and kidneys of senescent rats. Since the *in vitro* determination of the enzymatic activity measures the breakdown of protein, a higher rate of catabolism of tissue proteins in old animals may be inferred. The earlier study (age differences in enzymatic activities of tissues during protein depletion and repletion) may be criticized on the grounds that it did not measure protein metabolism in normal animals. Therefore, the rate of protein catabolism was estimated by injecting radioactive methionine into animals of different ages and measuring the rate of disappearance of S^{35} from the soluble protein fractions of various tissues. Although the rate of loss of radioactivity in the soluble protein fraction of liver tended to be higher in the older animals, statistical significance could not be established in this tissue nor in kidney, heart or muscle. Thus these studies again have failed to demonstrate any effect of age on protein metabolism.

Mitochondria from liver and kidney tissues of old and young rats have been separated. In both tissues succinoxidase activity per unit of washed mitochondria was the same for both old and young rats. The evidence thus suggests a senescent loss of whole mitochondria rather than a gradual loss of enzyme per mitochondrion. In these experiments, protein nitrogen content and turbidity of washed suspensions were used to estimate the number of mitochondria in the suspensions. The use of millipore filters for separating mitochondria is being explored to facilitate actual counting of the mitochondria. Studies are also being conducted to produce suspensions of isolated cells from other tissues such as the central nervous system for use in metabolic studies. Preliminary experiments show that incubation of minced cerebellum with papain and versene yields a suspension containing free Purkinje cells which can probably be isolated for metabolic studies and the preparation of mitochondria.

In past experiments, the age differences demonstrated in the concentrations of various enzymes in the kidney tissue of the rat have been relatively small (10 percent) in comparison to the large decrements in renal function observed in human subjects (60 percent). It is possible that aging affects the kidneys of these two species at different rates or that the enzymes chosen for investigation were not sensitive indices of renal function. Therefore, attempts were made to determine the effect of age on renal tubular transport in an *in vitro* system by measuring the accumulation of PAH by renal cortical slices from rats. Tubular function was estimated by the ratio of the concentration of PAH in the slice to that in the medium (S/M). The results demonstrated a tendency toward slightly lower S/M values in the senescent animals, but even this decrement was explained by a decrease in the number of cells as measured by the concentration of DNA in the tissue samples.

In another study, an estimate of age differences in total renal capacity was measured by the amount of renal tissue produced following unilateral nephrectomy in the rat. In addition to the total amount of tissue regenerated, the concentrations of succinoxidase, alkaline phosphatase, DNA, RNA and protein nitrogen were also determined. Although a greater degree of hypertrophy was found in the young animals (44 percent) as compared to senescent ones (33 percent), the concentrations of the various tissue components (DNA, RNA, protein N and enzymes) per unit wet weight in the hypertrophied kidneys did not vary by more than 10 percent from values found in the normal kidneys and no marked age differences were observed. At the present time, it is not possible to attribute the observed differences in the degree of hypertrophy specifically to aging since the older animals, as indicated by body weight changes, did not seem to recover from the operation as well as the young.

In view of the fact that the same tests have not been carried out in the rat as in man, clearance techniques for the estimation of renal function in the rat are being developed. At present it is possible to measure renal clearances in unanesthetized rats under conditions in which inulin and PAH plasma levels remain constant for thirty minutes and the bladder is adequately rinsed to assure complete urine collecting. In future experiments, it will be possible to compare the effect of age in man and the rat by the same renal function tests.

For many years, it has been well known that the life span of the rat and mouse can be increased by severe dietary restriction. In order to understand the mechanism of this phenomenon,

experiments were carried out to determine the biochemical and physiological differences between normal animals and those subjected to such dietary restriction. The results of this experiment demonstrated increased concentrations of succinoxidase in liver tissue and alkaline phosphatase in kidney tissue of the restricted animals. Since the previous study had indicated that the concentrations of these enzymes are lower in younger animals, these data do not support the concept that the restricted animal is similar to a chronologically younger animal. Similarly, the activity patterns measured in both suspension and wheel type cages also fail to support the hypothesis that the restricted animal is similar to a chronologically younger animal. Although dietary restriction has been shown to increase the life span in rats and mice, the application of this principle to species exposed to everyday stress is questioned in view of the work of McCay showing that the restricted dog succumbs much more readily to bacterial infection and parasitic infestation than does the normal animal. Since our experiments demonstrated that alterations in the concentrations of certain tissue enzymes do occur during dietary restriction, it may be possible to choose a diet which will support normal growth and still provide the necessary enzymatic alterations in the tissues to increase life span. Our first experiment in this direction demonstrated that it is possible to increase the concentrations of selected enzymes in various tissues by feeding growing animals diets which will promote optimum growth but which differ only in the quality (casein vs. whole dessicated liver) or quantity (20 to 32 percent protein) of the dietary protein. Thus far, these experiments have not been totally successful since the enzymes affected by this means were not identical to those altered by dietary restriction.

Intermediary Metabolism

The research activities of this section are concerned with the mechanisms of oxidative phosphorylation associated with the respiratory chain and with the substrate level reaction associated with the α-ketoglutarate oxidation.

The chemical reactions associated with the synthesis of ATP during oxidations in the mitochondrial electron transport chain are not known. The work carried out clearly implicates a dithiol grouping in the process. The evidence rests on the observations that arsenite, under special conditions, uncouples oxidative phosphorylation in liver mitochondria, exposes an ATPase and inhibits the exchange of P^{32}-phosphate with ATP. The effects are quite analogous to those of 2,4-dinitrophenol. The involvement of dithiols in respiratory oxidative phosphorylation brings out the similarity of the process to the "substrate level" phosphorylation in the oxidation of α-ketoglutarate where the primary energy-trapping reaction involves the reduction of the disulfide group of lipoic acid and simultaneous formation of a thiol ester. A related mechanism, involving the oxidation of a charge-transfer complex between a disulfide and a reduced electron carrier to the corresponding high-energy thiol derivative, has been proposed to explain the role of dithiols in the respiratory phosphorylation system.

The mechanism proposed above is partly based on the results obtained in an investigation of the mechanism of the dihydrolipoyl dehydrogenase reaction. This enzyme has been identified as a flavoprotein, and it carries out the terminal reduction of DPN by dihydrolipoate in the sequence of reactions resulting in the oxidative decarboxylation of α-ketoglutarate. Based on the inhibition of the enzyme by arsenite and cadmium ions, the involvement of a second oxidation-reduction "prosthetic" group, namely, a disulfide-dithiol, has been identified. An interaction between the flavin-adeninedinucleotide and the dithiol results in the appearance of an absorption maximum at 530 mμ in the reduced enzyme. A model for this interaction has been discovered in mixtures of reduced lipoate and flavinmononucleotide (FMN). In the absence of any enzyme, the two compounds form a dissociable molecular complex with an absorption maximum at the same position. On standing, the complex breaks down to the lipoic disulfide and $FMNH_2$. Neither the model complex nor the reduced enzyme show evidence for free radicals in the electron spin resonance spectrum. The complex formed from reduced lipoate and FMN is analogous to the complex proposed in the scheme for respiratory oxidative phosphorylation. Preliminary evidence suggests that the primary oxidation event in α-keto acid oxidation may involve an unknown grouping which comes into effect even before lipoate is reduced.

The dynamic turnover of mitochondria in rat

livers has been studied. S^{35}-methionine and C^{14}-acetate were injected in the animals and the rate of loss of label followed in four mitochondrial components (soluble protein, insoluble protein, lipid and cytochrome *c*). The results showed that all components had essentially the same half-life of 10.3 days which favors the conclusion that mitochondria are synthesized and broken down as units. The individual constituents do not appear to be replaced after the synthesis of the particle. No difference in turnover rates has been detected in senescent (20–22 month) compared to adult (12 month) rats.

Biophysics

The research program of this section is directed toward an understanding of cellular mechanisms and their relationship to aging. At present attention is being directed toward cellular processes in cultures of the protozoan *Euglena* which can be artificially "aged" and to the relation between structure and function in muscle enzymes with special reference to the role of –SH groups.

Experiments on the effect of steroid hormones on cultures of the protozoan *Euglena* have revealed items of importance. First, the increase in respiration caused by minute quantities of testosterone is noticeable only in the actual presence of the hormone, and does not appear to persist after the cells are washed. Second, the effect of testosterone is not evident unless a source of nitrogen is in the medium. It thus finally appears possible to make an hypothesis on a biochemical or biophysical level—e.g., that testosterone controls membrane permeability. This hypothesis is further suggested by the finding that vitamin D, also a steroid, enhances the growth of these cells in a low Ca^{++} medium, and work has now begun on the transport of Ca^{++} into these cells in the presence and absence of added vitamin D.

The effects of age and starvation on these protozoan cultures have also been examined in a preliminary fashion. Viable cells have been maintained for many months in the absence of either a carbon or a nitrogen source. Preliminary indications were that drastic changes occurred in both the RNA and DNA content of the cells. Prolonged checking of the analytical procedures for DNA analysis has shown that the DNA analyses were not reliable and a series of minor modifications has been worked out which will now permit proper measurement of the DNA content at various times of "aging." The RNA content decreases from about 30 μg/million cells to about 5 μg/million cells or less. During the lag period that occurs when the cells are put into complete medium the RNA is resynthesized. The length of the lag has been found to increase with the time during which the culture was "aged" and some quantitative data on the relation between time of aging and length of lag have been obtained. When the cells are "aged" in a medium that contains no sulfur it is found that they cannot survive for more than about one month. Thus the mechanisms which permit the cell to survive in the face of nitrogen or carbon depletion do not function for the case of sulfur depletion. The existence of these adaptive regulatory mechanisms suggests that experiments on the content of certain enzymes in these cells after various times of aging would be the most profitable direction for this work to proceed.

The study of the role of –SH groups in muscle proteins has provided several new insights on the nature of the enzymatic process in myosin. Earlier in this project it had been shown that Cu^{++}, Cd^{++}, and Zn^{++}, like parachloromercuribenzoate (PCMB), would enhance the ATPase activity of myosin if these reagents were present in low concentrations at high temperatures, e.g., 25° C). At high concentrations, the ATPase activity was inhibited, and at low temperatures inhibition occurred at any concentration of reagent. For ITPase activity only inhibition was observed. We have now studied the rates at which these reagents interact with myosin and the way in which the presence of the substrate—either ATP or ITP—affects this rate. From these studies it appears that there are at least two –SH groups on the active site. One –SH group is involved in the ability of myosin to differentiate between ATP and ITP, and may be involved in a small conformation change at the site. Reaction of the second –SH group with any of these reagents leads only to inhibition. Studies on the tryptic digestion of myosin have shown that the active site is located on a trypsin resistant part of the myosin molecule and that the site does not interact appreciably with other parts of the myosin molecule. Several differences between Cu^{++} and PCMB have also been found. In particular, the

use of Cu^{++} at several pH values has revealed the presence of an ionizing group, with an apparent pK near 7.4, that influences the enzymatic activity. The results of this work on myosin suggested that the adenine part of the DPN coenzyme molecule might interact with some dehydrogenase enzymes in a way similar to the interaction of the adenine part of ATP with myosin. Experiments with lactic dehydrogenase were begun with these hypotheses in mind. We have found a certain similarity between the effects of Cu^{++}, Ni^{++}, Cd^{++}, Co^{++}, and Zn^{++} on these two enzymes, and, in addition, have succeeded in demonstrating that there is an –SH group on lactic dehydrogenase, the access to which is controlled not by the adenine but by the phosphate group on DPN which is closest to the nicotinamide portion of this coenzyme. Further work is in progress.

Molecular Biology

The principal concern of this section in the past has been the function of metal ions in biological processes. During the last year, however, the focus of attention has been an artificial reaction of metals with biological molecules in order to elucidate their structure.

Structure of Nucleic Acids

It is believed at the present time that all of the hereditary information that brings about the development of an organism as well as its degradative changes on aging is carried in molecules of deoxyribonucleic acid (DNA). The material consists of four basic building blocks, adenine, cytosine, guanine, and thymine, of which hundreds are joined together in a chain by means of ribose and phosphate units. The information code is believed to consist of the sequence in which these building blocks are arranged; such sequence determination is therefore of utmost importance to an understanding of the hereditary code. Attempts to determine this sequence have, up to this time, been confined to enzymatic degradations. Unfortunately, however, no enzymes are available for splitting specific bonds in nucleic acids. The present effort is to develop a chemical reaction capable of such specificity.

Complexes of salicylaldehyde with copper, nickel, and cobalt have been found to react with adenine to produce substances that are different and more stable than the substances produced from cytosine, guanine, and thymine. Using various combinations of metals, salicylaldehyde, and the four bases, their ribosides and their riboside phosphates, it is indeed possible to distinguish all of these substances qualitatively and some of them quantitatively by simple color tests. So far, this differentiation has been possible only for the molecules that have been disassembled from the chain. The metals have been found to react also with ribose, making the differentiation on the chain more difficult, but various techniques are being used to overcome this difficulty.

Structure of Hemoproteins

Meanwhile, studies on the relation of rotatory dispersion of the hemoproteins to their structure have continued. Hemoproteins are proteins containing an iron complex, heme; the hemoproteins catalase and hemoglobin contain four hemes per molecule, whereas peroxidase and myoglobin contain only one heme per molecule. The rotatory dispersion is a plot of optical activity vs. wave length; this plot exhibits a "Cotton effect," i.e., an S-shaped curve, in the visible when the heme group is asymmetrically placed, but the plot is essentially parabolic when the heme is not asymmetric. All four of the molecules, catalase, peroxidase, hemoglobin and myoglobin, exhibit the Cotton effect when the molecules are intact, and they lose the effect when the natural folding of the protein molecule is changed in some manner into a more irregular shape. In the case of catalase and hemoglobin, these changes were shown to be accompanied by splitting of the 4-heme molecules into 1-heme quarters or 2-heme halves; here the phenomenon can be explained if each heme is symmetrically surrounded by protein and the presence of additional hemes in the molecule destroys this symmetry. However, in the case of peroxidase and myoglobin, the molecules cannot be broken down, and the rotatory dispersion changes can be explained only if one accepts the following generalization: The Cotton effect associated with the heme absorption is a measure of the ordered arrangement of the protein and

disappears when the structure of the protein has been destroyed.

This generalization has been applied in a study of the stability of the heme-protein site in catalase. The heme was removed and then added back to the protein. Four molecules of heme still reacted with each molecule of the catalase protein, showing that the site on the protein, to which the heme had been attached, was not destroyed in this procedure. However, the newly reconstituted hemoprotein showed no Cotton effect; hence the structure of the protein as a whole has been destroyed. Therefore, the site for heme attachment remains intact even when the gross structure of the protein is destroyed.

Metal Ions in Enzymatic Reactions

In the past we have studied the effect of metal ions on the substrates of enzymatic reactions. This year attention has been directed to the interaction of metals with proteins, specifically bovine plasma albumin (BPA). The most striking observation has been that the BPA molecule dimerizes in the presence of copper, but is not affected in this way by any other metal. Also, copper and cobalt shift the equilibrium between two forms of BPA, although manganese has no effect. A possible explanation of the specificity of manganese in many enzymatic reactions is that manganese, unlike other metals, will not change the structure of the protein.

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

INTRODUCTION

The year 1960 has seen both a widening of the scope of research activities and an intensification of the pursuit of promising lines of investigation in the National Institute of Allergy and Infectious Diseases. The coming year will see even more effective utilization of scientific resources, as new space becomes available and programs initiated this year begin to achieve their objectives.

Clinical investigations of the Institute have advanced in several ways. Of great significance for present and future programs has been the increased utilization of prisoner volunteers for clinical studies, through cooperation with the Federal Bureau of Prisons. Volunteers have been hospitalized in the Clinical Center, exposed to specific respiratory viruses, observed for clinical manifestations, and examined by precise laboratory techniques for evidence of infection. In a short time, these human volunteer studies have made available a vastly greater amount of detailed information concerning the respiratory syncytial virus and the Eaton agent than would have been possible by the usual clinical and epidemiological observations in the general population.

In connection with investigations of simian malaria, prisoner inmates have been inoculated with various strains of the parasite in the Atlanta Penitentiary. Some of them were transferred to the Clinical Center for precise clinical observations. This study also has permitted the extension of clinical and laboratory observations not possible in any other way. Other clinical studies underway for some years such as treatment of fungus infections, bacterial complications of cystic fibrosis, and drug resistance in staphylococcal infection have continued to add to specific knowledge of these diseases.

During the year an observation of great potential importance to public health was made by Institute malaria investigators and quickly substantiated by two other laboratories. Two scientists working with a strain of monkey malaria were accidentally infected, probably by a mosquito bite. This was the first clear-cut evidence that certain strains at least, of simian malaria, are pathogenic for man. An intensive investigation using all available resources quickly showed that man could be repeatedly infected by this strain through mosquito bite, that it could be transferred from man to man by blood, and that monkeys could be infected by mosquitoes fed on human cases. These observations formed the basis of a greatly expanded program on the pathology and biology of malaria, including the establishment of a field party in Malaya, the origin of the monkey strain infectious for man.

The Middle America Research Unit in Panama, established three years ago under the sponsorship of this Institute and the Walter Reed Army Institute of Research, has intensified its studies of the arthropod-borne viruses in the tropics. Laboratory procedures have been developed and diagnostic reagents prepared for working with a large proportion of the more than 125 arthropod-borne viruses. The Middle America Research Unit also has extended its studies to include special epidemiologic observations of poliomyelitis in Panama, and investigations of the role of mites in Central American virus infections. In conjunction with Gorgas Memorial Laboratory workers, Institute scientists recovered two strains of vesicular stomatitis virus from *Phlebotomus* flies. This is the first time this group of insects has been incriminated as a possible vector of this important virus.

Increasing interest in immunologic phenomena manifests itself in nearly all aspects of Institute research activities. The development of new immunologic techniques, such as immunoelectrophoresis and immunofluorescence, has stimulated new

approaches to new and old questions and attracted investigators into the field. The result has been a noticeable resurgence of clinical and scientific interest in allergic diseases. The Institute initiated a new program in clinical immunology during the year which will investigate such autoimmune diseases as lupus erythematosus, and thyroiditis, as well as certain clinical aspects of hypersensitivity and mechanisms of resistance.

The very important studies on respiratory infections which have been underway for some years continue. It is now clear that the Eaton agent is, as long suspected, an important cause of primary atypical pneumonia. Development of more precise laboratory techniques for isolation of viruses from animals, demonstrated that many laboratory mouse stocks are grossly contaminated with normally occurring latent viruses. At least five different viruses are now known to infect apparently normal mice. This has complicated studies on the relation of viruses to cancer, and confused much of the work in this field conducted over the last few years. It seems unavoidable that the development of pathogen-free animal stocks, particularly mice, will be essential for future investigations in cancer and in other diseases as well. The development of some means to eliminate or control these contaminating viruses from experimental animals poses a major and immediate problem to this Institute and to investigators elsewhere.

The year has witnessed also the planned development of research experience for junior investigators and of opportunities for permanent staff members to work in research centers outside the National Institutes of Health. The key to productive and significant research is, as has been pointed out repeatedly, the selection of imaginative, energetic and well trained investigators and the development of a stimulating environment in which these investigators have the freedom and resources to pursue their scientific ideas. One approach to this goal is to recruit competent interested young men soon after completion of their doctoral training. During the last year three such young men joined this Institute as a part of the NIH Research Associate Program and seven as part of the Clinical Associate Program. Six others were recruited to specialized research activities making a total of 16 new scientific staff members at the junior level. From these no doubt will come a number of our future permanent staff and all will become indoctrinated with research experience of great value to their eventual scientific development. Thus, the intramural activities perform an important role for training in research methods and goals to the future benefit of scientific endeavor, wherever these men may work—in universities, hospitals, or in the practice of medicine.

The Institute has pursued vigorously a policy of encouraging work assignments of its permanent staff in other well known research centers. One scientist is working at the Karolinska Institutet, Stockholm, Sweden; two are at the Pasteur Institute, Paris, France; one at the Max-Planck Institute for Virus-forshung, Tubingen, Germany; one in collaboration with the University of California and Institut de Recherches Medicales de la Polynesie Francaise, Papeete, Tahiti; one at the Wallaceville Animal Research Station, Wellington, New Zealand; and one with the Virus Laboratory, California State Health Department. Others have spent shorter periods of time in Malaya, Africa, and Brazil, pursuing specific research problems. A number of the staff have served on WHO Expert Committees and on special assignments, particularly in the areas of tropical medicine and parasitology. In this field, this Institute has probably the largest group of investigators of any other center in this country.

Research in tropical diseases will be expanded still further under the provisions of Public Law 480 which permits the use of foreign currencies to support specific intramural research projects in certain countries. Projects have been formulated and implementation of them during the coming year will permit the selected expansion of investigations important on a worldwide scale. These include problems in such diseases as malaria, schistosomiasis, fungus infections, arthropod-borne viruses, and filariasis. These international activities emphasize the expanding scope of our research responsibilities.

The Board of Scientific Counselors met twice during the year. The first meeting considered Institute investigations underway on respiratory diseases of viral etiology. The second meeting was held at the South Carolina State Hospital, Columbia, South Carolina where the Epidemiology Section of the Laboratory of Parasite Chemotherapy is located. Institute staff members

reviewed studies on simian malaria, chemotherapy of malaria, and intestinal parasite infections.

This report arranged by laboratory activities will summarize the major scientific accomplishments and advances achieved during the year in the direct intramural program.

LABORATORY OF CLINICAL INVESTIGATION

Clinical research activity has expanded during the past year largely as a response to enlarging the professional staff and cooperation with other Institute research units. A further period of growth will be needed to staff the clinical service in a manner consistent with optimum research productivity.

Infection of Volunteers with Respiratory Viruses

An extensive clinical study of acute viral respiratory disease was begun this year in association with staff members of the Laboratory of Infectious Diseases. The initiation of this project required much work but with the unstinting assistance of the Clinical Center, very satisfactory arrangements have been made to transfer to the Clinical Center, Federal prisoner-volunteers for study. This has been done with the permission and considerable assistance of Mr. James Bennett, Director, and Dr. Harold Janney, Medical Director of the Bureau of Prisons, Department of Justice. A team of custodial officers has also been assigned to the Clinical Center by the Bureau of Prisons to oversee the volunteers. The volunteers have uniformly cooperated with the program despite some extended periods of room isolation, frequent blood-letting, and other inconveniences. Many administrative arrangements have been developed so that the program has worked increasingly smoothly.

The results so far justify the investment in money and effort. It has been possible to produce in human volunteers a rather uniform "cold" with the respiratory syncytial virus. It may occur without respect to preinfection immunity, but the subsequent rise in complement-fixing antibody appears to correlate with severity of illness. Forty-six men have so far participated in this study.

Approximately 24 other volunteers have participated in studies with para-influenza 4 virus or Eaton (primary atypical pneumonia) agent. Future studies are planned with human influenza virus and with the recently defined group of REO viruses.

New Antifungal Drug

Beginning about four years ago, the Mycology Section of the Laboratory of Infectious Diseases began studies on an antifungal drug produced by Hoffman La Roche, designated as RO–2. This agent, an antimicrobial, was found to be the most active material ever tested *in vitro* and in animals against several of the pathogenic fungi, notably histoplasmosis and blastomycosis.

In the intervening years, 30 patients have been treated in the Clinical Center. Extremely favorable results have been observed in several patients severely ill with these diseases. From the standpoint of therapeutic activity, it appears that this drug is the best available for blastomycosis and histoplasmosis.

During the studies it was noted that the agent produced unusual hepatic changes. It was found that the dye, bromsulphalein, normally rapidly transferred from the blood to the bowel by the liver, was retained in high concentration in the blood in patients treated with RO–2. This effect appeared in a day or two after start of treatment, before tissue changes would likely occur, suggesting competition of the new drug for the liver excretory mechanism for bromsulphalein. After treatment was stopped, dye excretion promptly returned to normal or nearly normal. Liver biopsy has revealed changes indicative of minor hepatic damage in some cases. Because of the great importance of having a drug in addition to the relatively toxic agent, amphotericin B, for the treatment of fungal diseases, this new agent continues under investigation.

Simian Malaria

Following the demonstration of the infectivity of *Plasmodium cynomolgi bastianellii* for man, several inmates at the Federal prison in Atlanta volunteered for exposure to this agent. The need for careful clinical characterization of this disease in man led to the transfer of two infected

volunteers to the Clinical Center. Both men developed acute malaria which was carefully studied throughout its course. Significant alterations in urinary steroid excretion were detected, an unusual elevation of serum cholesterol occurred in one, and liver lesions not previously described, resembling but not identical with exoerythrocytic phase parasites, were demonstrated in both patients.

Penicillins and Penicillinase

Previous work here had defined nutritional requirements for penicillinase production by staphylococci and many parameters of its interaction with benzyl penicillin (penicillin G). During the year English investigators, working with the stripped molecule of penicillin, 6-amino-penicillanic acid, produced a new compound largely resistant to destruction by penicillinase. Working with this and several other penicillin derivatives, it was found that resistance to penicillinase is greatly influenced by steric positions of ethoxy groups on the side chain and that failure to be destroyed by penicillinase is associated with greater penicillinase inducing-capacity. Perhaps most significantly, this work has added to the now substantial indirect evidence that the major reason for present resistance of staphylococci to penicillin is the capacity of these micro-organisms to produce penicillinase upon contact with even low doses of penicillin.

Clinical studies with the penicillinase-resistant penicillin, dimethoxyphenyl-penicillin, have revealed that it possesses resistance to penicillinase *in vivo*, and that it is a powerful and effective anti-staphylococcal drug. After treatment of some patients for periods of three to five months, no penicillin-resistant staphylococci have been isolated. It has long been considered that chronic staphylococcal infection resembles tuberculosis and for the first time it appears that an agent is available which can be employed for extended periods of treatment without loss of effect, such as isonicotinic acid hydrazide in tuberculosis. If long-term therapy can thus be regularly given an enormous benefit will accrue to thousands ill with chronic staphylococcal disease.

Ascites in Mice By Injection of Adjuvants

In the past year a staff member has continued to study ascites induced in mice by the injection of adjuvant mixtures. This procedure has provided a much needed laboratory method for producing antibodies of a wide variety and a means of evaluating antigens. Recently, interest has been focused on the pathology and abnormal physiology of the lesion. It has been found that strain differences are associated with differences in susceptibility to ascites. Since ascites was found associated with a local plasma cell reaction, which in some strains of mice went on to plasma cell tumors, many implications toward problems in neoplasia have also been raised. These studies have become the basis for biochemical and pathologic studies with other Institutes.

Hypogammaglobulinemia

Staff members have shown that patients with hypogammaglobulinemia possess low, but definitely measurable, levels of antibody to the enteric viruses. The implication of this finding, in view of the normal resistance of these patients to viral infections, is that extremely low levels of specific antibody may provide adequate resistance against viral diseases. Subsequent, unpublished studies have indicated that these patients will develop circulating antibodies to Salk polio vaccine.

Bentonite Flocculation Test

The modification of the bentonite flocculation test for the detection of gamma globulin promises to provide the practicing physician with an accurate, convenient laboratory aid in the diagnosis of hypogammaglobulinemia. In contrast to the electrophoretic method, the results with the bentonite test can be known to the physician within a few minutes of arrival of the specimen to the laboratory. Other modifications of this technique will also give the levels of albumin and other protein constituents of blood and other fluids without resort to the more cumbersome method of electrophoresis.

The DNA-bentonite test for systemic lupus erythematosus has also been developed in the past year. This test measures the antibody in lupus serum which is directed against nuclear material. The specificity of this test is greater

than any previously described test for lupus. This test, however, is positive primarily in those patients with active disease and only rarely is positive when the disease is in remission. A modification of this test, the nucleoprotein-bentonite test, has retained all the attributes of the DNA test in regard to specificity, while achieving a much higher level of sensitivity for cases in remission. These promise to become important standard tests in the diagnosis of systemic lupus erythematosus.

Cystic Fibrosis of the Pancreas

Despite the commonly accepted point of view that antibiotics are helpful in this disease, observations of nasopharyngeal cultures failed to reveal any appreciable effect of antimicrobial treatment on *Staphylococcus aureus*. This does not negate a possible clinical benefit but does appear to minimize its value. It was coincidentally observed that *Escherichia coli* was not present in the nasopharyngeal flora of any child over the age of eight.

ROCKY MOUNTAIN LABORATORY

At the Rocky Mountain Laboratory research has continued directed toward both basic laboratory investigations and field studies of insect- and animal-borne diseases. In the first category are those projects concerned with the chemistry and surface properties of viruses, the highly intriguing relations of hypersensitivity and humoral immunity, and the basic relation of structural elements of microorganisms to the activity of the agents, both *in vivo* and *in vitro*. Field work is the foundation of projects related to studies of Q fever, tularemia, Colorado tick fever, and the ar-bo viruses. In addition, the combined efforts of the staff are directed to many other areas of research. Such projects include tuberculosis, influenza, poliomyelitis, and cryptococcosis.

Hypersensitivity

Studies of hypersensitivity at the Rocky Mountain Laboratory have been directed primarily toward clarification of the relations existing between delayed hypersensitivity and circulating antibodies and determination of the factors responsible for induction of contact hypersensitivity. It has been demonstrated previously that delayed hypersensitivity precedes circulating antibodies. This delayed hypersensitivity is directed toward protein. When circulating antibody appears, as occurs with relatively large amounts of conjugated protein, or when booster doses are administered, the specificity of the antibody response becomes oriented toward small configurations on the antigen molecule. Studies of immunity in neonatal animals have revealed that these animals, when injected with an antigen 12 hours after birth, develop circulating antibodies but fail to develop delayed hypersensitivity. Additional experiments suggest that this inability of neonatal animals to express such reactions is due to a deficiency in a skin reactive factor rather than an inability to respond to a primary injection of antigen.

Poliovirus

Continued studies of poliovirus have yielded considerable fundamental data. In cooperation with the group at the University of Minnesota, it was shown that agents such as octyl alcohol-chloroform and neutral hydroxylamine do not materially change the physical properties of purified infectious RNA of poliovirus but destroy over 99 percent of the infectivity of this material. The destruction of infectivity by uncoupling of a single link in a large particle (probably an acyl link of an amino acid with a phosphate group at the end of an RNA chain) suggests a possible approach to virus chemotherapy. Other studies have revealed that only 0.1 percent of RNA infectivity could be accounted for by residual protein, thus strengthening the concept that RNA does indeed constitute the infectious portion of the poliovirus moiety. By the use of chromatographic methods, it was also demonstrated that only certain of the avirulent strains of poliovirus can be differentiated from the virulent strains from which they are derived. This is in direct contrast to work reported by others. In studies of the infectivity of RNA it was found that the bulk of virus particles react with susceptible cells and that the relatively poor correlation between virus particles and PFU is due to the poor effi-

ciency of RNA at entering sites where it can influence virus production. A precipitation test for detection of antibodies against poliovirus has been developed which is more sensitive than the neutralization test presently employed. The antigen used is radioactive virus.

Endotoxins in Bacterial Fractions

Research on endotoxins derived from Gram-negative organisms has continued. As is so frequently the case, a fresh outlook on an old problem yields results of great value. The ideas held by Dr. Westphal, which attributed the activity of endotoxins to the presence of a firmly bound lipid ("lipid A"), were apparently generally accepted until presentation of the work done at RML. The finding that lipid A was not active and that deproteinized and "delipified" endotoxin was active stirred considerable controversy. In fact, the controversy was so intense that efforts to develop a vaccine against *Salmonella* infections have been diverted to settling this issue. Recent studies of the kinetics of inactivation of toxin by hydrolysis with hot acid have given data which should end this discussion. The old concept of "purified endotoxins" must be abandoned since there have been no previous toxins as good as those obtained at RML, and these are to be purified still further.

The purification of Vi antigen by curtain electrophoresis is a major advance in the study of this most important antigen. The demonstration that certain labile acetyl groups are responsible for the activity of Vi antigen resulted in production of material which was ten times more active than purified preparations prepared by mild acid hydrolysis. Emphasis should be placed upon the new chemical, physical, and biologic methods that have been devised to solve these problems.

Tuberculosis

The problem of immunity in tuberculosis has been studied intensively. Significant findings include the fact that mice may be satisfactorily immunized to subsequent pulmonary infection with virulent organisms by administration of small doses of avirulent organisms by the aerosol method. The demonstration that resistance is not due to interference strengthens the case for the value of immunization with living attenuated organisms for the prevention of tuberculosis. Since it has been shown that the delayed reactions elicited by protoplasm of various acid-fast organisms are specific in nature, it seems practicable to apply these findings to certain diagnostic problems in man. Use of fractions of tubercle bacilli in producing isoallergic encephalitis in guinea pigs show that the adjuvant effect lies in the cell walls and in a water-soluble protein prepared from the walls. This latter finding is important since it will allow the study of the adjuvant phenomenon from a molecular level.

Q Fever

The number of dairy cattle infected with *Coxiella burnetti* is large and is increasing, yet it is extremely difficult to detect cases of Q fever in man. In Idaho and Montana we have shown that a greater number of individuals residing on infected premises have antibodies against this organism than do those living on noninfected premises, yet no difference can be detected in the number of individuals who have symptoms compatible with clinical disease. The fact that organisms isolated from cattle have been uniformly of low virulence for experimental animals may account for the inability to find clinical cases of disease in those exposed only to cattle.

By the use of skin tests to eliminate allergic individuals from the study group, 190 inmates of the Montana State Prison were safely immunized without producing such reactions as have been previously reported. It is evident from these results, as well as from those previously obtained in laboratory personnel, that human beings can be safely vaccinated against Q fever if the precaution is taken to eliminate reactors by previous administration of specific skin-test antigen.

Methods developed for growing rickettsiae on modified Zinsser tissue cultures yielded relatively large volumes of organisms. These studies led to others involving purification of *C. burnetti* by sucrose gradients and by continuous-flow centrifugation in molar salt solution. These methods likewise made it possible to obtain certain chemi-

cal and physical fractions of these organisms. It was found that dimethyl sulfoxide could extract from Phase II *C. burnetti* a material which acted only as a hapten, but from Phase I organisms the extract obtained acted as a complete antigen. Lauryl sulfate also extracts complete antigen from Phase I organisms. Physically, the cell walls of these organisms can be separated from the protoplasm, and it has been noted that the cell walls are about 25 times more active in producing immunity than is protoplasm.

These studies on Q fever are of considerable significance. The laboratory and related studies have yielded information of both scientific and applied interest. It is apparent now that we can safely use our present vaccines for immunization of man and that it is feasible to produce large numbers of organisms which can be purified and used as vaccine or manipuated to give physical or chemical fractions which may be less toxic. The failure to find clinical cases of Q fever in man in the face of a rising incidence of infection in dairy cattle is highly interesting even if disappointing.

Other Rickettsioses

Studies of rickettsiae other than *C. burnetti* have been continued. By combining the methods presently used for fluorescent microscopy, a technique for sectioning arthropods has been developed which should be of interest to entomologists working in the field of embryology and anatomy and to medical entomologists, since thin sections in which the organs are not displaced can be obtained routinely. By applying the technique to the study of ticks infected with *R. rickettsii* it was found that the infection rate in local ticks varies from 15 to 28 percent. Not all of the ticks found infected by this method are infective for laboratory animals. The value of this type of study has yet to be fully appreciated.

The use of specific toxins has resulted in clarification of many of the problems related to the taxonomy of rickettsiae and has proved to be useful in ecological and epidemiological studies of this complex group of diseases. In further studies, potent immunogenic extracts have been obtained from certain of the rickettsiae. Their value as diagnostic and prophylactic agents is presently under consideration.

Bacterial Vaccines

Studies have been continued on vaccines for certain bacterial diseases. It has been found that while live Russian tularemia vaccine is capable of protecting mice more effectively than does ether-extracted vaccine derived from cell walls of *Pasteurella tularensis*, the protection produced by live organisms was not effective for long periods of time. Continued studies have emphasized the value of cell walls in producing immunity to infections with *Brucella abortus* in laboratory animals. It has also been found that live cells suspended in phosphate buffer and shaken with an excess of ether are killed but not disrupted. These cells constitute an excellent protective antigen which is less toxic (LD_{50} 7.5 mg.) than aqueous ether extracts obtained by conventional methods (LD_{50} 0.9 to 2.0 mg.).

Arbor Viruses

Studies of arbor viruses have yielded results of interest and suggest that emphasis on field studies would greatly increase the production of useful data. The California strain, described by Reeves and Hammon, has been isolated from a snowshoe hare in Montana, and serologic studies of hares obtained from Michigan indicate that the majority possess antibodies against this virus. In California it has been demonstrated that, although most infections in man with this agent are of the inapparent type, some infections result in serious disease. A virus closely related to Powassan virus was recovered from ticks from Colorado and is of importance since viruses of this group produce serious illness in man. Studies to date indicate that ticks probably are not the natural vector, but the relation to Powassan virus suggests that mosquitoes would most likely be the vector in nature. In studies of the complex relation of WEE virus with snakes and mosquitoes it has been possible to demonstrate that the virus can be readily overwintered in garter snakes and that mosquitoes can be infected by feeding on such snakes. While we have not been successful in isolating virus from snakes collected in the field, the laboratory data suggest that these or

similar animals could constitute a host suitable for overwintering of WEE virus. In Idaho and Oregon, WEE virus was isolated with considerable frequency during the summer season, while in North Dakota the virus did not appear to be active. Isolations of a considerable number of strains of *trivittatus* and *inornata* viruses were made.

Considerable research was performed to determine the level of viremia attained in wild and domestic birds infected with arbor viruses. After infection with WEE or St. Louis viruses, turkeys, ducks, chickens, and pheasants display levels of viremia which should cause infection in mosquitoes feeding on them. It is of interest, however, that in spite of considerable effort we have been unable to isolate arbor viruses from the bloods of vertebrates. Negative results were obtained in examination of 1,074 specimens collected in Montana, North Dakota, Oregon, and Minnesota during the spring of 1960. These studies fail to add weight to the contention that latent infections of birds are a factor in overwintering or of introduction of virus into endemic areas.

Colorado Tick Fever

Colorado tick fever continues to be a problem in the western United States. Without stimulation of physicians a large number of specimens for examination were received this year and virus was isolated from 49 of them. Our interest in the spectrum of symptoms has continued and we still see severe cases of illness due either to encephaliticis or bleeding tendencies. It was found that the complement-fixation reaction developed at the Rocky Mountain Laboratory is the simplest method for diagnosis of Colorado tick fever. Vaccine has been prepared and has been shown to be efficacious in mice. This type of vaccine has been used repeatedly in man without ill effects, indicating that a vaccine prepared from suckling mouse brain is harmless to man when repeated doses are given.

Ticks collected in Estes Park, Colo., were examined for the presence of Colorado tick fever virus. The incidence of infection was found to vary from 5 to 21 percent. This high incidence of infection in ticks accounts for the large number of cases of CTF reported in Colorado annually.

LABORATORY OF TROPICAL VIROLOGY

The activities of this laboratory are conducted at the Middle America Research Unit in the Panama Canal Zone and at Bethesda. The Middle America Research Unit is a joint research effort of the National Institute of Allergy and Infectious Diseases which has cognizance for studies on virus diseases and the Walter Reed Army Institute of Research which has cognizance for studies on fungus diseases.

Virus Isolates

During the first 12 months of a 3-year project on the ecology of arthropod-borne viruses in the tropical rain forest, conducted by the Gorgas Memorial Laboratory with the collaboration of MARU, major emphasis has been on virus isolation in suckling mice and hamster kidney cell cultures. Fourteen virus strains were isolated at MARU from 412 pools and 63,000 specimens provided by GML. Virus isolation rates were for *Phlebotomus*, 1:700 and for mosquitoes, 1:7000, although the rates varied greatly with species. Of the five *Phlebotomus* isolates, two of broad host range (including cell culture) and short incubation period are serologically identical. These viruses have now been identified as the Indiana type of vesicular stomatitis virus. The other three *Phlebotomus* and nine mosquito viruses are being related to each other, to known virus groups and to human and/or animal infection and disease.

Eastern Equine Encephalomyelitis

The prevalence of EEE antibodies in horses and man in two areas of suggested EEE virus endemicity has been determined, allowing an evaluation of the relative usefulness of several serological methods applicable to studies of this type. It was found that the incidence of EEE antibodies in 460 humans tested increased with advancing age (0.8 percent) under 10 years with progressive increase to 9 percent in the 41–50 year group). Complement fixation results on the same sera indicated the probable presence of other group A viruses.

Lizards of species common to this part of Panama were examined as a possible virus reservoir.

Specific EEE virus hemagglutination-inhibitors were found in some of their sera. The occurrence of viremia and HI antibody response following virus inoculation were experimentally confirmed by inoculation of lizards.

Encephalomyocarditis

Previously this laboratory described an outbreak of a fatal disease of swine caused by the EMC virus. The outstanding lesion in pigs dying during the outbreak was acute myocarditis. Since epidemiological observations suggested that natural infection resulted from ingestion of contaminated food, experiments were undertaken to reproduce the disease by feeding virus to young pigs. Viremia and virus excretion from the gastrointestinal tract were found to occur following the administration of brain from EMC inoculated mice. Infected pigs developed high titers of HI and neutralizing antibody during convalescence and had myocardial fibrosis at autopsy. Other studies included demonstration of EMC antibodies in a small number of city rats and rats caught on the affected farm, although wild rodents were found to be negative. Human sera were examined with interesting differences in the results depending on the donors' age: while a substantial proportion of the Panamanian population has been infected with EMC virus, the antibodies were found to be more common in persons of younger age.

Enterovirus Flora in Central America

For a period of 12 months the enterovirus flora of infants at an outpatient clinic in Panama City was systematically explored, establishing a base line of enterovirus fluctuation. The majority of viruses isolated belonged to the ECHO group, although in late 1959 and early 1960 poliovirus type 2 had become very prevalent. This was reflected in an uncommon occurrence of a small outbreak of paralytic disease due to type 2 poliovirus.

Other enterovirus studies have included 1) surveillance for the presence of type 1 poliovirus in Panama in late 1960 as a check on dissemination and threatened spread of this commonly epidemic type, 2) studies on a major epidemic of ECHO–9 virus which swept through the Republic of Panama and the Canal Zone and 3) initiation of a collaborative project on possible relation of enterovirus flora of Guatemalan children to their dietary status.

Mycotic Diseases in Panama

The research program on mycotic diseases has markedly increased local awareness of histoplasmosis in all of its clinical forms, as evidenced by recognition of three disseminated cases, two fatal and one successfully treated, within a period of 18 months. Until then only one fatal case had been described since Darling's original cases in 1906. Ecological and epidemiological studies led to isolation of *H. capsulatum* from eight additional soil samples, bringing up to 16 the total number of recent isolations from Panamanian soil. The fungus has been repeatedly recovered from the organs of trapped ground mammals, confirming its wide dissemination in nature.

Histoplasmin skin test continues to be a major tool for the study of epidemiology of histoplasmosis. Data on 9,200 children between six and 19 years of age have been obtained indicating, as expected, that the percentage of reactors increases progressively with age. The rate of histoplasmin sensitivity varies from 13 to 58 percent among 6-year-olds and from 68 to 92 percent among 19-year-olds, depending on location of their residence. A survey of 631 preschool children (6 months to 6 years) in the Canal Zone demonstrated an increase in hypersensitivity beginning with 3 years of age. A continuing similar study of Panamanian children in a city hospital is now in progress with information on over 800 already available.

Projects on other mycotic diseases have included diagnostic study and therapy of moniliasis, found to be a major superficial mycosis among both indigenous and transient population in the tropics.

Arbor Virus

At Bethesda new projects involved an interesting application of the technique of antiserum pool combinations to typing of arthropod-borne viruses, a wealth of data evaluating experimentally produced EEE virus infection in horses and a promising attempt to develop an inactivated

EEE virus vaccine for human use. The infected horses yielded specific antiserum which is being processed for prophylactic use in cases of human exposure under laboratory or natural conditions.

Accidental laboratory infection of a staff member with an arthropod-borne group C(Apeu) virus led to the first clinical-virological study of a syndrome produced by this important and common group of viruses of the western hemisphere.

LABORATORY OF BACTERIAL DISEASES

The research program of the Laboratory of Bacterial Diseases has continued in the same general areas as last year.

Intracellular Parasitism

These studies deal with possible changes in characteristics of infected and immune cells as the result of parasitism, and the effect of intracellular growth on the parasite. One such notable change of course is the production of specific antibodies by certain cells of immune animals. Effort has been directed toward the study of antibody production by cells *in vitro* and the macrophage was selected as a multipotential cell for such study. Macrophages obtained from the peritoneal cavity of guinea pigs immunized with egg albumin have been found to release antibody *in vitro* for a period of several days. This provides a system for further study of the nutritional or other requirements for continued antibody production *in vitro*, or even in serial cultures. Cells derived from macrophages have been carried in serial tissue culture for several months, retaining their phagocytic ability.

Brucellosis

Studies on brucellosis are conducted at a reduced tempo. There is continuing need to collaborate with other brucellosis research centers throughout the world to try and settle problems of classification and epidemiology of the Brucella. Currently we are doing some laboratory testing of brucellosis vaccine for human use prepared in Russia. There is present interest in this vaccine by the World Health Organization for its possible use in occupational and otherwise continually exposed groups.

Studies on the Staphylococcus are directed toward determining the factors responsible for pathogenicity, and toward development and standardization of tests for measuring relative pathogenicity of strains.

LABORATORY OF CELL BIOLOGY

The activities of the Laboratory of Cell Biology during the calendar year 1960 have been along three major lines: (A) The continued exploration of the metabolism of normal cultured cells, and an approach to the problem of metabolic controls; (B) the mechanism of viral synthesis; and (C) the study of cell cultures deriving from patients with hereditary metabolic disease.

Metabolism of Normal Cultured Cells

A number of significant observations have been made with respect to the amino acid metabolism of cell cultures. There has been no further elucidation of the pathway of serine synthesis; but the mechanism of crystine synthesis has been clarified, in that all the cell lines so far studied have been shown to use the classical pathway involving the demethylation of methionine to homocysteine, the condensation of the latter with serine to form cystathionine, and the cleavage of the latter to cysteine and homoserine. A dual pathway for proline synthesis has been indicated, one involving glutamine as the source of the carbon skeleton, and the other involving arginine by way of ornithine.

An intriguing recent observation has been the finding that a number of factors which are rigorously required by the cells for survival and growth can in fact be synthesized. Their nutritional requirement reflects the fact that they are lost from the cellular pool to the medium at rates which exceed the biosynthetic capacity of the cell; and with a sufficiently high cell population density, when the loss to the medium per cell is sufficiently reduced, the supposedly essential growth factors are in fact not required for survival.

In these cell cultures, unlike bacteria, the bio-

synthesis of amino acids is apparently not inhibited by the product of the reaction; and this mechanism of growth control is apparently not operative. Studies are in progress as to whether enzyme repression or feedback inhibition are effective controls in the biosynthesis of pyrimidines. A quite different control mechanism is perhaps indicated by the demonstration of a growth inhibitor in the supernatant medium of heavy cultures. The chemical nature of that inhibitor is under continuing study.

Studies on the mechanism of resistance to 2-deoxyglucose (2DG) have shown the presence in the resistant variants of compounds which inhibit the phosphorylation of 2DG to the metabolically active inhibitor, 2DG-phosphate. The relationship of that inhibitor to the observed resistance is under continuing study.

Viral Synthesis

A number of important new observations have been made with respect to the mechanisms of viral synthesis. The puzzling wide disparity between the number of physical particles in viral suspensions, and the number of plaque-forming units, i.e. particles capable of initiating infection in susceptible cells, has been partially resolved with the demonstration that after the viral particle has been absorbed by the cell, it may undergo several alternative fates. A large proportion are rapidly eluted into the medium, essentially intact but no longer infectious, presumably reflecting a minor alteration in the protein coat. Some particles remain unchanged within the cell. Others are degraded intracellularly, in that the nucleic acid is exposed and becomes susceptible to intracellular ribonuclease. Only a small fraction of the absorbed viral particles are stripped of their protein and initiate infection.

In the case of poliovirus in the HeLa cell, although the viral protein and RNA are synthesized concomitantly, a partial dissociation has been achieved with appropriate inhibitors of protein synthesis, which completely block the formation of mature virus, but not of infectious RNA. This is of particular importance in relation to the supposedly obligatory relationship between protein and RNA synthesis in growing cells. Of interest also is the fact that metabolic inhibitors which effectively block the synthesis of cellular DNA and of DNA viruses have no effect on the formation of poliovirus. It would therefore appear that poliovirus RNA may be used directly as a template for the formation of virus, without the necessity for intervening DNA synthesis.

In contrast to the situation with poliovirus, in the case of vaccinia, there was a marked lag between the formation of the viral nucleic acid (DNA) and that of the mature virus.

Galactosemia Cell Cultures

An exciting new development has been the successful cultivation from patients with a hereditary metabolic disease (galactosemia) of cells which in culture demonstrate the metabolic defect characteristic of the disease. This suggests an entirely new experimental approach to problems of human genetics.

LABORATORY OF GERMFREE ANIMAL RESEARCH

Animal Studies

A series of observations has been made on the behavior of *Entamoeba histolytica* in the germfree host. In earlier studies with standardized techniques, amoebic lesions were not produced in the germfree animal following inoculation. In fact, the parasite failed to live in the intestine beyond five days. Recent changes have been made in the manner of rearing and handling the amoebae *in vitro* prior to inoculation which seemed to result in more vigorous organisms. The latter have produced lesions in the absence of bacteria, although the type and severity are still not typical of those encountered with a bacterial associate. Thus, it would appear that the latter is not the only determinant of the course and the pathogenesis of the infection.

Some studies are preparatory to an analysis of the nature of the nutritional effects observed in certain parasitisms. For instance, the intestinal mouse parasite, *Nematospiroides dubius*, does not require a bacterial flora to develop from an infective larva to the adult form in the host; but apparently, it does require a flora to develop from the egg to infective larva. Another observation

is that the sex of the host, although noted by several workers to be a factor in the outcome of infection by this parasite in conventional (contaminated) animals, does not appear to be a factor in the germfree host.

Guinea Pigs

In studies on the growth and biology of germfree guinea pigs, a staff investigator has obtained several advanced pregnancies in animals maintained on irradiated diets, although no fetus was carried to term. It is to be recalled that germfree guinea pigs have not yet been bred with success. The importance of the intestinal flora to this species was pointed up by the finding that conventional (contaminated) guinea pigs reproduced normally on this same irradiated diet.

In a collaborative project with an investigator at the University of Pennsylvania, it has also been shown that the use of large dosages of a cathartic, or the application of tourniquet shock, increased the number of red cells of germfree chickens coated with human B-like antigens following monoinfection with *Escherichia coli* 0_{86}. These studies are providing information on the manner in which red cells of one type may acquire antigenic characteristics of other cell types, especially B.

Mouse Colony

The germfree mouse colony has been undergoing an intensive serologic study including an assay for the presence of certain so-called "natural antibodies" against a variety of bacteria. Such antibodies or antibodylike reactivities for organisms like *Staphylococcus*, *E. coli* and *Salmonella typhosa* have been found to occur in a variety of uninoculated conventional animals and are presumed to originate from encounters with the viable organisms or related antigens. Animals which have lived for many generations free from contact with live bacteria are almost the *sine qua non* for establishing finally the validity of these ideas. Studies thus far, with the Communicable Disease Center and investigators in the National Cancer Institute, have shown that germfree mice 2–3 months of age were free from antibodylike reactivity toward *Staphylococcus* and *E. coli* antigens. Reactivity, however, toward *S. typhosa* was obtained in several instances, although no evidence of the presence of the latter was found in the germfree colony. Thus, this finding strengthens sporadic reports that nonbacterial substances (perhaps in this case dietary components) can cause "cross" reactions with this organism.

Tumors

The germfree animal colony and a conventional colony derived from the same stock now has existed for approximately 2 years. Some of our exbreeders, are one to two years of age. Whenever a germfree or conventional animal not on an experiment dies, especially if it is 6 months or more of age, it is examined thoroughly for gross evidence of malformations or tumors. Among approximately 50 such animals so-called spontaneous lung tumors have occurred in some of the germfree as well as the conventional mice. While the numbers of animals are obviously small, the incidence has been markedly *higher*, thus far, among the conventional animals (those exposed to external contamination) than among the germfree. This seems to be particularly true among animals 6 to 12 months of age.

LABORATORY OF PARASITE CHEMOTHERAPY

This country's commitment of $38 million in fiscal year 1961 toward a program of world-wide malaria eradication and the long-term interest in malaria by most of the senior staff resulted in a research effort, during the past year, largely directed toward problems in that field. Special emphasis was given to the study of simian malaria in man and in monkeys because malaria in simians might be a real deterrent to the eradication program. Clinical facilities for volunteers at the Atlanta Penitentiary were enlarged and the staff increased. A laboratory was established at Kuala Lumpur, Malaya, in cooperation with the Malaya Institute of Medical Research and the United States Medical Research Unit. Studies on several aspects of the simian-human-malaria problem have been in progress there since mid-August.

As a result of the above development, it was

decided to move the Section on Cytology, now located at Memphis, to Chamblee, Georgia, early in 1961. This arrangement will bring the simian hosts closer to the human volunteers at the penitentiary, and the insectary maintained by the Section will be geared to accommodate the work at Chamblee and at the prison.

Malaria—Human

Plasmodium falciparum (McLendon strain): Chloroquine (300 mg, base) and primaquine (45 mg, base) given together beginning three days after mosquito bites and weekly thereafter for a total of eight doses, resulted in suppressive cure in 5/5 subjects. Controls were positive 11 to 15 days after infection. After two days of parasitemia, each control was given the above drug combination which was repeated weekly for a total of three doses. Parasites were removed promptly and cure was obtained based on no evidence of infection during 227 days of observation.

Primaquine, at daily doses of 0.75 mg, had some sporontocidal effect upon *Plasmodium falciparum* gametocytes but none against those of *P. vivax* (one case). Therapeutic doses (1.4 gm in three days) of amodiaquine had no sporontocidal effect against gametocytes of *P. falciparum* (one case). The effect referred to is against the development of the malaria parasites in the mosquito.

A strain of *Plasmodium falciparum* from Colombia, South America, was found to be resistant to chloroquine. This finding is of utmost importance in terms of malaria eradication.

Plasmodium vivax (Chesson strain): A drug combination of primaquine (45 mg) and pyrimethamine (50 mg) given weekly beginning seven days after mosquito bite and continuing for a total of four doses, gave suppressive-cure in 4/5 subjects; the other subject developed a patent infection 240 days after infection. Pyrimethamine (50 mg) given alone, as above, produced suppressive-cure in 1/4 subjects; the other three came down on days 82, 83 and 84. Five controls all came down 12 to 13 days after infection.

The Russian 8-aminoquinoline, quinocide, was compared with primaquine and found to be distinctly inferior as a curative drug against early and late primary attacks of Chesson vivax malaria particularly from the standpoint of the occurrence of second and third relapses.

Another 8-aminoquinoline, Win 5037, was studied in five subjects. Toxic effects and failure to cure made further investigation unwarranted.

Plasmodium malariae: The results of a 14-year study of the biology of *Plasmodium malariae* were drawn together for publication. The highest infectivity for mosquitoes occurred during the eighth to tenth weeks of the primary attack. Although the infection rate of mosquitoes was ordinarily low, the relatively long period during which mosquitoes could be infected may explain the persistence of *P. malariae* in nature. The ability of the symptom-free malarious patient to infect mosquitoes at a rate similar to that of the symptomatic patient makes eradication difficult.

Malaria—Simian

Plasmodium cynomolgi bastianellii: In early May, two accidental sporozoite-induced infections with *Plasmodium cynomolgi bastianellii* occurred at our Memphis Laboratory. This happening was of signal importance because it showed that simian malaria, contrary to the generally held opinion, was infectious to man. In that light, full scale study of human infections was undertaken at our Atlanta Penitentiary installation.

Two infections were induced in inmate volunteers by inoculation of infected blood obtained from one of the accidental sporozoite-induced infections in man. Twenty inmate volunteers were infected by bites of *Anopheles quadrimaculatus* or *Anopheles freeborni* which had fed on infected monkeys. The prepatent period ranged from 14 to 29 days and the parasite density ranged from 5 to 500/cmm. The most constant symptom was headache and the most significant signs were fever, splenomegaly and hepatomegaly. Infections were allowed to run their course, generally without treatment.

Anopheles freeborni were infected from two patients but attempts to infect volunteers by their bites have yielded equivocal results. The finding that *P. c. bastianellii* will grow consistently and produce clinical illness in man suggested the possibility that malaria is a zoonotic disease, that is, a disease which man can acquire from animals with which he is associated. Whether or not such transfer occurs in nature is not yet determined, but should it occur, it would be of greatest sig-

nificance to the world-wide malaria eradication program.

Plasmodium cynomolgi cynomolgi: Eleven inmate volunteers were bitten by *Anopheles freeborni* infected with *P. c. cynomolgi* on 8 September, and to date (14 December) three have exhibited evidence of infection (i.e., fever). Parasitemia has been demonstrated in only one, on the 58th day after mosquito bites. These results show that this strain infects man far less readily than *P. c. bastianellii.*

Field Studies in Malaya

Three staff members, Drs. Eyles, Dobrovolny, and Mr. Clinton S. Smith, were detailed to Malaya during the year where they engaged in the study of simian and human malaria in cooperation with the Malayan Institute for Medical Research and the U.S. Army Medical Research Unit at Kuala Lumpur.

The epidemiology of monkey malarias is being studied and the feeding habits of some of the *Anopheles* determined. By injection of uninfected monkeys with sporozoites from natural infections, it was determined that *Anopheles hackeri* is a natural vector of *Plasmodium knowlesi.* This is a most important discovery, especially since the vector of this parasite has been sought for repeatedly during the last 25 years.

Studies of malaria in aborigenes associated with monkeys have been made. Blood passed from aborigenes to monkeys have thus far produced no patent infection in the monkeys.

EE Stages and Drug Action

Studies were continued on the direct effect of drugs on the exoerythrocytic stages of primate malaria. When sulfonamides were used with pyrimethamine to exploit the possible synergism of the two drugs, monkeys developed parasitemia 30 to 40 days after inoculation with sporozoites even though all parasites observed in liver biopsies were damaged. The curative efficacy of quinocide, the Russian drug, was compared with primaquine. Even when administered at twice the dosage used with primaquine, quinocide was less effective. Chloroquine had no observable effect upon the liver forms of *Plasmodium cynomolgi.* Young parasites appeared in the blood in large numbers on the 8th, 16th, and 24th day indicating the existence of secondary exoerythrocytic generations.

Insect Tissue Culture

Blood cells from caterpillars and cells of the ovariole sheath of several species of moth pupae have been cultivated in several different media. The virus of St. Louis encephalitis has been maintained in cultures of hemocytes from larvae of the catalpa sphinx for ten days. Oöcysts of *Plasmodium gallinaceum* attached to the midgut of *Aedes aegypti* have shown growth *in vitro* and sporozoites have been produced.

Biochemical Studies

It was shown that mosquitoes infected with malaria have higher levels of ribonucleic acid than uninfected mosquitoes. Chromatographically, the acid-hydrolysate of ribonucleic acid from a pyrimethamine-resistant strain of *Plasmodium falciparum* differs from the acid-hydrolysate of ribonucleic acid from a pyrimethamine-susceptible strain. Bephenium hydroxynaphthoate inhibited glutamic acid transaminase of *Nippostrongylus muris.* Bephenium chloride and quinacrine reduced the rate of glucose absorption by the tapeworm *Hymenolepis diminuta* but low concentrations of dithiazanine iodide stimulated glucose absorption by this cestode.

Intestinal Parasites

Epidemiological studies on the inmates of a mental institution show a high persistence of *Trichuris* and hookworm for six years, with an apparent decrease in Strongyloides. To test dithiazanine and tetrachlorethylene, alone and in combination, heavily parasitized mental patients were given the drugs for about one year. A large number of worms were removed but the cure rate was low and transmission was not stopped. Bephenium hydroxynaphthoate and bephenium chloride were used with good results against hookworm, *Ascaris* and *Trichuris.*

Schistosomiasis

The activity of griseofulvin observed in mice infected with *Schistosoma mansoni* was not well

developed in hamsters or monkeys. A series of tetracycline analogues which show an affinity for microfilaria did not combine with schistosomes and were without activity. One of these analogues was significantly more active against microfilariae of *Dirofilaria immitis* than tetracycline.

In many tests, the efficacy of stibophen (Fuadin) therapy on mature *Schistosoma mansoni* infections in mice was increased up to 16 times by feeding a balanced semi-synthetic diet. The toxicity of the drug was not similarly increased. The enhancement of curative action by the purified semi-synthetic diet was thought to be due to the absence of, as yet unidentified, inorganic salt(s) that interfere with drug activity. It was found in mice fed on the purified semi-synthetic diet that higher blood levels of the drug were maintained for a longer period than when the same amount of Fuadin was injected into mice fed on the commercial pellet diet, suggesting that the increased cure-rate was due to higher blood drug level. Similar drug advantage was observed in mice given tartar emetic while on the purified diet.

LABORATORY OF PARASITIC DISEASES

This Laboratory continues to emphasize fundamental studies on parasites and parasitic diseases. No important changes in the program were instituted during the year. The program of the laboratory is well diversified considering the size of the staff and the competencies of the various staff members cover a large proportion of the field of parasitology.

Although the emphasis is on basic studies, this does not imply a narrow viewpoint and the laboratory is well aware of the many practical problems parasitic diseases create throughout the world. The laboratory is often called upon for help and advice concerning prevention and control of parasitic infections and so must maintain competence, and a reputation for competence, to deal not only with basic problems of parasitism but also problems of prevention and control of parasitic diseases. Therefore, the laboratory continues to carry on a variety of activities which help it maintain its international reputation and increase its capacity to cope with problems of parasitism. Such activity also returns benefits in the forms of ideas for laboratory research and clues which may explain puzzling laboratory findings.

Toxoplasmosis

Studies on toxoplasmosis in New Zealand sheep have shown that the prevalence is high. New information has been obtained concerning the distribution of the organisms in the tissues and their persistence there. After inoculation the distribution of the parasite in tissues is erratic and the parasites rapidly disappear from tissues other than the muscle and placenta. Since residual infection occurs in muscle, mutton may serve as a source of human infection. Congenital infection with *Toxoplasma* is an important medical problem, therefore it is of special interest that the sheep studies have indicated that inoculation of sheep 60 days before pregnancy did not result in congenital infection or abortion but inoculation at 30 days pregnancy caused abortion or foetal death with absorption. Infection at 90 days pregnancy was less likely to be dangerous to the foetus.

The status of resistance or immunity to *Toxoplasma* continues to be puzzling, since living organisms fail to protect completely animals against challenge, especially when the challenge is great, and because low grade parasitemia may persist for months in mice and rabbits in the presence of high serum antibody levels. The observation that cysts of *Toxoplasma* probably form in tissue cultures provides a new opportunity to study the manner of cyst formation and the factors that lead to cyst formation.

Amoebiasis

The work on the preservation of living *Entamoeba histolytica* and other protozoa has practical significance since success would permit retention of strains without continuous sub-culturing. This is a relatively new field and techniques are still evolving. The work so far has shown that this approach is feasible since four species have been frozen and stored for periods ranging from one to four months depending on the species involved. *E. histolytica* has been kept at −197° C. for 24 hours, suggesting that almost indefinite storage at this temperature may eventually be achieved.

Laboratory culture of *E. histolytica* continues to receive attention since it is so important to learn more concerning its nutritional requirements and its pathogenicity in the absence of other organisms. It is noteworthy that satisfactory axenic culture of this species has been achieved for the first time. The protozoa are cultured in a complex diphasic medium containing no cells but including chick embryo extract.

The substitution of a species of Crithidia for *Trypanosoma cruzi* in cultures of *E. histolytica* provides a more economical and rapid way of producing large cultures of the amoeba. Demonstration of the value of the Coulter Counter for the enumeration of protozoa in suspension adds a valuable tool for quantitative work and suggests this method may be applicable for counting other organisms of similar size such as tissue culture cells.

Parasitic Infections in Germfree Animals

The use of germfree animals in worm-parasite studies continues to reveal the value of this tool and adds to our knowledge of the peculiar nature of the germfree state. The technique seems to be particularly useful for studying conditions that influence natural resistance and nutritional relationships of parasite and host. For example, it was found that the roundworm, *Nematospiroides dubius*, develops as well in germfree as in conventional mice but while in conventional mice the worm recovery is much higher from the male animals, the recovery from germfree mice is the same for both host sexes. The cause of the difference is unknown. Also, it has been shown that the feces of germfree mice do not support development of *N. dubius* larvae and that bacteria in the feces provide important factors for larval development. There was further evidence that the alteration in levels of serum protein components in germfree animals is due to dietary factors.

Sterile Culture of Worms

Studies on the sterile culture of worms continues to produce fundamental information on the nutritional requirements of the parasites and brings closer the day when we can use the axenic animals for immunologic and therapeutic studies. Survival studies using relatively advanced larvae of *Nippostrongylus muris* has produced important results. The intent has been to try, by addition of elements to the medium, to induce the larvae to reach the adult stage. Starting with a salt mixture, dextrose was added until the optimal level was reached. Then casein was added and survival time rose to 11 days, but there was not development of the larvae. Addition of a yeast extract to this mixture not only increased survival but permitted growth to the adult stage. Thus, a much more simple medium than used before has been evolved and the achievement of a defined medium for culture of *N. muris* adults is much closer. A similar approach is being used in attempts to culture microfilariae of *Dirofilaria immitis*.

Nutrition and Schistosomiasis

Although the study of the relation of nutrition to schistosomiasis in Puerto Rico is still incomplete, it appears that enrichment of the diet does not affect the number of eggs passed in the feces. However, it is interesting to note that the enriched diet did cause a loss of hookworms and whipworms from the intestine. This has a bearing on the problem of the existence of hookworm infection without hookworm disease. In laboratory studies conducted in Bethesda the enhanced efficacy of stibophen in mice receiving a semisynthetic diet was shown to be due to the absence from this diet of as yet unknown inorganic salts.

Dual Virus and Helminth Infections

Interaction of two pathogenic organisms in the same host has had relatively little attention in spite of some very provocative work done in years past. A study of simultaneous infection with encephalomyocarditis virus and *Trichinella spiralis* in rats has produced striking and significant results. While the virus alone does not injure adult white rats when given intraperitoneally, in the presence of *Trichinella spiralis* infection many of the rats are crippled and die. This potentiation of virus pathogenicity is not due to nonspecific stress but seems to be related to the presence of the worms on the muscles. The virus can be recovered from the muscle of *T. spiralis*-infected rats but not from muscle of rats without

T. spiralis. The reason for the influence of the worm infection on the activity of the virus is unknown. The phenomenon offers an opportunity to study some of the fundamental factors in the pathogenesis of both the virus and the worm parasite. It also provokes the question as to what effect this worm infection may have on other virus infections.

Ammonia Toxicity in Mice

The study of liver damage in relation to ammonia toxicity in mice has revealed that low oxygen in breathed air greatly enhances ammonia toxicity. The mechanism of this effect is not clear. Though hepatic coma is usually considered to be related to ammonia toxicity none of the substances which exacerbate hepatic coma in man increases ammonia toxicity in mice. In fact, six of ten decrease it. Ammonia toxicity in mice was greatly reduced by hypothermia and this suggests that the same measure may be useful in treating hepatic coma in man. Finally, mouse liver damage was induced in eight different ways but none caused any change in the animal's response to intravenous ammonia.- Thus, though high blood ammonia levels seem to be related to liver damage, the causal relationships are by no means clear.

Helminths

Fundamental physiological studies have focused on the calcareous corpuscles of tapeworms and on the phospholipids of tapeworms. The calcareous corpuscles are amorphous but, on heating, dolomite, brucite or apatite may be formed. Electron microscope pictures of corpuscles heated with KOH reveal the presence of well-formed crystals. The glycerol containing phospholipids of *Taenia taeniaeformis* are about half lecithid and half cephalin. Sphingomyelin is present and more than one cephalin is known to occur in the larvae of this tapeworm. Hexose-containing phospholipids occur in both larvae and adults.

Study of the mechanism of energy metabolism of sub-cellular elements has dealt, among other things, with the mechanism by which mitochondria which are depleted of high-energy phosphate intermediates are stimulated to oxidize substrates when ATP is added. This is a complex, though fundamental, bioenergetic system for which a better understanding is needed. Addition of ATP not only restored succinate oxidation but also caused reduction of intra-mitochondrial DPN. The succinate oxidation involves an energy-requiring reaction and this energy is apparently added at one site in the respiratory chain and used at another for reducing pyridine nucleotide.

LABORATORY OF BIOLOGY OF VIRUSES

The basic objectives of this laboratory continue to be the same as last year. It is obvious from this annual report that four out of five units have projects with the same general objective—investigation of mechanisms and localization of animal virus synthesis within the infected cell. Each of these units is also interested in the infectious nucleic acid of viruses. In view of the complexity of this problem and the important implications of any information that is obtained, this "duplication" is quite justified. Actually, it is not duplication since different approaches are used and different virus-cell systems are studied.

The electron microscope has been installed and is now used not only by the Biophysical Unit but also by other units of our laboratory and by units of the Laboratory of Infectious Diseases. With studies on the structure of viruses and a project concerned with the genetics of animal viruses added to the biochemical and biological studies, there is now fairly complete coverage of the important facets of basic virus biology.

Intracellular Location of Poliovirus

By use of radioautographs and staining with fluorescein-tagged antiviral antibody, the intracellular location of poliovirus antigen—presumably viral protein—during the cycle of virus multiplication has been determined. Demonstrable antigen first appeared one hour after infection and was diffusely distributed through the cytoplasm. At three hours, just before the appearance of new virus, it was present throughout the nucleus with a tendency to be concentrated around the periphery of the nucleolus. At five to seven hours, particulate accumulation of antigen in the cytoplasm was noted. Incorporation of radioac-

tive-tagged amino acid into cell protein ceased shortly after the start of infection, whereas incorporation of thymidine into RNA continued until after three hours and tended to localize in the nucleoli.

Mutants of EMC Virus

Plaque type mutants of EMC virus have been found, segregated and characterized. The stability of the mutants has been determined and the plaque type shown to be a function of the viral RNA. It has been shown that the difference in the size of the plaques formed by these mutants is brought out by an inhibitor present in the agar overlay used on the plaque plates. This inhibitor resides in the agaropectin fraction of the agar and can be separated from the agarose fraction which then permits both plaque type mutants to form similar sized plaques.

Polyoma Virus

By the use of a serum protection test in newborn hamsters, evidence was found that polyoma virus transforms normal cells to tumor cells quickly and directly without extensive virus multiplication being necessary. Furthermore, no evidence could be found to suggest a lysogenic relationship of virus to tumor cell. All attempts to show the presence of infectious or masked virus or of virus antigen in transplantable polyoma-induced tumors have been negative. It appears that once the virus initiates the tumor it is no longer required for tumor growth and maintenance.

Tetracycline Fluorescence

The discovery has been made that when the antibiotic tetracycline stains tissues in such a way that they fluoresce under UV light, this fluorescence is localized in the mitochondria of the cells. This makes a convenient vital stain of these subcellular elements for further studies. There appears to be some similar localization of the antibiotic fluorescence in certain bacteria.

TMV Model

A complex model of tobacco mosaic virus has been constructed on theoretical grounds, and on checking this model against known biochemical and biophysical properties of the virus a remarkable consistency is found. Certain refinements of electron microscopic technics have produced photographs of this virus which reveal previously not seen fine structure also consistent with the theoretical model.

LABORATORY OF IMMUNOLOGY

Since the activation of the Laboratory of Immunology in 1957, the program has been concerned, principally, with basic research. However, for some time an important need has been felt for the initiation of clinical studies in immunology and allergy. In September 1960 the Clinical Immunology Section was activated and as space permits, will be expanded and will work in close collaboration with the Laboratory of Clinical Investigation on clinical studies involving immunological aspects of such diseases as lupus erythematosus, nephritis, and chronic thyroiditis, in which an auto-immune basis is suspected.

Allergic Thyroiditis

Experimental allergic thyroiditis was produced in Strain 13, inbred, histocompatible guinea pigs by immunization with a single dose of guinea pig thyroid extract in complete Freund's adjuvant. Thyroiditis developed as early as five days after immunization, was present in all animals at 16 days, and by seven weeks was consistently present and generally severe. Delayed skin test hypersensitivity was found as early as five days after immunization in nearly all animals, and was present in all animals with thyroiditis at seven weeks. At seven weeks after immunization, anti-thyroid antibodies were present, and antibody titres correlated with the presence and degree of thyroiditis. This correlation was not found at certain other times after immunization. The presence of delayed hypersensitivity was correlated with experimental allergic thyroiditis, while the presence of circulating antibody did not correlate with thyroiditis. These observations constitute the earliest production of experimental allergic thyroiditis and the most severe disease at the time intervals studied.

House Dust Allergens

Studies on the chemical and physical properties of house dust extracts that are used clinically for the diagnosis and treatment of house dust allergy have been studied to identify the components responsible for the specific skin reactions produced in house dust sensitive individuals. It has been found that the house dust extracts consist of a heterogeneous mixture of acidic polysaccharides. The heterogeneity has been demonstrated by electrophoretic and ultracentrifuge sedimentation analysis and also by the multiplicity of cross reactions obtained with antisera to the various pneumococcal polysaccharides. The chemical composition of the various fractions has been shown to be roughly 5 to 20 percent polypeptide and 80 to 95 percent polysaccharide, containing about equal amounts of uronic acid (probably glucuronic acid), D-glucose, D-galactose, D-mannose with lesser amounts of L-rhamnose and L-arabinose.

Genetics of Gamma Globulin

Agar-gel immunochemical analysis of sera from rabbit litters, with precipitating antibodies prepared in rabbits, has shown that seven antigenic determinants of the gamma globulins are genetically controlled by at least two gene loci with each specificity exhibited when the appropriate allele is present. Since the gamma globulins are soluble proteins which have properties of both an antigen and an antibody, they should be subject to quantitative estimation and cytological localization. This immunogenetic system, therefore, may be uniquely suited for the study of certain basic problems in genetics, embryology, immunology and protein chemistry.

In other studies, antibodies to human serum proteins were prepared in monkeys since this animal, being a closely related species, might be more discriminating for minor antigenic differences than a distantly related species. Three "slow" gamma globulins were found, instead of the one usually detected with horse or rabbit antibodies. Two of these were shown to be related to myeloma proteins. The quantitative estimation of these gamma globulins in serum should be helpful in the early diagnosis and study of diseases, such as multiple myeloma, which involve qualitative and quantitative changes in the gamma globulins.

Hypersensitivity

The genetically distinct guinea pigs of inbred Strains 2 and 13 have proved to be a very important immunological tool. After studies established the fact of skin compatibility in the two strains, experiments were conducted to transfer cells with a measurable biological activity. Transfers of tuberculin sensitivity were undertaken by the intraperitoneal injection of living lymphoid cells from compatible donors. The almost quantitative transfers between inbred guinea pigs were a reflection of the continued viability of the active cells in the recipients.

Two models are being developed to study the mechanisms of immediate and delayed hypersensitivity in the inbred guinea pigs; protracted anaphylactic shock and, the massive local hemorrhagic reaction, respectively. It has been shown that there are differences in susceptibility to hypersensitivity reactions. Strain 2 guinea pigs were more resistant to death by bronchospasm and tended toward a protracted syndrome in anaphylactic shock. Both Strain 2 and 13 guinea pigs required more mycobacteria than did random-bred Hartley guinea pigs for inducing "delayed" sensitivity to egg albumin, using Freund's adjuvant.

Human Serum Auto Antibodies

Fractions of human serum separated by anion-exchange cellulose column chromatography were studied by immunoelectrophoresis. The conditions for elution of eighteen immunologically distinguishable human serum proteins from the columns were determined. Gamma globulin obtained under the appropriate conditions by this method was found to be pure; rabbits immunized with this fraction made antibodies to none of the other serum proteins. By the use of anion-exchange cellulose columns, it has been found possible to separate the 7S from the 18–19S antibody activities in sera of patients with thyroiditis and lupus erythematosus. Initial results indicate that the addition of immunoelectrophoretic characterization of these and other sera will be extremely helpful in our aim of characterizing the antibody activities found in human serum.

Fluorescent Antibody Staining of Malaria Parasites

The fluorescent antibody staining of the human malaria parasite, *Plasmodium vivax*, has been recorded for the first time. A globulin fraction of convalescent serum from a patient having a long-standing infection with *P. vivax* was labeled and the fluorescent antibody applied to thin blood films containing the parasite. The organism was visible by virtue of its specific immunofluorescence. Fluorescent antibody studies were conducted on *P. cynomolgi bastianellii*, the monkey malaria parasite which, recently, has been shown to be transmissible to man. Considerable morphological detail was observed at fluorescence. Preliminary studies on the staining reactions, as based on degrees of fluorescence, indicate that *P. vivax* and *P. cynomolgi bastianellii* parasites may have common antigens and that the two species may be closely related

LABORATORY OF INFECTIOUS DISEASES

In 1960 the Virus and Rickettsial and Epidemiology Sections of this laboratory continued integrated and comprehensive efforts to define the importance of virus infections in disease. Field investigations of human and animal virus infections were made possible through collaborations with a number of other organizations, including the Bureau of Medicine, USN; the District of Columbia Children's Hospital Research Foundation; the District of Columbia Welfare Department; the New York City Health Department; the National Cancer Institute; the National Institute of Allergy and Infectious Diseases; the Laboratory of Clinical Investigation, NIAID; and in Paris, France the Laboratoire des Virus, Hopital Saint-Vincent-de-Paul; and Le Centre Claude-Bernard de l'Hopital Saint Louis.

Natural events and opportunities afforded by our collaborators shaped the course of most field studies. Technical breakthroughs in the laboratory made it possible to take fuller advantage of these opportunities to study natural disease and thus acquire not only new information about specific virus infections, but also to move nearer our ultimate goal, namely, a clear view of the numerous viral causes of human diseases sufficiently comprehensive to make concerted efforts to control them appear feasible and worthwhile.

Virus Pneumonia

Pneumonia and other lower respiratory tract infections continue to represent major causes of death and a large segment, presumed to be viral in origin, is still uncontrolled. Until recently it was wholly undefined. During 1958 and 1959 our studies at Children's Hospital and Junior Village helped define the relative importance of adenoviruses, para-influenza viruses, and influenza viruses in causing lower respiratory illnesses of childhood. The data suggested that as much as 40 percent of croup bronchiolitis and pneumonia were explained by these viruses. In 1960, using more sensitive methods, we were able to explain a much larger percentage of such illnesses, chiefly because we were now able to assess the very significant contributions of respiratory syncytial virus (RS) to the respiratory disease problem. Early in the year large outbreaks of RS virus were intensively studied both at Children's Hospital and Junior Village. Over 80 strains of RS virus were isolated from children with pneumonia and 60 percent with bronchiolitis yielded RS virus, whereas virus was recovered from less than one percent of comparable control patients without respiratory illness.

Retrospective analyses of serologic surveys of respiratory illnesses in Children's Hospital since 1957 suggested that perhaps 20 percent of all lower respiratory illnesses observed during the last three years was due to RS virus. Thus, considering the contributions of adenoviruses, para-influenza viruses, influenza viruses, and "PAP" virus it now appears that 50 to 60 percent of the more severe respiratory illnesses of young children can now be explained and, hopefully, controlled. Except for influenza virus (which contributed probably less than 5 percent of the total), the LID respiratory virus unit personnel played key roles in the discovery of the first representatives of each of the other virus groups—adenovirus, para-influenza, and RS. Delineation of still undefined viral causes of the respiratory disease syndrome represents the major challenge to respiratory disease investigators for 1961.

During 1960 several experimental but commercially prepared killed vaccines containing various

combinations of adenoviruses (6 types), para-influenza viruses (3 types), and Coxsackie B viruses (5 types) were tested in Junior Village. The evidence suggests that while modestly antigenic, the vaccines had insufficient potency to be regarded as satisfactory for larger scale studies.

Primary Atypical Pneumonia

The etiologic role of PAP (Eaton's virus) in primary atypical pneumonia suggested earlier by Eaton and Liu, was finally fully established in 1960. In cooperation with the Bureau of Medicine, USN, the continuing "epidemic" of virus pneumonia in Marine recruits at Parris Island was studied in several ways. Serological studies showed that 51 percent of 530 pneumonia cases had antibody rises to PAP virus; only 6 percent revealed contemporary infection with adenoviruses. Serologic studies of infection showed PAP virus to be much more common than disease; approximately 30 recruits were infected for each case of pneumonia, information vitally important to fuller comprehension of the natural history of this important virus.

In 1959 treatment of Parris Island pneumonia cases with broad spectrum antibiotics (tetracyclines) appeared to reduce the severity and the duration of the Eaton pneumonias. In 1960 the efficacy of a new tetracycline drug, demethychlortetracycline, was tested in a well-controlled double blind study including 290 pneumonia patients. The drug greatly reduced the severity and duration of pneumonitis and fever in those shown to have serologic responses to PAP virus. These findings, based on accurate laboratory diagnosis, fully confirm earlier but controversial reports of the efficacy of tetracyclines in atypical pneumonias. It also adds further support to the importance of the Eaton virus as a cause of virus pneumonia.

An additional link in the chain of evidence establishing the PAP virus as an important cause of pneumonia was achieved recently in collaborative studies with the Laboratory of Clinical Investigation, NIAID. Volunteers inoculated intranasally with PAP virus grown in tissue cultures reacted with a wide gamut of respiratory signs and symptoms, including pneumonitis characteristic of PAP.

Common Colds and Viruses

Recent studies have served to clarify and enlarge existing concepts of the etiology of common mild respiratory illnesses in adults. It is now quite clear that instead of a few specific closely related viruses, numerous viruses belonging to different groups each contribute in part to the syndrome called the "common cold." Thus the newer viruses (adenoviruses, para-influenza viruses, respiratory syncytial virus and others), together with older agents (influenza viruses and certain bacteria), each contribute only a small proportion of the milder respiratory ailments of adults. They contribute a larger segment of more serious diseases, particularly in children. Very recent reports of common cold viruses from England, together with the prior reports of agents with somewhat similar properties in this country, served to focus our attention on these viruses in 1960. Together with investigators elsewhere, it was found that most, if not all, of these agents—the British HGP and FEB, the American 2060, JH, Coe and PETT viruses which grow selectively and rather "fussily" in human epithelial cell lines, really represent "fastidious" enterovirus strains which have (as do almost all Coxsackie A's and some ECHO viruses) special growth requirements. These viruses, as do a number of still unclassified agents found in Junior Village during the past several years, have properties very similar to the Coxsackie A viruses; indeed, several have been shown, on the basis of serologic markers and/or by suckling mouse pathogenicity, to be indistinguishable from Coxsackie A viruses.

New Serological Test Procedures

The laboratory section of the Epidemiology Section concentrated on the development, application and evaluation of *in vitro* test procedures for the identification of new viruses as well as for detecting virus infection as expressed in antibody responses. Thus, using conventional complement fixation (CF) and newly developed hemagglutination inhibition (HI) procedures it has been possible for our group to type thousands of virus isolates belonging to the adenovirus, myxovirus, enterovirus, and reovirus groups. As was true during the past several years, LID in 1960 again described and characterized more new

representatives of these viruses than all other virus laboratories in the world combined. This was made possible during 1960 because each of our various virus research units contributed new diagnostic techniques. One group developed additional specific HI procedures for identifying adenoviruses and adenovirus infections; and for reoviruses and enteroviruses as well. Similarly, another group developed tissue culture procedures for isolating Eaton's PAP virus, while others not only discovered several "new" mouse viruses in tumor virus study systems, but developed serological procedures for recognizing their presence.

Serologic Reagents

But the availability of simplified procedures are of very little use unless the necessary reagents are also available. Although many virus research laboratories could do the tests, few laboratories are able to produce the necessary reagents. The magnitude and cost of producing and certifying them promise to continue to exceed any possible resources available. This fact has had a very depressing effect on research efforts aimed at the study of viruses as causes of disease, and serves as yet another deterrent to early delineation of the common virus diseases as public health problems. Consequently, with the help of NINDB and Microbiological Associates, LID in 1959 and 1960 accepted responsibility to develop and evaluate more than a hundred commercially produced virus antigens. LID, of course, has been active in the certification of virus prototypes and furnishes many to the Virus Registry of the American Type Culture Collection. It is also collaborating with the Enterovirus and Adenovirus national committees in setting up standards for large scale production of certified antiserums for serotyping and classification of viruses, perhaps the highest priority need of all virus laboratories concerned with human infection and disease.

Reference Laboratory for Viruses

Wholly through the operation of circumstances, the Virus Section of LID has become virtually the chief (in many instances only) reference laboratory for many of the newer viruses, including adenoviruses (about 30 human and several animal serotypes), myxoviruses (five new parainfluenzas occurring in three species), reoviruses (three serotypes in four species), many of the newer and some older enteroviruses (5–10), salivary gland viruses (from four species), and new mouse viruses (six), the latter frequently found in tumor virus study systems.

Until virus reagents desperately needed for many extremely common viruses are made available either commercially, through government agencies, or both, LID as the sole custodian of many of these agents cannot avoid responsibility for assisting other excellent virus laboratories to identify their viruses, and on a pro-tem basis at least for keeping order in the general virus field. Unfortunately it has no specific commitment to provide such services and even worse, no specific budget to cover them, so that the involuntary, constantly growing and unavoidable service functions must be done at the expense of research missions.

However, it must be admitted that the simpler virus diagnostic techniques and the availability of a complete supply of viral reagents in the laboratory (developed out of necessity) facilitate not only epidemiologic studies of naturally occurring virus infection but also enable it to evaluate the significance of the data furnished by other laboratories who come for technical assistance.

Cancer Viruses

Studies of cancer viruses can be subdivided into several categories: (a) Laboratory studies of the properties of cancer viruses and development of laboratory tools for detecting and working with them; (b) field studies of the behavior in nature of those tumor viruses for which suitable detection tests are available; (c) studies of extraneous viruses ("background noise") now preventing high caliber virologic practice in the study of animal tumor viruses and obscuring interpretation of nearly all current observations on them; and (d) the study of general virus experiences in relation to human cancer—the "background noise" in the human cancer problem—which must be done eventually if the role of viruses in human cancer is to be defined.

The approach to these various interdependent studies is based on the following beliefs: (1) That the conventional methods of standard virology must be applied to cancer virus research if sig-

nificant progress is to be made; (2) the study of cancer viruses obviously cannot be separated from general virology; and (3) that the "biologic point of view" rather than attitudes fostered by preoccupation with categorical disease, represents the best approach to a real understanding of the natural history of cancer viruses just as it does to other viruses.

Mouse Polyoma Cancer Virus

New *in vitro* survey tools developed during 1959 (CF, HI, and MAP) were evaluated and applied in 1960 in studies of polyoma virus growth and excretion, its experimental epidemiology, and its natural history. This interesting and versatile cancer virus causes tumors not only in all strains of *Mus musculus*, but also in hamsters, rats, rabbits, and guinea pigs (Stewart and Eddy). Of equal interest is the fact that it can be studied and surveyed with the same facility as ordinary viruses, such as influenza and polioviruses. Virus isolation and serologic procedures, combined with epizootiologic studies have produced the following interesting observations:

Polyoma virus was found to be widely disseminated in mouse colonies nearly everywhere. Infection was found to be more commonly present than absent in laboratory strains raised in experimental or commercial laboratories and in wild strains found in city tenements. However, the basic ecology or natural cycle appears to exist in rural areas—on farms and in feed mills in small towns.

A full year's surveillance of *Mus musculus* infestation and polyoma infection of crowded tenements in Harlem revealed that virus infections persisted without exception in numerous separate foci. Three epidemiologic factors seem most important, namely, large mouse populations capable of furnishing adequate supplies of young susceptible mice, the extensive contamination of the tenement environment (virus was demonstrated in sweepings from areas showing signs of mouse activity), and finally the overcrowding which insures the continuous and extensive use of communal nesting areas (also demonstrated to be contaminated by virus). Apartment houses having smaller and less dense mouse infestation were generally free of infection and remained so during the study.

Systematic studies of polyoma in rural environments were undertaken during the last quarter of 1960. However, it appears from preliminary data that here may be found the *basic natural cycle* of mouse polyoma. *Mus musculus* infestation and polyoma infection of *Mus* was found to be most intense in feed granaries on the farm and in cereal grain storage elevators in mills. As many as 30 percent of several hundred mice trapped in these environs showed persistent evidence of polyoma infection, many of them apparently excreting virus in their urine. The virus has been found on cereal grains in the vicinity of mouse nesting areas, which appear to be very numerous in the granaries so far examined. The actual extent of cereal grain contamination by mouse excreta containing polyoma and no doubt other microbes must still be evaluated; however, present evidence suggests that it probably is very extensive, if not appalling.

Since natural infection of wild mice is not limited to polyoma virus, but includes a number of other viruses known or suspected to infect man and domestic animals, the extension of these preliminary findings will likely prove very interesting.

Extraneous Cancer Viruses

In 1960 the "background noise" problem in cancer virus research grew to almost "deafening" proportions and, in the opinion of LID virologists, constitutes the number one obstacle to intelligent and truly effective research on cancer viruses.

Nearly every animal tumor virus system currently under study was shown to be contaminated with extraneous agents and several viruses widely proclaimed as "tumor" viruses turned out to be fellow traveling ordinary viruses. To list a few examples: Friend leukemia was found contaminated with polyoma and mouse adenovirus; Gross leukemia by polyoma, K virus and mouse adenovirus; Schwartz leukemia with polyoma, K virus and mouse adenovirus; Moloney leukemia with mouse hepatitis and mouse reovirus; the polyoma itself became contaminated with mouse adenovirus, hepatitis and salivary gland viruses.

LID virologists showed that the "seeds" of the "background noise" viruses are commonly present in the animals used for the induction of

tumors, and of course in the subsequent passage materials as mentioned above. The extraneous viruses most commonly encountered in cancer systems were the newer ones, such as polyoma, K virus, mouse reovirus and adenovirus; but this in part may be due to newly developed easily applied survey tools for these agents. Other viruses encountered less often (perhaps because of comparatively less sensitive tools) were mouse hepatitis, mouse salivary gland virus, the newly discovered "thymic agent" (TA). Except in newborns, most of these viruses occur subclinically and latently.

Medical Mycology

Investigations on pathogenic fungi have included broad fields of research and although definitive goals have been reached in most of them, all will be continued in order to further exploit productive lines of investigation. In most cases new or additional species of pathogenic fungi will be used in investigations, or techniques will be altered to permit further development of experimental studies.

The antibiotic X–5079C was found to be fungistatic but not fungicidal and its apparent low degree of *in vitro* activity due to its decay in culture medium. The yeast form of *Histoplasma capsulatum* is much more sensitive to X–5079C than the mycelial form and an assay method, sensitive to 1 ug/ml using *H. capsulatum*, was developed. X–5079C has low toxicity for HeLa cells and is active against *H. capsulatum* grown in HeLa cells.

A second strain of *Coccidioides immitis* has been converted to serial culture in the spherule form. Quantitative measurements show the ability of various carbon and nitrogen sources to support growth of spherule and mycelial forms of strain M–11 of *C. immitis*. Only mannose is utilized as readily as glucose by spherules. Mannose and fructose support growth of the mycelial form as well as does glucose. A substrate which preferentially supports growth of the spherule form was not found in this study.

Spherules were utilized to immunize mice. An increased survivor rate in the immunized mice was noted after challenge with a lethal infecting dose and an earlier clearance of organs (negative cultures) in the immunized mice was observed after challenge with a sublethal dose.

Cryptococcus Neoformans

By titrating and plating out organs of experimentally infected mice, it was found that several minutes after *Cryptococcus neoformans* was injected either intravenously or intracerebrally into mice, the largest numbers of yeast cells had been retained in the lungs. The fungus population in the lung then decreases and 2–3 days after infection multiplication in the brain is apparent. Although the interval from infection to death of infected mice varies with the strain of *C. neoformans*, the numbers of yeast cells per gram of brain tissue are approximately the same regardless of strain.

Studies of the saprophytic occurrence in natural habitats of fungi which cause mycoses have continued. *Cryptococcus neoformans* has been isolated from many additional collections of pigeon guano. When this material is collected from old pigeon nests and from roosting sites in hay mows of barns and upper floors of buildings, Histoplasma has never been found. There is increasing circumstancial evidence that a presently unstudied pneumonic form of cryptococcosis has occurred in men heavily exposed to such material and that such epidemics have been erroneously diagnosed histoplasmosis.

Emmonsia Crescens

In collaboration with an investigator at the Rocky Mountain Laboratory a new species, *Emmonsia crescens*, was described. This fungus differs from the first species of Emmonsia (*E. parva*) *in vivo* and *in vitro* at 37° C. by its multinucleate condition (instead of uninucleate), its ability to produce the *in vivo* form *in vitro* at 37° C., and its greater size. *E. parva* conidia when inhaled or incubated at 37° C. increase in diameter from 2–4 u to 400–480 u. This 10^6-fold increase in volume of a single cell is very unusual in the fungi.

Staphylococcus Studies

It has been established that staphylococcal penicillinase is associated with particulate mate-

rial in the cell and thus an explanation has been given for the refractoriness in preparation of this enzyme by conventional methods. New and more potent inhibition of *Staph. penicillinase* have been uncovered and the hope remains and is heightened for the ultimate finding of a chemically useful inhibitor. Further, sea water has been found to possess strong inhibitory activity against *both* penicillin-sensitive and penicillin-resistant staphylococci (phage type 80/81).

Real progress has been reported in the understanding of iron metabolism in the staphylococci. As a direct result of continuing work dealing with mechanisms of the development of nonspecific immunity and in particular the function of the iron-transporting protein of plasma, siderophilin, fundamental observations on the effects of iron deficiency on the growth and metabolism of *S. aureus* have been reported. Work on the biology of the staphylococci so long neglected during the "antibiotic era" is cardinal to effective new therapy of staphylococcal infections.

Streptococcal M Protein

Progress has also been made on the search for better methods of isolation and purification of M protein of streptococci. These results are of obvious importance in the understanding of Group A streptococcal virulence. Further, highly interesting observations have been reported dealing with the mechanism and significance of the long-chain test for determination of anti-streptococcal immunity.

Bacterial Metabolism

Real understanding of the intimate mechanisms of energy metabolism in *Hydrogenomonas* in particular and other bacteria and higher forms in general is closer as a result of work performed in this section this year. In an enormously complicated field, progress has occurred in the definitions of the essential reactions.

Detoxification studies on potentially useful chemotherapeutic agents have continued and new and promising leads have been uncovered for agents active against bacteria, fungi, parasites, and, it should be added, against cancer as well. Several of the aforementioned detoxified compounds have passed preliminary screening processes performed by the Cancer Chemotherapy Center.

Pinpointing of the enzymic locus of discrimination among hydrogen isotopes by pseudomonas has been reported this year. The area lies in formic acid metabolism.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

BASIC RESEARCH

Introduction

During the coming calendar year, NIAMD will attain its tenth birthday. The intramural structure has completed a period of growth, which despite occasional growing pains, has been in the main satisfying to its personnel and productive of good science. Both the pains and the science have been reviewed in earlier issues of this annual report.

We now look forward toward an era of limited physical growth. The laboratory and clinic space assigned to us is approximately saturated with men, with machines or with both. Indeed, in some areas the degree of crowding is such as to interfere with highest production. We can anticipate at this time but one increment in our space allotment, that portion of Building 2 currently occupied by The National Institute for Dental Research. The intended disposition of this space will be considered subsequently.

The fact that the limitation of physical space is now in sight and that a limitation in personnel must inevitably follow should not, of course, be taken to indicate a cessation of scientific and intellectual development. Indeed, we may expect that decline in architectural and personnel distractions will direct an increasing fraction of our cumulative energy toward our prime business which is the conduct of research.

Administration

Over the past decade the administration of this Institute has been largely concerned with problems of new recruitment, addition and fortification of sections and branches, acquisition and refurbishing of space, and purchase of the essentials of research equipment. Henceforward, attention will have to be directed chiefly toward replacement of lost personnel and of obsolescent equipment.

It is our belief that in treating with excellent scientists, the best administration is the least administration. The mandate presented to the administrator is to tempt to his Institute research leaders of the very highest quality available, to supply them with the materials, support and environment for optimal productivity, and then, to leave them alone. The only important control which administration may effectively exert over the choice of areas of research is in the selection of the disciplines in which senior recruitment is undertaken. Thereafter, one must have sufficient confidence in the scientist selected to place in his hands the choice of what experiments he shall perform. If he has been properly chosen, he will be best qualified to make this decision. The administrator of first-rate scientists has two important responsibilities; firstly, to resist the temptation to meddle in the science of his staff, and secondly, to make no compromise with quality.

In relation to the latter point, the accompanying opinion is expressed by the President's Science Advisory Committee: "In the advancement of science, the best is vastly more important than the next best. Mediocre research is generally worse than useless * * * It is therefore of first importance that national support * * * should aim at sustaining and reinforcing outstanding work wherever it may be found." [1]

Research and Education

A recent report on scientific progress in the United States contains the following statement: "Yet in the long run it is dangerous to separate research in any field entirely from education. If a research field is to be attractive to good young men, it ordinarily needs roots in the universities. The pool of graduate students in our universities is the pool from which the scientists of the future

[1] This quotation and the one which follows are from "Scientific Progress, The Universities and The Federal Government," Statement by The President's Science Advisory Committee, The White House, Washington, D.C., November 1960.

must come. These young people do not easily study what is not taught; they do not often learn the meaning of research which does not exist in their environment. A scientific field which has no research life in the universities is at a grave disadvantage in recruiting new members. As learning and teaching require research, so research, in the end, cannot be sustained without teaching. Hence it is always important for research installations to maintain effective connections with students."

It is the thesis of this report that basic research and graduate education go best together. This thesis is in harmony with what many of us believe. It has been our hope to take steps, insofar as these are compatible with government regulations, to permit scientists in this Institute to reap the benefits normally reserved for members of university faculties. Among these the following may be listed:

1. To facilitate access by the scientist staff to that most valuable and industrious population of junior investigators, the pre- and post-doctoral fellows.

2. To permit to our scientists the continuous rejuvenation which comes from repeated exposure of an aging faculty to class after class of fresh and refreshing youngsters.

3. To gratify, for those who need such gratification, the achievement of that "immortality" which derives from seeing one's teachings transmitted to succeeding generations of scientists.

These and other related goals must be attained if NIAMD is to remain a most attractive environment for our most productive scientists. To this end NIAMD has participated actively in all ventures in and about Bethesda relating to the teaching-learning function. The research Associate program is blossoming and in our Institute eight such Associates are presently housed, a new class of four being admitted each July. This program has attracted wide and favorable attention in the best teaching hospitals and medical schools and the number of applicants each year is burgeoning. The courses sponsored by the Graduate School of the U.S. Department of Agriculture at NIH also continue to grow in enrollment. NIAMD participates by supplying both students and teachers in this unusual educational adventure. In addition, members of our scientist staff offer courses at local universities and sponsor at Bethesda Ph.D. candidates enrolled in these schools. All these arrangements, though less than ideal, serve to satisfy, to some extent, the goals mentioned above. Various categories of summer students have also been accommodated.

Laboratory of Molecular Biology

This is to be a new laboratory. Ultimately, five Sections are envisioned, tentatively to be named Metabolic Enzymes, Chemical Genetics, Physical Chemistry, Molecular Structure and Microbial Genetics. Two of these five sections will represent groups presently in other Laboratories of our Institute who will move and expand in a new environment. Section Chiefs for two of the remaining three groups have been recruited and active steps are under way in the filling of the remaining vacancy.

The guiding philosophy in the staffing of this new laboratory has been to collect a group of outstanding, energetic and enthusiastic investigators with common goals but diverse technical backgrounds. All the groups will have interests in and about the biological role of macromolecules such as nucleic acids and proteins. They will all be concerned, more or less, with the biogenesis, structure and function of these molecular species. They will bring to this interest the skills and ideas of the crystallographer, the spectroscopist, the enzymologist, the geneticist and others. It is hoped and expected that the interchange of ideas among these groups of investigators will result in a whole which is far greater than the algebraic sum of its parts.

Organizational Matters

The retirement of Dr. Ralph Lillie required a review of the Laboratory of Pathology and Histochemistry which he had headed for many years. Dr. L. L. Ashburn was persuaded to become the new Chief of the Laboratory, assisted by Dr. Gert Laqueur. To accommodate the needs of the several scientists, sections were rearranged and Section Chiefs were designated as follows: Dr. Spicer, Biophysical Histology; Dr. Highman, Pathologic Anatomy; Dr. Brecher, Hematology; Dr. Glenner, Histochemistry; Dr. Sokoloff, Rheumatic Diseases.

The retirement of Dr. Nathan Eddy deprived the Section on Analgesics of the Laboratory of

Chemistry of its chief. Happily, Dr. Everett May, long associated with Dr. Eddy, will serve as chief of the section, renamed Section on Medicinal Chemistry.

The Pediatric Metabolism Branch under Dr. Paul di Sant'Agnese is now well launched. This clinical branch has found common research interests with several of our basic science laboratories and its members have entered upon a number of interesting collaborative ventures.

A number of distinguished Visiting Scientists have come to NIAMD during the past year. Dr. E. G. L. Bywaters of London spent 3 months working with members of the Arthritis and Rheumatism Branch. Dr. Ephraim Racker of New York visited for several months with members of the Laboratory of Biochemistry and Metabolism, where the influence of his vast knowledge and fine critical judgment was greatly appreciated. In the same laboratory, Dr. Ernst Simon of Rehovot, Israel, has spent a year in many active collaborations with various members of NIAMD in and about his own research interests. Dr. Hin Tjio of Saragossa, Spain has been working in the Laboratory of Pathology and Histochemistry. His unique contributions in the area of chromosome anatomy have brought to him many consultations and proposed collaborations.

Intramural scientists have also participated in a variety of extracurricular functions. Among these may be mentioned Dr. Bunim's delivery of the Heberden Oration and Dr. Heppel's presentation of the 10th N.I.H. Lecture.

Laboratory of Nutrition and Endocrinology

The primary emphasis of the work in the laboratory is directed toward the elucidation of the mode of action of nutrients and hormones. To this end intact animals are intensively studied to determine the effect that dietary or environmental factors may have on the animals' physiological economy. At the termination of these studies, a variety of tissues are examined biochemically or histologically to secure a more exact description of the role played by nutrients and hormones. It is anticipated that these studies will also provide clues and suggest additional investigations for those scientists who are interested in the more restricted areas of biochemistry or physiology.

During the past year, the laboratory has increased its efforts in the field of biochemistry. Because of the large amount of biochemical work being done on each animal, there has been a reduction in the space devoted to the care of the animals and an accompanying increase in laboratory area.

Nutrition

Vitamin E and Factor 3. Shortly after vitamin E was first reported as an essential nutrient for normal reproduction of rats, this vitamin became the center of a controversy. A number of investigators claimed that some animal species did not require the vitamin at all. Later, other workers showed that the functions ascribed to vitamin E could be assumed by a variety of compounds all of which were effective antioxidants. Although somewhat abated, the controversy still continues with the arena reduced from the intact animal to individual tissues or cellular components.

A few years ago, Dr. John G. Bieri showed that chicks could be raised to adulthood and could produce fertile eggs when fed a diet devoid of any detectable vitamin E. After a year on the deficient diet, the tissues of the birds contained no vitamin E as shown by analysis. In continuing this work, Dr. Bieri developed an *in vitro* test system using the auto-oxidation of liver cell mitochondria as an index of tissue vitamin E deficiency. The lipids in mitochondria from vitamin E-depleted animals readily auto-oxidize whereas mitochondrial lipids from animals fed an ordinary diet containing vitamin E do not. If the diet is devoid of unsaturated fats and vitamin E there is a substantial reduction in peroxidation.

The addition of vitamin E or a number of antioxidants directly to the mitochondrial system reduces the peroxidation seen in the *in vitro* test. The addition of selenium or cystine to the *in vitro* system prepared from chick livers has no effect but when these compounds are fed to the animals, their liver, heart, and kidneys showed antioxidant activity. These observations suggest that when selenium or cystine are fed, the chick

forms a substance which functions to reduce the level of peroxidation.

The chick has a very limited capacity to store the biologically active form of selenium in its body. This was shown by Dr. Bieri when he fed three groups of day-old chicks different levels of selenium in a vitamin E-free diet for one week. At the end of this period, the selenium was removed from the diet. All groups developed symptoms of a combined vitamin E and selenium deficiency at approximately the same time regardless of the level of selenium present in the diet during the one week feeding period.

Dr. Bieri has concluded from his own work and that of other scientists that the primary function of vitamin E in the chick is that of a tissue antioxidant. This vitamin is important in this respect not only in reducing the oxidation of lipids in the diet and gastrointestinal tract but in the tissues of the animal as well.

In the course of the above work an analytical method was developed for chromatographically separating α-tocopherol from the unsaponifiable fraction of animal tissues. By this means as little as 2 μg. of α-tocopherol can be detected. The procedure can be applied to 0.4 gm. of tissue. Ubiquinone (or coenzyme Q) can also be determined by this method.

In January 1957, Drs. Klaus Schwarz and Calvin M. Foltz showed that dietary liver necrosis in rats could be prevented or cured by adding very small amounts of certain inorganic selenium compounds to the diet. The concentrates of Factor 3 prepared from natural products contained selenium in an organic form. Smaller amounts of Factor 3 than of inorganic selenium were required in the diet to prevent dietary liver necrosis. A variety of organo-selenium compounds synthesized by Prof. Arna Fredga of the University of Upsala and assayed by Dr. Schwarz indicated that optimal Factor 3 potency occurred in di-seleno, dibasic acids containing 4 or 5 carbon atoms. The straight chain compounds were more active than branched chains. The di-seleno acids are converted in the animal to seleninic acids before they become biologically effective. The most active compound is benzyl-Δ-monoselenovaleric acid which is close to the activity of Factor 3.

In an attempt to determine how selenium functions, Dr. Fiorenze Stirpe (visiting scientist) studied the incorporation of isotopically labelled valine into the tissues of rats maintained on the necrosis-producing ration (Torula yeast ration). This work indicated that the rate of incorporation of the valine into protein was enhanced in the animals with necrosis but it was not affected by dietary supplements of liver protective substances such as Factor 3 or vitamin E. The increase of amino acid uptake was related to increased rate of protein turnover which appears within a few days after the rats are put on the Torula yeast diet.

Work by Dr. Trygve Tuve with fresh Baker's yeast showed that the cells incorporate trace amounts of radioselenite when incubated in the presence of glucose. After a short lag period, the cells incorporate the selenium at a steady rate which depends upon the level of selenite in the medium. Most of the radioactive selenium (92 to 94 percent) appeared to be in a "protein-bound" fraction (precipitable with trichloroacetic acid). Chromatographic analysis of hydrolysates of this fraction showed an organoselenium compound that differs from the selenium compounds previously found in proteins. Fractionation of the trichloroacetic acid soluble material of the cells indicated the presence of another new organoselenium compound. The selenium was not present as an analogue of cystine, methionine, gluthathione, or selenium, di-, or tetra-cysteine. The two unknown compounds were also detected in rat urine following an injection of radioselenium. These substances were also present in the trichloroacetic acid extracts of liver and kidney homogenates.

There are a number of areas in the world including the Pacific Northwest where lambs and sheep develop white muscle disease resulting in large economic losses to the farmers. The addition of small amounts of selenium to the rations fed the pregnant dams in these areas eliminated the difficulty. Pasture grasses from the Northwest were shown by Drs. Schwarz and John R. Schubert (guest worker) to contain adequate amounts of selenium, but it was not always available to the animal.

In previous studies, vitamin E-deficient rat liver homogenates showed a marked decline in the rate of oxidation of α-ketoglutarate and succinate. Work during the past year by Dr. Laurence M. Corwin showed that the same is true for

fumarate, malate, pyruvate with a catalytic amount of malate, α-hydroxybutyrate, and glutamate. Vitamin E when added either to the diet or to the Warburg flask completely prevented the decline in the rate of oxidation of most of the above systems. Compounds such as glutathione, BAL (British antilewisite), menadione, N,N'-diphenyl-p-phenylenediamine, and methylene blue which maintained the integrity of free sulfhydryl groups were also effective in overcoming the decline in oxidation. The effects are not related to auto-oxidation, since the metabolic impairment can be prevented by levels of the protective compounds which are not effective in preventing peroxide formation. Additional work suggested that tocopherol shields the vicinal dithiol groups in the dehydrogenases and other sulfhydryl groups essential for the enzyme activity and thus spares them from inactivation by inhibitors such as cadmium and arsenite. Since microsomes are required for decline to occur, the nature of the microsomal agent actively precipitating the respiratory decline was studied. The agent in the microsomes is not stable to boiling nor can it be removed by EDTA (ethylenediaminetetraacetic acid) and subsequent dialysis.

Folic Acid. During the past year significant progress has been made in the isolation of the folic acid derivative that occurs naturally in liver. A major breakthrough in this problem occurred when it was demonstrated by Drs. John Keresztesy and Kenneth O. Donaldson that tetrahydrofolic acid could be enzymatically converted to the prefolic acid form as evidencer by chromatographic and microbiological assay (performed under the direction of Mr. Howard A. Bakerman). The enzymatic system requires a reduced diphosphopyridine nucleotide-menadione reductase system. These studies indicate that this prefolic compound is a reduced tetrahydrofolic acid derivative. An attempt is being made to increase the scale of the enzymatic reaction so that sufficient amounts of the reduced tetrahydrofolic acid can be isolated and identified.

Although the folic acid activity of many cells and tissues is known, very little information is available about the different folic acid compounds which are present in the cells and tissues. Dr. Milton Silverman has developed procedures for the quantitative identification of the known folic acid-like compounds. With these techniques, Dr. Silverman has shown that in some leukemic cell lines (with Dr. Lloyd Law, NCI) the major folic acid form is N^{10}-formyltetrahydrofolic acid (80 percent). The other components consist of small amounts of N^{5}-formyltetrahydrofolic acid and unsubstituted tetrahydrofolic acid. In contrast to the situation in the tumor cells, the liver of the host mouse contains primarily the prefolic acid compound being studied by Drs. Keresztesy and Donaldson with small amounts of the tetrahydrofolic acid compounds mentioned above. These results suggest that the functional form of folic acid in the tumor cells is a monoglutamate derivative reduced to the tetrahydro level. This is in contrast to a generally accepted proposition that the physiologically active forms of folic acid exist as polyglutamates. Similar studies are underway on several bacterial species. This work (with Dr. Bernard T. Kaufman and Mr. Bakerman) has shown that these cells not only contain most of the known folic acid compounds but also several derivatives which have not been completely characterized.

In the presence of reduced triphosphopyridine nucleotide, folic acid is reduced to tetrahydrofolic acid by a protein fraction from chicken liver. N^{10}-formylfolic acid is also reduced to the tetrahydro level by chicken liver extracts. However, the mechanisms involved in these two reductions appear to be different. Although the requirement for TPNH (triphosphopyridine nucleotide) is common to both systems, the reduction of the formylated derivative is stimulated by the presence of free folic acid and citrate or versene. The enzymatic components involved in the reduction of N^{10}-formylfolic acid are being purified by Drs. Silverman and Kaufman.

A number of folic acid antagonists have been used in the treatment of a variety of diseases and in studies directed toward the elucidation of the metabolic function of folic acid. Dr. Roy Kisliuk (Visiting Scientist) succeeded in reducing some of the folic acid antagonists (aminopterin and amethopterin) to their tetrahydro derivatives. These compounds appear more closely related to the active form of the vitamin than the previously used antagonists. The reduced antagonists proved more effective in overcoming the activity of folic acid than the unreduced forms both in bacterial cells and in chicks (Dr. M. R.

Spivey Fox). These results with the observation of other investigators (that aminopterin and amethopterin can be reduced by cells) suggest that *in vivo* these inhibitors exert their effects after reduction to the tetrahydro form.

Vitamin B_{12}. Previous work by Dr. Fox showed that a severe deficiency of vitamin B_{12} could be produced in the chick by feeding it a diet high in fat, low in methionine, and free of the vitamin. The deficient chicks exhibited poor growth and an impaired metabolism of histidine as shown by a high excretion of formiminoglutamic acid. Dietary supplements of either vitamin B_{12} or methionine overcame both signs of deficiency. The intact methionine molecule is required for these effects since homocystine, cystine, choline, or betaine were inactive. Thymidine, which can replace vitamin B_{12} under some conditions, had no effect on the excretion of formiminoglutamic acid. The high fat level in the deficient diet was necessary for poor growth but not for the excretion of formiminoglutamic acid.

Germ-Free Studies. The germ-free animal presents a unique opportunity to secure unequivocal answers to a variety of questions relating to the possible contribution of the intestinal flora to the physiological economy of the animal. In addition thereto, this opportunity has resulted in problems which are uniquely related to germ-free animals. One of these relates to the difficulty in meeting completely the germ-free animals' nutritional requirements. Work by Dr. Floyd S. Daft, Mr. Ernest G. McDaniel, and Mr. James C. Smith, Jr., has resulted in the development of diets which produce reasonably good growth in the guinea pig. These animals, however, develop caeca which in some cases become so large that they represent 43 percent of the total body weight (in a 21-month-old-male). In conventional animals, the weight of the caecum is usually 2 to 3 percent of the body weight. Since there is a possibility that autoclaving the diet may destroy certain essential and as yet unreocgnized nutrients and that these nutrients may be required in maintaining the caecum at normal size, a number of germ-free guinea pigs were fed supplements of raw fresh eggs, whole human blood, raw tissue, and feces from germ-free rats. These natural products were tried since they were sterile originally and for this reason they could be brought into the germ-free tank without being autoclaved. None of these substances was effective in reducing the size of the caecum. There was actually an indication that the guinea pigs consuming the rat feces showed an aggravation in the enlargement of the caecum. There is a possibility that the rat feces fed the guinea pigs may have increased the amount of trypsin in the lower part of their intestinal tract. This is based on the observation of Dr. Bengt Gustaffson (visiting scientist) that the feces from germ-free rats contain large amounts of trypsin whereas the feces from conventional animals contain none of this enzyme.

The enlarged caecum is not the result of the germ-free state since a number of conventional guinea pigs that were removed from their mothers by caesarian section and fed nothing but autoclaved diets showed similar enlargement of their caeca. The removal of the major portion of the caecum from day-old germ-free guinea pigs at first appeared to keep the caeca small but until the animals are sacrificed no conclusive answer will be available. An interesting incidental observation of the latter study was the high mortality (80 percent) among the conventional guinea pigs on whom the caecaectomy was performed compared to the low mortality (20 percent) among the germ-free animals. The conventional animals died within less than 24 hours of the operation showing severe shock so it is doubtful whether infection can explain the differential mortality.

Protein. Dr. J. N. Williams, Jr. is studying the effect of a prolonged dietary protein deficiency on the changes in the composition of the liver of rats. Within 10 days after the animals were put on the protein-free ration, they began to show changes, some of which progressed throughout the 72 days of the experiment. The number of cells in the liver was estimated from the concentration of deoxyribonucleic acid. On this basis, the number of cell per unit weight of liver almost doubled after 72 days of protein deficiency. This change was primarily a reflection of inanition and not of protein deficiency *per se* since the same change was shown by the animals fed a complete diet in amounts equal to that consumed by the protein-deficient rats. The nitro-

gen content of the individual liver cell in the protein-deficient group was reduced to 60 percent of that in the rats fed a complete diet on an *ad libitum* basis; the comparable value for the pair-fed controls was 85 percent. The total number of liver cells in the protein-deficient and pair-fed control animals showed a progressive reduction throughout the 72-day period to 70 percent of the *ad libitum* controls. The ratio of liver weight to body weight increased markedly in the protein-deficient rats while little change occurred in the pair-fed controls.

The deprivation of dietary protein should influence the animals' ability to synthesize the enzymes involved in energy transformations. To evaluate this proposition, Dr. Williams determined the activity of the individual components of the succinic oxidase system in his protein-deficient rats. The activity of succinic oxidase and succinic dehydrogenase per liver cell decreased to one-half of normal by the 10th day of the protein deficiency. Thereafter, the cells appeared to resynthesize these enzymes so that by the end of the experiment their activities were about 75 percent of normal. Throughout the study, there was no change in the antimycin A-sensitive activity (a measure of the flow of electrons between cytochromes-*b* and c_1) or of cytochrome–*c* concentration within each cell. About 85 percent of the cytochrome oxidase activity was lost from the cell after 46 days on the protein-deficient diet. In spite of the large reduction in the cytochrome oxidase activity, there was still sufficient enzyme to maintain succinic oxidase activity at a high level. These studies indicate that each individual enzyme of the same mitochondrial system responds differently to a protein deficiency.

Dr. Peter G. Condliffe, during the tenure of a fellowship sponsored by the National Foundation, spent one year at the Carlsberg Laboratory in Copenhagen. While there he studied the exchangeable hydrogen atoms in orosomucoid which is a glycoprotein present in human plasma to the extent of 0.05 to 0.1 percent. It is the principal protein excreted in the urine of patients with nephrosis. Dr. Condliffe found that at neutrality and 37° all available hydrogens exchanged within two hours but a number, approximately equal to the number in the peptide moiety, do not exchange instantaneously. The latter hydrogens probably represent bonds involved in the secondary structure of the molecule. An helical structure would explain this observation. Confirmation of this hypothesis will be sought in studies of the optical rotatory dispersion of orosomucoid. During the course of this work a new method was developed for the preparation of the protein which involves the use of an alcoholic fraction of plasma protein-VI (Cohn's fraction). After the alcohol is removed and the protein dissolved in water, the solution is dialyzed against a phosphate buffer. The equilibrated solution is put through a diethylaminoethyl cellulose column which adsorbs the orosomucoid. Elution is accomplished with a sodium phosphate solution.

Lipids. Drs. Robert O. Scow, Sidney S. Chernick, and Martin Rodbell (NHI) found that when an emulsion of cottonseed oil labelled with C^{14}-tripalmitin was perfused through the liver of a rat, the disappearance of the labelled tripalmitin was proportional to its concentration in the perfusate. This was true for concentrations up to 7.4 milliequivalents of ester per liter. Above this level, the liver removed the lipid at a constant rate of 10 microequivalents per gm. of liver per hour. The labelled ester removed by the liver was recovered primarily as neutral and phospho lipids (60 to 90 percent) and to a lesser extent as ketone bodies (11 percent) and carbon dioxide (2 percent). Although both the Kupfer and parenchymal cells removed the triglyceride without de-esterification and metabolized it, the parenchymal cells accounted for 75 to 80 percent of the activity.

Plasmalogens comprise a group of phospholipids which give rise to a long chain aldehyde, a fatty acid, glycerol, a base, and phosphoric acid on acid hydrolysis. Previous work by Drs. Carl E. Anderson (Visiting Scientist) and Williams indicated that liver contains an enzyme which splits the vinyl ether linkage by which the aldehyde is attached to the plasmalogen. To facilitate this work, a method was devised for the separation of the plasmalogens from other aldehydrogenic compounds in the lipid extract. Silicic acid chromatography under special conditions proved satisfactory for this procedure. With this technique in hand, it is now possible to proceed with the enzymatic study.

Glucose Tolerance Factor. Previous work by Drs. Walter Mertz and Schwarz showed that when rats were raised on certain diets, their rate of removal of intravenously injected glucose was reduced from a control value of 4 to 4.5 percent to 2.8 percent per minute. They found that when trivalent chromium was added to the diet, the removal rate of the glucose was restored to the control value. The epididymal fat pads removed from rats showing the reduced rate of glucose removal utilized less glucose than was true of the pads from rats fed the control diets. The glucose uptake could be increased to normal by the addition of chromium to the excised tissue. The activity of a number of different chromium (III) complexes in the tissue assays was almost identical to the activity seen in the *in vivo* experiments. These findings indicate that differences in the glucose tolerance factor (GTF) activity of the chromium complexes in the *in vivo* studies cannot be explained solely on differences in intestinal absorption.

The work of Dr. Mertz indicated that insulin was required in the *in vitro* system to show the stimulatory effect of chromium on the incorporation of labelled glucose and to a lesser extent of acetate into the lipids of adipose tissue. Labelled galactose was used to determine whether chromium had any effect on the transport of nutrients across the cell membrane. In such studies the adipose tissue from rats raised on the GTF-deficient diet showed a reduced rate of galactose entry. The addition of 0.01 μg. of chromium increased tissue galactose by 56 percent after one hour of incubation. At that time the tissue water in the controls was 67 percent saturated with galactose whereas it was 100 percent saturated in the chromium-supplemented tissues. These studies suggest that chromium may function close to the site of action of insulin, i.e., in increasing the entry of sugars into cells.

Obesity. The obesity produced in a variety of strains of rats by feeding a high-fat diet has a genetic component to it as shown by the work of Dr. Richard S. Yamamoto. He found that a hybrid produced by crossing the Sprague-Dawley with the NIH black rat did not become obese when fed the high-fat diet even though the progenitors of the strain did. The level of fat in the diet has no effect on body composition, plasma lipids or liver composition of these hybrid rats. An extension of these studies showed that an obese strain of mice (STR/1N) had higher levels of lipids in their plasma than mice of the lean strains (DBA/2JN and A/LN). The difference in the lipid levels was primarily due to neutral lipids. Genetic studies (with Dr. Leon Sokoloff) showed that the obesity and high plasma lipid levels were dominant traits. These studies also showed that the high hemoglobin levels and hematocrit in the A/LN mice are dominant traits as shown by crossing these mice with the STR/1N mice which have low blood levels. In all groups, the hemoglobin level was directly related to the hematocrit.

Guinea Pigs. The guinea pig has a higher requirement for dietary protein than most other laboratory animals as shown by the work of Dr. Mary E. Reid (Guest Worker). Maximum rates of growth were secured when the diet contained 35 percent of protein either as casein or as a purified soy protein. Good growth was also secured when the diet contained 20 percent protein, provided the casein was supplemented with 1 percent L-arginine or the soy protein with 0.5 percent DL-methionine. These studies suggest that, as far as growth is concerned, the guinea pig has a high requirement for certain amino acids and not for protein *per se*. Evidence is also being accumulated by Dr. Reid that as the level of dietary protein is increased, the ratio of the weights of the liver, testes, and spleen to body weight increases.

Rabbits. Previous work by Dr. Olaf Mickelsen and Miss Jeanne M. Reid indicated that although rabbits did not manifest the typical deficiency symptoms when fed a thiamine-free diet for long periods, they did show a reduction in urinary and fecal excretion and reduced levels of the vitamin in the brain. In an extension of this work, young rabbits were fed a thiamine-free diet supplemented with the vitamin antagonist pyrithiamine. Six of the 7 rabbits developed typical neurological symptoms beginning on the 18th day. Four of these rabbits died within 24 hours after first exhibiting the symptoms; the two remaining animals showed dramatic responses to thiamine injected intraperitoneally. Pyrithiamine increased the urinary excretion of thiamine, of-

fering additional support for the hypothesis that this antagonist releases the vitamin from body cells.

Endocrinology

Experimental Diabetes. One of the major problems still confronting the clinician who treats large numbers of diabetics is the sudden and apparently uncontrollable ketosis which develops in some patients. Considerable effort has been devoted by Drs. Scow and Chernick to the elucidation of the physiological factors in the diabetic rat which produce severe ketosis. Earlier work by this group showed that when insulin was withheld for 17 hours or more from the hypophysectomized-pancreatectomized rat, severe ketosis developed following the administration of glucocorticoids. Growth hormone had no effect in these animals. However, when these diabetic rats were treated with insulin, and shortly thereafter injected, then both growth hormone and glucocorticoids were necessary to produce ketosis. Both hormones were also needed to produce severe ketosis in the fasting pregnant rat. In both cases, insulin quickly suppressed the ketosis. These findings suggest that growth hormone acts as a ketogenic agent by masking the action of the traces of tissue insulin but this occurs only when the level of insulin is low.

The above findings have been confirmed in the rat made diabetic by phloridzin. In this animal either insulin or bilateral nephrectomy (to prevent the loss of sugar) prevented the development of ketosis. The phloridzin-diabetic rat also required both glucocorticoids and growth hormone before severe ketosis developed. This animal differed from the pancreatectomized rat in that there was no accumulation of lipids in the blood and liver when the blood ketone levels exceeded 40 mg. percent.

The work of Drs. Scow, Chernick, and Ernst Simon (visiting scientist) suggests that the diabetogenic effect of mannoheptulose is due to its suppression of insulin release from the pancreas. When 400 mg. of mannoheptulose, a seven carbon sugar isolated from avocados, were injected into a rat, it immediately developed increased levels of blood glucose and ketone bodies. There was no change in the blood lipids. The effect of the mannoheptulose waned within 2 hours at which time its blood concentration fell below 40 mg. percent. Mannoheptulose is an isomer of sedoheptulose, an important intermediate in the pentose shunt. Sedoheptulose differed from mannoheptulose in not raising the blood sugar level. Sedoheptulose resembled glucose in lowering the blood ketone level. When fasting rats were given glucose by stomach tube, the blood glucose level was increased and the blood ketone level decreased. When mannoheptulose was given with the glucose, the blood glucose level was greatly augmented but the blood ketone level remained elevated until the mannoheptulose had practically disappeared from the blood. Insulin injected into the fasting rats lowered both the blood glucose and ketone levels even when mannoheptulose was given. These findings indicate that exogenous insulin can overcome the action of mannoheptulose and that the latter has no effect on the action of insulin once it is released from the pancreas. Additional studies indicated that mannoheptulose had no effect on the blood levels of both glucose and ketone bodies in the pancreatectomized-diabetic rat.

Dr. Scow is developing a technique for infusing the pancreas *in situ*. In the rat, this involved removal of the spleen and all the pancreas except that which receives its blood supply from the splenic artery. A polyethylene tube was inserted into a small branch of this artery near its origin. With this setup it soon became evident that the solutions injected into the artery did not mix completely with the blood, consequently only a small portion of the remaining pancreas was being infused. When this difficulty is overcome, the preparation will be used to study the effect of mannoheptulose infusion on insulin release from the pancreas.

Dr. Scow and Andre Robert (Guest Worker) have developed a technique for perfusing an isolated segment of adipose tissue. A long finger of fat attached to the mesometrium of female rats proved most satisfactory for this purpose. Several problems have developed such as hemolysis, blood clotting in the apparatus and edema of the perfused tissue. All but the last have been overcome.

The work of Drs. Evelyn Anderson and Robert W. Bates during the past year suggests that the action of orinase on the release of insulin is still

unsettled. In some experiments with dogs, orinase has increased the level of insulin in the peripheral blood while in others, orinase has had no effect. One of the major problems in this study is the method of extracting the insulin from the blood prior to bio-assay. A number of extracts have killed the test animals and for this reason the extraction procedure is currently under intensive study.

Pituitary Hormones. For the past 7 years Drs. Robert W. Bates and Peter G. Condliffe have prepared their thyroid stimulating hormone (TSH) concentrates from two lines of pituitary tumors in mice. These tumors now show a decreasing concentration of TSH. Attempts have been made over the past 2 years to locate new tumor lines with a high concentration of TSH. Of some 30 primary pituitary tumors that have been transplanted, only 4 or 5 have high concentrations of TSH. Mice with the latter tumor lines have enlarged bile ducts and elevated blood levels of TSH.

A disappointing observation in this work is the instability of the dry TSH concentrates prepared from bovine pituitary. These concentrates regardless of whether they were stored at room temperature or in a vacuum dessicator at 1°C. all showed a 50 percent reduction in their activity. It is not known as yet whether or not the instability of these preparations is species-related as is the case for the prolactins. Prolactin preparations from sheep pituitaries are more stable than those from bovine glands, especially during fractionation in acid ethanol or acetone.

The plasma of rats having large transplantable "mammotropic" pituitary tumors has been shown by Dr. Bates to contain as much prolactin on a dry weight basis as does the tumor powder. This is the first clear evidence that mammary development in rats with tumors is associated with increased prolactin levels in the blood.

Dr. Bates has shown that the injection of thyroxine and prednisone or these combined with either growth hormone or prolactin had only a small effect on body weight gains and food intake of hypophysectomized pigeons. When all four substances were given, 20 μg. of the mixture produced a greater effect than 1 mg. of either growth hormone or prolactin alone. The relative increases in weight of the gastrointestinal tract, liver, pancreas, and kidney were similar to but greater than the increase in body weight which confirms the splanchnomegalic effect of pituitary hormones in pigeons.

Even when the pars distalis is removed from pigeons, the injection of such varied substances as insulin and formaldehyde will still produce a hypertrophy of the adrenals. The work of Dr. Richard A. Miller (visiting scientist) showed that when the infundibular process was removed with the pars distalis, thereby removing the source of ACTH, the weight of the adrenals doubled spontaneously in a certain percentage of the pigeons and in all birds if formaldehyde was injected.

Evidence has been secured by Drs. Evelyn Anderson and Richard C. Grindeland (guest worker) that rats with mammotropic pituitary tumors continue to grow after hypophysectomy and that the rate of corticosterone production continues at a high rate. These observations suggest that these pituitary tumors elaborate growth and adrenocorticotropic hormones.

Steroids. The work of Dr. Hildegard N. Wilson shows that patients with adrenocortical carcinoma have (1) a 3- to 10-fold increase in the total amount of Δ^5-pregnenolone available for steroid hormone synthesis; (2) an increased proportion of the total synthesis going into the "androgen" pathway, especially in a virilized patient; (3) a large increase in dehydroepiandrosterone which could not be transformed further and was excreted as such; (4) an increase in the proportion of 11-deoxycortisol which was not hydroxylated on carbon 11 to form cortisol; (5) an increase in the proportion of progesterone not converted to cortisol. These studies suggest that certain enzymes may not be functioning properly in adrenal carcinoma tissue thus preventing the synthesis of certain corticosteroids in amounts equal to that produced by normal adrenal tissue.

The urine of three patients with Klinefelter's syndrome studied by Dr. Wilson showed a reduction in androgens and cortisol metabolites. These patients with hypogonadism, gynecomastia and high titers of gonadotropins presumably have an enzymatic defect which affects the early stages of steroid synthesis either in the adrenals or gonads.

It was shown by Drs. Anderson and James

Davis (NHI) that aldosterone secretion is not controlled by a humoral substance elaborated in the midbrain as postulated by other workers. Dogs with lesions in the midbrain secrete from the adrenal gland normal amounts of aldosterone. The rate is increased following hemorrhage.

A micro method for the assay of ACTH has been developed by Miss Frances E. Wherry and Drs. Anderson and Bates. This method is 10 times more sensitive than presently available methods. It is based on the increase in corticosterone in adrenal venous blood of the hypophysectomized rat following an intravenous injection of ACTH. Methods for the extraction of ACTH from plasma are being developed so that the levels of this hormone in plasma can be determined.

Laboratory of Biochemistry and Metabolism

Carbohydrate Metabolism: Reactions involving carbohydrate polymers.

Glycogen isolated from animal tissues following extraction with cold acid has been found to be of appreciably larger average molecular size than that isolated by extraction with hot concentrated alkali. Isolation and identification of the products has revealed that, anaerobically, the chief action of hot concentrated alkali upon glycogen consists of a series of degradative steps starting at the free reducing and of the polysaccharide molecule and proceeding along the one short straight chain of α1,4-linked glucosyl residues. The products formed are chiefly free isosaccharinic acid and a polydisperse series of polysaccharide acids. Quantitative measurements of the isosaccharinic acid split off by alkali from glycogen samples of known average molecular size, were found to be in fairly good agreement with the amounts expected from calculations based upon idealized molecules of similar size having the highly branched treelike structure which has been predicted from enzymological studies. Borohydride reduction of the terminal aldehyde group has also been found to render the glycogen molecule stable to alkali degradation. The apparent stability of "glycogen" to concentrated alkali is due to the elimination of the reducing end of each molecule and its replacement by a saccharinic acid residue (M. R. Stetten).

Pursuant to a study of the mechanism by which sugar moieties are transferred by some transferase enzymes several critical experiments have been performed. Glucose-1-0^{18}, prepared by exchange of glucose with H_2O^{18}, was incubated with raffinose and levansucrase according to the following equation:

$$\text{glu-1-}0^{18} + \text{gal-0-glu-0-fru} \xrightarrow[\text{levansucrase}]{} \text{gal-0-glu} + \text{glu-}0^{18}\text{-fru}$$

The sucrose formed was labeled with 0^{18} showing that fructose is transferred as fructosyl. Dextran produced in the dextransucrase reaction was devoid of 0^{18} showing that glucose is transferred as glucosyl, as follows:

$$n\ \text{glu-}0^{18}\text{-fru} \xrightarrow{\text{dextransucrase}} (\text{glu})_n + n\ \text{fru}$$

These results support the prediction of Koshland to the effect that the transfer of an enzyme-specific moiety occurs at the bond between the anomeric C atom of that moiety and the bridge 0 atom. Whereas Koshland studied only hydrolytic enzymes, in which water is the acceptor of the group transferred, the present studies are the first to be done on transfers from carbohydrate donors to carbohydrate acceptors. It is concluded that a glycosyl-enzyme complex is the intermediate structure in these transfers:

$$\text{gl} - \text{R} + \text{Enz} \xrightarrow{\text{transferase}} \text{gl} - \text{Enx} + \text{R}$$

(F. Eisenberg and S. Hestrin)

Studies on the mechanism of formation of various mammalian mucopolysaccharides have revealed the presence of a previously unrecognized chondroitin sulfate polymer in human umbilical cord and attempts to isolate and identify it are in progress. In addition, it has recently been demonstrated that red blood cell hemolysates from patients with Hurler's disease are unable to metabolize UDPG and that the acivity is restored by freezing. The addition of magnesium, in small amounts, overcomes the original defect in this tissue and renders it unaffected by freezing. Further investigation of the levels of free and bound magnesium in normal and pathologic hemolysates is planned (J. Hickman).

A study of the pattern of enzymatic degradation of a polyuronide has been undertaken. This polymer, alginic acid, which contains a repeating

unit consisting of D-mannuronic- and L-guluronic acid is utilized for growth by an unidentified soil organism. Extracts of this organism, purified approximately 40-fold, catalyze the formation of a series of partially unsaturated oligosaccharides leading to the formation of a new ketouronic acid, 2-keto-3-deoxy-6-aldehydro-hexonic acid. This compound appears to be a 5-epimer of a similar monosaccharide recently reported elsewhere as a product of the bacterial utilization of hyaluronic acid and chondroitin sulfate. Further studies on the mechanism of the reaction as well as the subsequent fate of this metabolite are planned (J. Preiss).

The elucidation of the structures of the various oligosaccharides present in human milk which serve as growth factors for *Lactobacillus bifidus*, and the elucidation of the reactions by which some of these factors are incorporated into the cell wall of this microorganism will be investigated. It is felt that such an approach will also provide information relating to the mechanism of action of penicillin, an agent known to block the incorporation of N-acetyl muramic acid into bacterial cell walls (P. O'Brien).

Carbohydrate Metabolism: Reactions involving small molecules.

The previously described L-gulonic (DPN) dehydrogenase from hog kidney has been reinvestigated and purified 100-150-fold. Studies with this enzyme have led to the successful isolation and identification of β-keto-L-gulonic acid as an intermediate in the conversion of L-gulonic acid to L-xylulose. The purified enzyme was shown to possess an unusual substrate specificity in that it reacts with all of the hexonic, pentonic and tetronic acids in which the hydroxyl group on the β-carbon possesses a laevo configuration. In addition, the β-keto function of acetoacetate and 2,3-diketo-L-gulonate has been shown to be reduced stereospecifically with the resultant formation of L(+)β-hydroxybutyrate and 2-keto-L-gulonate respectively. Neither of the latter two compounds had been previously implicated in mammalian metabolism. A specific decarboxylase acting upon β-keto-L-gulonate has been purified from guinea pig liver acetone powder and L-xylulose identified as the unique product of this reaction (J. D. Smiley and J. Winkelman).

An investigation of the enzymatic reactions involved in the formation and metabolism of L-ascorbic acid in mammalian tissues has been completed. The enzymatic utilization of vitamin C has been shown to involve an oxidation to diketo-L-gulonic acid which is decarboxylated to yield L-xylonic and L-lyxonic acid. L-xylonic acid in turn is converted to the 5-carbon analogue, L-erythroascorbate and this in turn oxidized and decarboxylated to yield L-threonic- and L-erythronic acid (G. Ashwell, J. Kanfer, and J. J. Burns).

Biosynthesis of GDP-L-Fucose.

The pathway of the biosynthesis of this nucleotide sugar has been studied and a novel intermediate, GDP-4-keto-6-deoxy-*D*-mannose, has been implicated in the biosynthetic conversion of GDP-*D*-mannose to GDP-*L*-fucose. In addition GDP glycero-*D*-mannoheptose has been isolated and identified from yeast (Dr. V. Ginsburg).

Nucleic Acids.

Secondary structure, due to hydrogen bonding between specific base pairs is of increasing importance in this field. In protein biosynthesis it is believed that small polynucleotides with attached amino acids go to specific places on the RNA template, and thus the amino acids are lined up in an ordered way (adaptor hypothesis).

Several recent projects are concerned with hydrogen-bonded complexes between oligonucleotides and polymers. This continues work begun last year. The deoxynucleotide, (d) pApApA forms a complex with the ribose polymer, poly U. This is a model for the manner in which DNA could complex with RNA and control its synthesis. The effect of noncomplementary bases was investigated. Thus, pApApApApU bonds with poly U so that every "U" of poly U complexes with every "A" of the oligonucleotide. The terminal, noncomplementary "U" of pApA pApU apparently rotates out of the way (M. N. Lipsett, L. A. Heppel and D. F. Bradley). Results like these are significant in assessing effect of mutation and abnormal bases in nucleic acid helices.

The problem of nucleotide sequence, the primary structure of the RNA chain, becomes ever more pressing. More and better enzymatic reagents are needed for its solution. It was discovered here that alkaline phosphomonoesterase

is the best available agent for specific removal of terminal phosphate from RNA and other polynucleotides. With its help the Whitfeld stepwise degradation method has been applied for nucleotide sequence in S-RNA. However, traces of nuclease still contaminate the enzyme and further purification is necessary (R. J. Hilmoe and D. R. Harkness). Studies on specificity of *Staph. aureus* nuclease, wheat germ nuclease and spinach ribonuclease were carried out—all for the purpose of using them as reagents in elucidating RNA structure (L. A. Heppel).

Another investigation seeks to find out how big a polynucleotide has to be before it takes on the properties of a polymer such as nucleic acid. That is, at what stage is a secondary structure, involving hydrogen bonding between bases, acquired? The evidence is that a hexanucleotide can acquire such an ordered, secondary structure (M. F. Singer, L. A. Heppel, G. Rushizky and H. A. Sober).

Studies on the mechanism of action of polynucleotide phosphorylase are being continued. New results on the primer requirements have just been obtained (M. F. Singer). Biosynthesis of RNA in a fraction from *E. coli* (A. L. Stevens) showed an apparent requirement for DNA.

Studies on the structure of polynucleotides have been continued using, primarily, infrared spectroscopic techniques. Evidence that the I + C polymer may have a DNA-like structure has also been obtained (T. Miles).

Steroids

Studies on mechanisms of hormone action. Several recent studies have increased our understanding of how hormones are able to exert an effect on metabolism by altering the activity of enzymes. Thus, certain estrogenic steroid hormones inhibit a key enzyme—glutamic dehydrogenase. It was found that their ability to inhibit crystalline beef liver glutamic dehydrogenase is due to their ability to alter the structure of the enzyme by dissociating it into subunits. The subunits possess a different catalytic activity, alanine dehydrogenase, from that of the parent tetramer. The conversion of the tetramer to the subunit is catalyzed by steroid hormones and the reverse reaction is facilitated by adenosine diphosphate (G. M. Tomkins and K. L. Yielding).

Aldehyde Dehydrogenase. A steroid-sensitive aldehyde dehydrogenase has been discovered and studied. It is strongly inhibited by such compounds as progesterone and stimulated by diethylstilbestrol, cortisone and several steroids. This is the only case of an enzyme whose activity has been altered in several directions by different steroid hormones. The role of this enzyme in galactose metabolism has also been studied (E. S. Maxwell).

The cytoplasmic fraction of liver contains two DPN-linked aldehyde dehydrogenases. Only one of these is steroid sensitive. Whereas progesterone and the androgens inhibit this activity, the estrogens, and to a lesser extent cortisone, inhibit when the substrate concentration is rate limiting, but stimulate when the substrate concentration is nonrate limiting. The mechanism of inhibition appears to be different from that of stimulation. The dual nature of the estrogen effect may represent a prototype for a homeostatic mechanism by which accelerated metabolism occurs when substrate is present in excess and decelerated metabolism occurs when substrate is limiting (E. Maxwell and Y. Topper).

In light of the recent finding that vitamin A acid can substitute systemically for other vitamin A forms, it was of interest to find that vitamin A aldehyde is irreversibly oxidized to the acid by aldehyde dehydrogenase, and that the K_m for the aldehyde is about 10^{-6} M. The kidney enzyme is quite sensitive to estrogens, and this may account for the fact that female kidney contains much less of the vitamin than does the male kidney (T. D. Elder and Y. Topper).

In continuation of studies on galactose metabolism, it has been found that stimulation of the epimerase reaction leads to acceleration of the transferase reaction in hemolysates (T. D. Elder, S. Segal, and Y. Topper).

Metabolism of Steroids. It has been shown that the liver represents a heterogenous cell population with respect to the steroid 4–5 double bond reductastes (R. F. Bakemeier and G. M. Tomkins). This finding may be related to current ideas about antibody production in reticuloendothelial systems. In other work, it was found that

there are at least nine separate steroid 4–5 unsaturated β reductases (A. N. Weinberg and G. M. Tomkins). These are TPNH-dependent enzymes catalyzing an irreversible reaction. Some of their physical properties have been studied. They have similar, but not identical, mobility on diethylaminoethyl cellulose chromatography and in density gradient centrifugation. The pH optima are different and they have a different substrate specificity.

Regulatory Mechanisms and Hormones

In attempts to delineate the site of the diabetogenic action of mannoheptulose (MH) the following observations have been made. This seven-carbon sugar does not affect the insulin effect on glucose uptake by isolated rat diaphragm; thus, the peripheral action of insulin is unaltered by MH. The pattern and rate of I^{131}-insulin degradation by perfused rat liver is unchanged by MH, indicating that the hormone is not more quickly destroyed in the presence of this sugar. A rise in blood ketone bodies is caused by MH even when glucose is simultaneously injected. Sedoheptulose, an isomer of mannoheptulose, does not elicit these effects. It has been tentatively concluded that the site of diabetogenic action of MH is the pancreas. A gluconogenic effect of MH appears to be mediated through the adrenals (E. Simon, R. Scow and S. Chernick).

The nature and locus of binding of insulin by target tissues, and the characteristics of the enzymic degradation of this hormone by mammalian liver are under investigation.

Chemical and electrophoretic data obtained with a fluorescent derivative of insulin indicate that the product contains 1.25 fluorescin radicals per molecule of protein. Results of rabbit bioassays indicate that the derivative possesses ½ to ⅔ of the activity of the native hormone. Preliminary experiments involving the use of fluorescence microscopy have demonstrated that the derivative is strongly bound by leucocytes but not by erythrocytes (F. Tietze and G. Mortimore).

Following treatment of insulin-I^{131} with highly purified liver "insulinase" approximately 15 percent of the total radioactivity of the substrate becomes soluble in trichloroacetic acid in contrast to nearly 100 percent with crude liver homogenate. This result may indicate that the purified preparation represents only one component of a complex that constitutes, in its entirety, the enzyme system known as "insulinase" (H. Katzen and D. Stetten).

Oxytocin and vasopressin stimulate glucose oxidation by mammary gland *in vitro*. The effects of these hormones are additive to that of insulin, but not to each other (T. Goodfriend, J. Cohen, and Y. Topper).

The chemical nature of the humoral agents which accumulate during uremia and the chemical basis for the changes in cerebral function in uremia are being studied. It has been found that normal rat brain cells, when suspended in serum from uremic patients, generate significantly greater yields of $C^{14}O_2$ from glucose-C^{14} than do the same cells suspended in normal serum. This finding provides an assay for an agent in human uremic serum which alters the metabolism of nervous tissue. Attempts to concentrate and ultimately to identify the agent or agents responsible are being pursued. If this goal is achieved a rational therapy for uremia may result (N. Cummings, D. Stetten).

Pyridine Nucleotides and Other Coenzymes

A number of TPNH-dependent enzymes have been shown to be inhibited by the end product, TPN. The biological significance of this finding has been explored and it is felt that this inhibition, rather than being fortuitous, represents an important biological control (G. Ferro-Luzzi and G. M. Tomkins).

In continuation of studies designed to study the metabolic fate of intracellularly generated reduced pyridine nucleotides the synthesis of cholesterol and fatty acids by rat liver slices has been investigated. The synthesis of cholesterol (digitonin-precipitable steroids) from acetate-2-H^3,C^{14} results in a product with an isotopic ratio of H^3/C^{14} of 0.4. This is in strikingly good agreement with that derivable fro mtheoretical considerations, namely 0.44 ± 0.04. Similar experiments utilizing acetate-2-H^3,C^{14} incorporation into fatty acids revealed an isotope ratio of 0.24, to be contrasted with a theoretical value for stearic acid synthesis of 0.11. Which of the considerations utilized in calculating the theoretical value for stearic acid is in need of modification has not been determined.

The metabolism of TPNH and DPNH generated intracellularly has also been studied. This was accomplished with the use of glucose-1-C^{14}, H^3 and glycerol-2-H^3 as substrates. It has been shown that these two reduced pyridine nucleotides are not equivalent in the cell. TPNH is utilized in fatty acid and cholesterol synthesis to an extent 10 to 20 times greater than DPNH.

Utilizing the H^3/C^{14} ratios in fatty acids and cholesterol from glucose-6-C^{14},H^3 and acetate-2-C^{14},H^3 an estimate has been made of labilization of hydrogen in the Embden-Meyerhof pathway. The results indicate that ⅔ of the hydrogens bound to C–6 of a molecule of glucose are exchanged during conversion to acetyl coenzyme A. Presumably this occurs at the phosphoenolpyruvate-pyruvate reaction (B. Bloom and D. Foster).

The biosynthesis of thiamine and its phosphorylated derivatives by baker's yeast is being investigated. An enzyme which catalyzes the condensation of the two-ring moieties of thiamine phosphate according to the following equation:

$$\text{"Pyrimidine"-PP} + \text{"Thiazole"-P} \xrightleftharpoons{Mg^{++}} \text{Thiamine-P} + \text{PP}$$

has been isolated and purified about 1,000-fold. The reaction has an equilibrium constant of approximately 10, and is inhibited by pyrophosphate noncompetitively. Procedures have been devised for the synthesis of hydroxymethyl pyrimidine and the corresponding mono- and pyrophosphate derivatives (I. Leder).

Protein and Amino Acid Synthesis

Cell-free extract which makes small, but apparently significant, quantities of the inducible enzyme penicillinase from *B. cereus* has been obtained. The requirements for this system are under study at the moment (M. Nirenberg).

Histidine Biosynthesis

The first step of histidine biosynthesis in *Salmonella* has been shown to be the reversible condensation of phosphoribosyl-pyrophosphate and ATP to form N-1-(5′-phosphoribosyl)-ATP and pyrophosphate. The condensation product has been isolated and its structure proved. The enzyme catalyzing the reaction has been described and named phosphoribosyl-ATP pyrophosphorylase. It is lacking in one class of histidine mutants. The enzyme is strongly inhibited by histidine. None of the other enzymes of the pathway is inhibited by histidine and the inhibition of the first enzyme by the end product seems to be another example of *feedback inhibition* control.

In addition to feedback inhibition another mechanism, *repression*, is capable of regulating the amount of histidine synthesized in *Salmonella*. Repression results in control of the rate of synthesis of the enzymes of a pathway by the end product of the pathway (B. N. Ames and R. G. Martin).

Enzymatic Utilization of Model Compounds

Continuing studies on the mechanism of enzyme catalyzed aldehyde oxidation have resulted in information concerning the sites of substrate binding to protein. Employing techniques of enzyme digestion and competitive inhibition, it has been concluded that aldehyde substrates are bound to closely juxtaposed SH groups of the enzyme, whereas pyridine nucleotides are bound at sites other than sulfhydryl groups. The fact that each of 10 different aldehyde dehydrogenases tested are sensitive to inhibition by arsenite, a specific inhibitor of disulfhydryl systems at low concentration, strengthens the case of disulfhydryl involvement for enzyme catalyzed aldehyde oxidation. The precise manner in which such sulfhydryl groups participate is in the realm of speculation but probably involves a series of disulfide interchange reactions.

An enzyme thiooxidase has been purified from a mold, *Piricularia oryzae*, which catalyzes the oxidation of conjugated sulfhydryl compounds. The enzyme catalyzes the oxidation of catechols as well and is analogous in many respects to polyphenol oxidases. Because the thiol substrate is oxidized to a disulfide and no further, thus differing from polyphenol systems which oxidize catechol extensively, the mechanism of the oxidation may be studied without interference from competing reactions. Here, too, are indications for the involvement of closely linked sulfhydryl groups (W. B. Jakoby, and G. Aurbach).

Laboratory of Physical Biology

The Laboratory of Physical Biology comprises five sections which devote attention to basic research in disciplines varying from physics and

chemistry to physiology and often cooperative ventures involving several disciplines. In a general sense, the several projects are related by the attention given to the material aspects of life processes, but specifically with the energetic and structural interconversion which is responsive to the various environmental factors, both immediate and remote, that interact with organisms. Much cooperative work was carried out with personnel of other Institutes and some at laboratories made available in off campus research centers.

In the area of photobiology studies on the basic phenomena of photosynthesis are concerned with the primary step in photomechanisms. Early transient phenomena are of special interest. Dr. Olson has developed a spectromicrophotometer for observing fluorescent spectra and absorption changes at half second intervals. The transient formation of a yellow fluorescent component in what (max. emission 540 mμ) has been demonstrated to be nonenzymatically produced, to require interface distribution, and to depend on availability of oxygen. The process is seen to be reversible and similar to others in activated components of protochlorophyll and numerous porphyrins.

Photoreception in animal tissues has been shown by Dr. Hagins to result in the release of selectively concentrated potassium ions in the receptor cells. This is accompanied by an electrical impedance fall which is subject to suppression by procaine but without effect on the externally observed action current. A model has been devised which accounts for most of the findings in terms of known properties of electrically excitable cells.

The effects of destructive radiation have been studied by Dr. Greenblatt, who, in cooperation with Dr. Elkind of the Cancer Institute, has shown that clonal techniques of studying the survival and the changes in chromosome properties can be used to great advantage in cell cultures of the Chinese Hamster. A model was devised which demonstrates critical points in the phenomena of chromosomal injury which are subject to further experimental proof by the techniques devised in this study and in cooperation with Dr. Royce Lockhart.

By means of nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR), Dr. Becker has shown the molecular structure of a number of substances of biological interest in many cooperative studies performed with scientists of both NIH and nongovernmental institutions. Information obtained from the techniques of NMR and EPR supplements that obtained by infrared spectroscopy and provides detailed information on structure, particularly on molecular interactions and the character of molecules containing impaired electrons, including free radicals and paramagnetic atoms and ions. A substantial addition to the number of accurately determined hydrogen bond energies now in the literature has been made. This work has thrown support to some tentative theories and doubt on other long held generalizations regarding hydrogen bonding. Studies of porphyrins, of interest to Dr. Watson (University of Minnesota) have provided information on the position of functional groups and tautomerism of the NH protons in certain metal-free porphyrins. A free radical intermediate in the oxidation of chlorpromazine *in vitro* is being studied to elucidate its apparent second order decay reaction demonstrated by the methods used in this project. Many other NMR spectra have been made for other investigators and found helpful and even instrumental in solving challenging structural problems.

The use of dipole moment, dielectric polarization and spectroscopic absorption, rotatory dispersion and dichroism by Dr. Charney has resulted in several different methods of elucidating the role which intermolecular forces play in molecular organization . The importance of atomic polarization rather than molecular characteristics in causing the apparent dipole moment of p-quinone was demonstrated. The binding of protoporphyrin to Bovine serum albumin was studied to elucidate the control of the stoichiometry of protein binding by copper. Interest has been developing in a method of studying polar molecules in the glycerol-water-salt solution glass formed at liquid nitrogen temperature. Nucleosides exhibit characteristic and specific differences in the sharpening of the spectral bands under these conditions. In addition, the effect of solvents on spectral absorption has been shown as a function of Van der Wall's constant "a" and related to the ratio of dipole moment and polarizability.

Studies of steroid photo reactions have been continued by Dr. Brackett with Dr. Sharpless to show the quantum requirements for ergosterol transformation. The data are consistent with those of Havinga and give in addition values at three longer wavelengths. The work also provides further information on the time course of transformations involving tachysterol and precalciferol which have been the subject of previous studies. The application of analog computer techniques with the assistance of Mr. Hahn has facilitated the otherwise tedious reduction and analysis of data so that much more information may be brought to bear on the proof of current concepts. Instrument development in all areas has been directed toward automation and computer analysis.

Observations of the optical rotatory dispersion in the ultraviolet region by Drs. Weiss and Charney suggest that the Cotton effects of ergosterol and its stereo isomers cannot be explained solely through action of the asymmetric carbon atoms adjacent to the dienic chromophore, implying a contribution by the latter and thus a deviation from planarity in the dienic system.

The application of flash photometry to ergosterol studies by Dr. Adams has shown that thermal reactions are of importance in the elucidation of the complex isomerizations which take place in irradiation by various means.

Dr. Ziffer has shown that a differential assay of vitamins D_2 and D_3 is available through the finding that these products are transformed to their pyro- and isopyro-calciferol isomers respectively during gas chromatography.

In the past year the formation of a new section studying the effects of high energy radiation on substances of biological interest and on organisms themselves has proceeded slowly with the difficult problem of setting objectives with feasible modes of attack. Under the direction of Dr. Liddel the means for thermal neutron exposure were devised by Dr. Malich in the installation of a 400 Kev positive ion accelerator which is currently able to yield well over 100 times the product of the Polonium-Beryllium source available earlier. Calibration of the neutron flux has been carried out in such a manner as to give absolute values of neutrons per unit volume at various positions in the exposure chamber. The use of this source for exposures both of substances of biological interest and living organisms is currently being arranged.

In anticipation of other objectives of this section, Dr. Kempner has continued his studies of radioactively labeled compounds and their synthesis in organisms such as yeast cells. His studies with uracil and fluorouracil show that both are incorporated only into RNA and not into DNA. This allows the use of fluorouracil in the study of the selective action of the cells between these two compounds at various stages of synthesis of RNA. In comparison with previous work using other analogs in protein synthesis, it is seen that the nucleic acid synthesis mechanism has the ability to select pyrimidines in at least three observable steps.

The use of controlled low voltage electrons to localize various functional sites in large molecules has been exploited by Dr. Preiss. He has shown that basic globular proteins which have been subjected to dry, *in vacuo* bombardment acquire the ability to induce an excess turbidity in polynucleotide solutions. This turbidity is a non-monotonic, protein characterizing function of the dose and an analytical treatment of the data has been made correlating experimental examination of the significance of primary, secondary, and tertiary structures. The importance of isoelectric pH and molecular weight of the proteins has been shown and the importance of the number of S–S bridges is under study. The general method promises much application in an area otherwise singularly inaccessible to quantitative assessment. The comparative effects of thermal and faster neutron radiations are under study.

Studies in physico-chemical aspects of semipermeable membranes have been pursued by Drs. Sollner, Caplan, Shean and James with the findings that self-exchange and allo-exchange of ions can be very satisfactorily described by current theoretical treatments devised here. A new approach in the study of perm-selective cation exchange membranes has centered on the pronounced frequency dependence of the resistance of such membranes. In the case of highly charged membranes, new light has been thrown on the anomalies which classical and current theory cannot resolve. The inclusion of the "contractile effect" of polyelectrolytes in such considerations can improve the rationalization of

procedures which otherwise give such anomalous results. Studies on nonstructural, homogeneous ("oil") membranes are underway showing much greater ionic selectivities and reaction rates than previously described.

In another area of Molecular Biophysics the investigation of macromolecular organization of substances of biological interest is under study through electron microscopy and X-ray diffraction by Dr. Labaw and Mr. Mosely. They have shown that in the study of fine structure it is possible to see striated images from periodic objects in which the period may be one-half, equal, or twice the actual period of the object. Definition of the conditions producing such phenomena has been accomplished for some organic substances in an attempt to facilitate the correct interpretation of such fine structure images, particularly those near the limit of resolution.

Studies in Physical Biochemistry have continued the analysis of the various phenomena concerned with the relationship of structure and function at the molecular, cellular, and organ level. Dr. Laki and his group continue to analyze the mechanical, chemical, and energetic factors in the special protein complexes that account for the unique behavior of muscle, blood clotting and a number of allied systems in which the interconversion of energy and structure takes place through highly specialized properties of discrete parts of oftentimes huge molecular aggregates. Muscle is one example of a number of biological transducers which has received much study here but other aspects such as the mode of action of enzymes are actively being pursued as in the case of thrombin, carboxypeptidase, A, B, myosin ATPase, and others. In addition, the phenomena of the polymerization of proteins has remained of interest since it lends insight on the formation of networks which merge into the visible structures which can be directly observed.

It was found by Dr. Bowen that in glycerol treated muscle fiber ADP and phosphate ion suppress the work done by fiber-bundles in ATP induced contractions but that this is less effective than the suppression of ATP splitting by homogenized fiber. He also showed that the diffusion of ATP in fiber bundles could be determined by measuring the extra tension developing in various sized bundles of ATP tensed muscle on the addition of ADP.

Studies on myosin by Dr. Carroll show that free diffusion measurements of dog heart myosin correspond to inhomogenious material in both normal as well as failing hearts and the deduced molecular weight (5.0×10^5) must therefore be considered an average value. Dr. Laki found that tryptic digestions of myosin showed "fingerprints" in which the count of arginine containing spots gave an estimate of a minimal molecular weight in the neighborhood of 2.0×10^5. Such a weight indicates multiple aggregation into larger myosin molecules through orderly side to side and end to end associations.

Drs. Stewart and Bowen showed by labeling techniques that inorganic phosphate binding is present in myosin ATPase but find that it is probably not a contributing factor to the high initial rate of action.

The dissection of myosin into three distinct components by specific chemical reagents has been used by Drs. Kominz and Carroll in studying the mode of synthesis and structure of this protein. Comparative studies are in progress.

In studies on F-actin, Dr. Laki has found by using labeled ATP that F-actin binds only one ADP molecule but a varying number of inorganic phosphate molecules depending on the concentration of phosphate in the medium.

The equilibrium constant for dimerization of human mercaptalbumin with mercury or mercurials was shown by Dr. Simpson to have a value two orders of magnitude smaller than formerly estimated and that this presented an interference problem in reactions with other cations.

In the study of fibrin-fibrinogen transformation, Dr. Gladner, in cooperation with Dr. Folk of NIDR, showed that the complete sequence of amino acids in peptide B of co-fibrin is: N-acety . thre . gly . phe . pro . asp . tyr . So_4 . asp . glu . gly . gly . asp . asp . arg . pro . lys . val . gly . leu . gly . ala . arg.

They also have shown that very highly purified carboxypeptidase B from pig pancreas has a molecular weight of 34,000 and that it contains one gram atom of zine per mole of the enzyme. It consists of a single polypeptide chain. Dr. Carroll showed that pyruvate and α-ketoglutarate dehydrogenation complexes from *Escherichia coli* are large multienzyme units of 4.8 and 2.4 mil-

lion grams, resp., capable of carrying out a series of reactions involving several coenzymes.

In the field of amino acid analysis a new approach has been successfully made by Dr. Saroff in collaboration with Dr. Karmen of NHI by the adaptation of gas chromatography. The key to this success was the preparation of N-trifluoroacetylmethyl esters of the amino acids. The method has yielded good results with valine, methionine, serine, glutamic acid, phenylalanine, hydroxyproline and cystine.

Drs. Irreverre and Viswanatha have established the presence of hydroxylysine in trypsin and also detected it in chymotrypsin. Dr. Irreverre found that an interesting α-amino acid, homocitrulline, was present in the urine of infants. It is not found in adults or older children.

Physiological studies which range from mammalian species through invertebrates form a varied approach to the effects of environmental and other physical factors on biologic functions. A continuation of Dr. Park's investigation of the cyclical phenomenon of gonadal development in fresh water *Hydra* shows that it is independent of population density over a 25-fold range but budding is inversely related to crowding. The effect of environmental factors either directly or via secondary mechanisms is being further investigated.

Studies on the biochemical transformations during insect metamorphosis by Dr. Levenbook have shown that blood citrate titers found in larvae of 17 species are high before metamorphosis but fall steeply during this stage only to increase to a peak during mid-pupal life before the decline to adult levels. Dr. Buck has shown by studies of response latency that the neuroeffector linkage of the firefly lantern has three steps in electrical stimulation. This is viewed as involving a conventional neural conduction at adequate stimulation levels; an excitation of an intermediate unit at higher stimuli, probably the tracheal end-cell, which can be considered analogous to the motor end plate of muscle; and, third. a direct excitation of the photocyte. Dr. Keister has shown that blowfly adults are able to maintain respiration at hypoxic levels lower than possible for larvae or pupae but that in all stages temperature and hypoxia influence both normal and basal metabolism in a parallel manner.

Studies on the effect of hypoxia in higher forms have been carried on by Dr. Altland as in the past and are directed to the effects on the blood and circulatory tissues. In collaboration with Dr. Highman, it has been shown that hypoxia plays a recognizable role in the experimental production of pulmonary arteriosclerosis and in calcium deposition in atherosclerotic lesions in the aorta and pulmonary vessels. These lesions are correlated with very high serum cholesterol values in cholesterol-fed rabbits exposed to high altitudes. Altitude tolerance studies in chickens show that they have a lower tolerance than any small warm blooded animals previously studied. The altitude exposure of dogs results in an increase in certain serum enzymes beyond normal levels revealing a hitherto unexplored physiological mechanism involved in hypoxic stress. In conjunction with Dr. F. Smith, NCI, it was discovered that there is an increase in the titer of antisheep erythrocyte hemolysin in rats exposed to high altitude and it is felt that this may provide a new experimental method for increasing other antibody titers.

Studies on the circulatory reaction of sensitive mammalian species to plasma expanders by Dr. L. Marshall have shown that moderate doses of dextran increase the incidence of fatal insulin convulsions in rats while a larger dose is protective. A relation to the significant hyperglycemia found in the latter under conditions of consciousness may be inferred. During pentothal anesthesia such a hyperglycemia is greater than in conscious rats and this coupled with the reported finding that high blood glucose suppresses dextran reactions, and the previously found vasodepression enhancement through pentobarbital may explain why the lack of demonstrable reactions to dextran are seldom seen under anesthesia in rats. It was shown that after insulin, vasodepression followed intravenously administered dextran in rats but less severely than in normals. Similar relations are observed in edema formation, but hematocrit values show that after insulin treatment there may be greater generalized fluid loss to the tissues than in the so-called "target areas" in which it seems to be reduced. In dogs receiving insulin there is seen an at-

tenuated reaction to polyvinyl-pyrrolidone (PVP) given intravenously. Under pentobarbital anesthesia, the rise in blood sugar following PVP injection is no greater than when conscious, correlating with earlier findings that this anesthesia introduces no alteration in reaction.

The section has also continued some work on pulmonary physiology and currently Dr. Specht and Mr. Brubach are studying the use of heavy gas mixtures for demonstrating the efficiency of lung ventilation and in a new application of Archimedes principle for determining the density of the body by this heavy atmosphere. The latter method is alternative to and perhaps more sensitive than current clinical procedures.

Laboratory of Chemistry

Medicinal Chemistry

Work has continued on the synthesis of analagesic drugs. The analgesic drug Phenazocine (NIH 7519) developed in recent years through the work of this section is now in clinical use for injection as Prinadol (Smith, Kline & French Labs.) in this country and as Narphen (Smith & Nephew, Ltd.) in England. Studies in our laboratories indicate a broad spectrum of clinical utility for oral preparations and marketing of an oral form seems imminent (Drs. Eddy, Cochin and May).

The benzomorphan class of compounds continue to show high analgesic potency and low physical dependence capacity in monkeys. One of the more interesting benzomorphans under study has shown excellent carryover of pain-relief from mouse to man. In this series, compounds in which the 5 and 9 alkyl groups are *trans* (equatorial-equatorial), are at least 10 times more active than the much more readily obtainable *cis*-counterparts (Drs. N. B. Eddy and E. L. May).

A new and improved synthesis (Dr. E. M. Fry) of the benzomorphan class of neuropharmacologic agents has been developed. This synthesis, a 3-step sequence starting from pyridine alkohalides, is based on the 1.2-shift of the benzyl or substituted benzyl group of 1,2,5,6-tetrahydropyridine quaternaries (Stevens rearrangement) and is particularly applicable to 4-substituted and unsubstituted pyridines not otherwise practicably amenable to the benzomorphan synthesis. By this new method and by the Grewe procedure, a new (5,9-diethyl) benzomorphan and its diastereoisomer at C–9 (the latter in minute yield) have been synthesized from 3,4-diethylpyridine (Dr. E. L. May and Mr. J. H. Ager).

There has been further demonstration of the importance of electrical effects in the stereochemical control of addition of H–H and CH_3–H to the carbonyl group of 9-oxobenzomorphans. Either possible diastereoisomeric carbinol, to the exclusion of the other, may be obtained depending on whether the proximate nitrogen is quarternary or tertiary (–). These carbinols (analogs of 14-hydroxydihydrocodeinone and morphinone) and particularly their 0-acetyl derivatives are potent analgesics (Dr. Seiichi Saito and Dr. E. L. May).

Codeine has been transformed by an interesting sequence of reactions into an analog of phenazocine containing a morphine-like oxygen bridge. Continued research on the analogs (at the ester function) of acetyl choline has revealed that when nitrogen is diphenethyl substituted, maximum analgesic activity is seen with the acetyl. In a 5-step synthesis an analog in which one of the N-phenethyl groups was replaced by p-$AcOC^6H^4COCH_2$ was obtained, although its isolation was complicated by its remarkable sensitivity to solvolytic cleavage (Dr. J. G. Murphy).

In the immunochemical field vinylglucoside (via the Hofmann elimination reaction) and p-hydroxystyrene have been synthesized and preliminary polymerization and copolymerization studies performed. It is believed that such polymers and copolymers may serve as the backbone for attachment of determinant groups in the total syntheses of antigenic substances (Mr. T. D. Perrine).

It has been found that thyroxin will significantly depress N-demethylase activity when given once a day at 0.5–0.75 micrograms per kilogram of rat over a thirty day period and 4 mg./kg. of morphine administered twice daily for one week results in significant reduction of N-demethylase activity. The longer the drug is given, the lower the dose at which one sees significant reduction (Dr. J. Cochin).

Carbohydrates

Further studies of the sugars in the avocado and *Sedum* species have shown that, in addition to the D-*glycero*-D-*manno*-octulose reported earlier, the avocado contains a second octulose and also two nonuloses. *Sedum* extracts also appear to contain a second octulose and the same two nonuloses. Nine-carbon sugars have not heretofore been encountered in nature (Drs. Richtmyer and Sephton).

Condensation of products formed from glycosides by periodate oxidation, followed by reduction, yields the glycosides of aminosugars. By this route kanosamine, 3-amino-3-deoxy-D-glucose, the sugar moiety in the antibiotic kanamycin, was synthesized (Dr. H. H. Baer).

β-Sedoheptitol (D-*glycero*-*D*-*gluco*-heptitol) has been condensed with formaldehyde and the structure of the resulting tri-*O*-methylene derivative has been proved to be 1,3:2,4:5,7-tri-*O*-methylene-D-*glycero*-D-*gluco*-heptitol (Mr. E. Zisis).

The synthesis of 1-substituted aldoses through the reaction between 1-thio-aldose derivatives and various heavy metal salts, a process discovered here last year, has been investigated from various aspects. One result of this work is the discovery of a wholly new synthesis of 3-deoxyribose nucleosides (Dr. Pedersen).

Methods for the synthesis of labile 1-acylaldoses have been explored. Among other pathways, that through fully benzylated aldofuranosyl halides has been investigated, with the D-ribofuranose and L-arabinose series as examples (Dr. R. Barker).

The discovery that the disaccharide sophorose, 2-*O*-β-D-glucopyranosyl-D-glucose, is a contaminant in U.S.P. dextrose and also a potent inducer for the formation of at least one enzyme has directed attention to this rare sugar. In order to make the substance available for biochemical research, a synthesis, giving the sugar in 30 percent yield, was evolved (Dr. B. Coxon).

Metabolites

The program on the development of nonenzymatic methods for the cleavage of peptide bonds in proteins, peptide hormones and enzymes at present occupies the majority of the members of the Section on Metabolites. With a two-fold aim in mind independent methods for "auditing" primary sequences of proteins established by the "accounting method" of overlapping sequences of tryptic peptides have been developed and the selectivity of the chemical agents employed has been utilized to modify enzymes and proteins and to correlate chemical reactivity of special functional groups or centers with the secondary or tertiary structure of the protein.

The most important discovery made during the year 1960 in this area was the *selective cleavage of peptide bonds next to methionine*, which was initially accomplished by addition of alkyl halides to yield tertiary alkylmethionine sulfonium derivatives which, on heating in aqueous solution, broke down under cleavage with release of an N-terminal pipetide and a peptide with C-terminal homoserine (lactone) (Drs. Lawson, Gross and Foltz).

A major improvement was the discovery of cyanogen bromide as the agent of choice for cleaving methionine peptides *at room temperature.* In this case the intermediate methionine cyanosulfonium salt breaks down by an intramolecular process smoothly and rapidly at neutral or acidic pH to yield, in addition to the aforementioned peptide fractions, methyl thiocyanate which was assayed by gas chromatography (Dr. E. Gross).

The new methionine cleavage has been applied to *bovine pancreatic ribonuclcase.* The formation of a novel heptadecapeptide, a *chemical tail peptide* (in contrast to the enzymatic 20-residue S-peptide produced by the action of subtilisin) was observed which had the original N-terminal lysine in addition to a C-terminal homoserine (lactone). By electrophoretic separation and by dinitrophenylation technique the topography of the cleavage of the 4 peptide bonds next to the 4 methionines in ribonuclease has been determined. In the light of these new results, the standing controversy between the Rockefeller Institute and the National Heart Institute on the positions of glutamic acid and serine occurring either in positions 18 or 11 has been answered for native ribonuclease in favor of position 11 for glutamic acid, the position favored by Anfinsen and his group (Dr. E. Gross).

Preliminary results on the application of cyanogen bromide to the *α-chain of human hemoglobin*, which contains 2 methionines, have been obtained in collaboration with W. Konigsberg

of the Rockefeller Institute and point to successful and selective cleavage (Dr. E. Gross).

Work on the structurally extremely difficult cyclic antibiotic *gramicidin-A*, containing over 40 percent tryptophan, has been continued and put on a more secure basis by the preparation of 60 grams of the pure gramicidin-A in collaboration with the Research Laboratories of the Schering Corp. in Bloomfield, N.J. (Drs. S. Ishii and E. Gross).

Selective oxidation of all the tryptophans in gramicidin and selective partial cleavage of peptide bonds next to tryptophan has been achieved by the use of N-bromosuccinimide in alcoholic aqueous solution in yields approximating 50 percent. Countercurrent distribution of the reaction mixture gave *ethanolamine* as the major water-soluble fragment and at least 3 lipophilic ninhydrinpositive fragments of lactonic character, one of which was further purified by reverse phase paper chromatography (Dr. E. Gross).

In extension of the investigation on the action of N-bromosuccinimide on trypsinogen and its derivatives T. Viswanatha, LPT–NIAMD, and W. B. Lawson made a similar study of the action of N-bromosuccinimide on *chymotrypsin*. In addition to the oxidation of tryptophan residues, one of the tyrosine residues is also affected by treatment of the protein with N-bromosuccinimide under the conditions employed in these studies. The oxidative modification of the enzyme resulted in a loss of catalytic activity toward typical protein and ester substrates. Such partially inactivated enzymes still possessed the ability to incorporate phosphoryl or acetyl groups upon reaction with diisopropyl phosphofluoridate or *p*-nitrophenylacetate respectively. Oxidation of chymotrypsin with N-bromosuccinimide seems to retard both the acylation and deacylation steps in the catalytic process without affecting the K_m value. A direct measurement of the rate of deacetylation of C^{14}-acetyl chymotrypsin has been made.

Contrary to data previously reported from other laboratories, the oxidative splitting of tyrosyl-cysteine bonds has been achieved in high yield. The N-bromosuccinimide method, when applied to S-carboxymethylribonuclease, gave consistently higher yields than with the intact enzyme. Conditions for the differential cleavage of tryptophyl and tyrosyl peptide bonds with N-bromosuccinimide have been worked out for mixtures of tryptophyl and tyrosyl model peptides (Drs. J. G. Wilson and L. A. Cohen).

With regard to the difficult task of utilizing the imidazole ring of histidine as a potential functional group for neighboring group effects that may labilize and cleave C-histidyl peptide bonds some progress has been made. The easily removable N-toluenesulfonyl group attached to imidazole makes it stable to oxidative degradation. In the N-bromosuccinimide cleavage of S-carboxymethylribonuclease the oxidative cleavage of a histidylphenylalanine bond is a possibility (Drs. Wilson and Cohen).

A great number of model peptides containing hydroxyproline, an important building stone of collagen, have been synthesized (Dr. J. Francis) and investigated with regard to conditions for non-enzymatic cleavage. In the course of this investigation, a large body of data on the fundamental reaction mechanisms of peptides derived from cyclic and open γ- and σ-hydroxy and unsaturated amino acids have been accumulated. The study of N-tosyl and N-benzoyl-allyl- and -methylallylglycine amides and peptides has contributed to an understanding of the mechanism of the iminolactonization reaction observed with N-bromosuccinimide in phosphate buffer at pH 7 (Dr. N. Izumiya).

As an outgrowth of the studies on hydroxyproline its dehydration product 3,4-dehydroproline, which is not accessible from hydroxyproline, has been synthesized by reduction of pyrrole-α-carboxamide with phosphonium iodide in fuming hydriodic acid. The amide of this new amino acid is optically unstable. This has led to an unprecedented combination of enzymatic resolution with asymmetric transformation. This amide was resolved by leucine amino peptidasee of hog kidney. Ammonia was liberated from the amide and the optically active L-amino acid was formed in yields substantially greater than the theoretically possible 50 percent of L-isomer. Evidently yields of 100 percent of L-isomer were formed which under the conditions of enzymatic resolution partially reverted to racemic material. This type of resolution presents the kind of model which has been discussed but never realized in theories dealing with the origin of life and optically active matter in nature where normally 100 percent of L-amino acids, but as a

rule no D-forms, exist (Dr. A. V. Robertson).

In collaboration with the Laboratory of Clinical Biochemistry, NHI, a new spectrophotometric *assay method for L-amino acid oxidase* from snake venom has been developed based on the quantitative conversion of 3,4-dehydroproline to the strongly absorbing pyrrole-2-carboxylic acid by this enzyme (Dr. Robertson).

In collaboration with New England Nuclear Corp. L-proline-3,4-H^3 has been made available to biochemists for metabolic studies. There has always been a great need for such a selectively tritiated optically pure proline (Dr. Robertson).

In collaboration with the Laboratory of Clinical Biochemistry and Dr. E. Katz, Special Fellow from Rutgers Univ., the *modification of actinomycin* through addition of dehydroproline and many of its derivatives to the culture medium of *Streptomyces griseus* is being investigated. The aim in this study is the elaboration of less toxic and more carcinostatic actinomycins and to gain more information on the enzymes that incorporate such foreign amino acids as pipecolic acid, azetidinecarboxylic acid, etc. into the peptide part of actinomycin (Dr. Robertson).

In collaboration with Dr. A. Berger, The Weizmann Inst. of Science, Rehovoth, Israel, *poly-3,4-dehydro-L-proline* has been made by polymerization of N-carboxy-3,4-dehydro-L-proline anhydride. The residue rotation and mutarotation of this polymer was determined and compared with those of poly-L-proline, which is assumed to have a righ-handed helical configuration in solution (Dr. Robertson).

A simple qualitative visual test for the *detection of monamine oxidase in tissue slices* has been developed; it is based on the slow conversion of dehydronorkynuramine to indigo by oxidative deamination and intramolecular cyclization to indoxyl which is rapidly autoxidizeed to indigo, whose substantivity without addition of further aromatic substituents is, however, not sufficient for a useful histochemical staining method (Dr. Y. Kanaoka).

In cooperation with Dr. Axelrod, NIMH, *studies on catechol-O-methyltransferase* and on the equilibrium with the products arising from the action of microsomal O-demethylase have been carried into the *mescaline series* utilizing as substrates mescaline, mescalol and mescalone on the one hand, and derivatives of 3,4,5-trihydroxyphenethylamine on the other hand. So far 4 partly phenolic metabolites of mescaline have been observed and identified by synthesis. The pharmacological aspects of these metabolites are being worked out in cooperation with the Sterling-Winthrop Research Institute (Dr. J. Daly).

In collaboration with the Laboratory of Clinical Biochemistry, the *mechanism of the action of monamine oxidase* is being investigated. It has been found in this cooperative investigation that dimethyltryptamine N-oxide is degraded by monamine oxidase at a rate significantly depending on the partial pressure of oxygen present (Dr. Y. Kanaoka).

In collaboration with Regis Chemical Company, Chicago, and aided by a special grant from Psychopharmacology, *kynuramine dihydrobromide* has been made available to clinical and biochemical laboratories as the standard substrate of choice for the rapid and routine spectrophotometric assay of monamine oxidase. By the same arrangement *homocarnosine*, the new peptide of γ-aminobutyric acid with L-histidine, which has been isolated from brain tissue in the Laboratory of Clinical Biochemistry, has been made available in research quantities. Similarly, serotonin as the crystalline acid oxalate, has for the first time been made available to biochemists and clinicians with none of the drawbacks that the previous serotonin-creatinine complex had.

In collaboration with Dr. S. L. Friess and Chief Durant of the Naval Medical Center the *labilization of ester bonds in aminocyclitol derivatives* has been further investigated. The symmetric and asymmetric di- and tri-O-acetates of N,N′-tetramethyl-2-deoxystreptamine have been synthesized, purified and subjected to kinetic analysis of their hydroylsis rates. A combination of field, charge-transfer and overall conformational effects combine to impart to some of these esters a liability which, with a half-life time of 19 minutes, surpasses that of *p*-nitrophenyl acetate by more than 100 times. These models deserve consideration in connection with model substrates for cholinesterase (Drs. Kny and Daly).

Cooperative studies with the Laboratory of Clinical Biochemistry, NHI, on the *mechanism of hydroxylation of dopamine to norepinephrine*, and of tyramine to octopamine utilizing selectively tritiated precursors have yielded evidence

for the existence of an *enzyme-substrate intermediate* in which the tritium atoms at the methylene group next to the aromatic nucleus exchange with the solvent. The elaboration of this phenomenon into a quantitative assay for dopamine-β-oxidase (norepinephrine synthetase) is in progress (Dr. Y. Kanaoka).

In collaboration with the Laboratory of Clinical Biochemistry procedures have been developed for the detection of *inhibitors of monamine oxidase in vitro* and *in vivo* and for the determination of the duration of their central and peripheral action. These procedures have been applied to the detection and study of several new classes of inhibitors (Dr. M. Ozaki).

It has been demonstrated in several studies that the presence of tertiary butyl groups *ortho* to a phenol greatly increases the contribution of dienone structures to the resonance hybrid. The effect of such a difference on spectral and ionization properties has been reported as well as changes in the rate of several reactions. Stable phenolic hypobromites have been detected and methods have been elucidated for stabilizing free radical intermediates analogous to those occurring in the biosynthesis of thyroxine (Dr. L. A. Cohen and Mr. W. B. Jones).

Steroids

Anthrasteroid rearrangement: The two isomeric anthrasteroid alcohols (acid catalyst, *p*-toluenesulfonic acid·H_2O) have the secondary hydroxyl in ring A (either position 2 or 3); dehydroxylation leads to hydrocarbons isomeric at C_{14}; the mechanism accommodating the formation of these isomers must be complex and is at present completely obscure. By rearrangement of 3-desoxydehydroergosterol, depending on the conditions, either the 14α- or the 14β-anthraergostatriene is formed. Finally and most surprisingly, the *14*α-anthraergostatrienol acetate (as only product) was obtained by boiling dehydroergosterol in acetic acid (Dr. O. Tanaka and Mr. J. Steele).

Thiol analogs of corticoids: A new method for converting thiocyanoderivatives to thiols *via* the acetyldithiocarbamates has been developed and applied to 3α,9α-oxido-11β-thiocyano-5β-androstan-3β-ol-17-one-3-methylether and the corresponding corticoid (Dr. Y. Ueda).

Stevioside: The only uncertainty left in the structure of stevioside is the position of the carboxylic group, i.e., whether at C_4 or C_{10}. Experiments are being conducted to degrade the two di hydrosteviols to the corresponding 13-desoxy primary alcohols and to compare these directly with the analogous degradation products from Djerassi's cuauchicicine (Dr. U. Beglinger).

Steroidal Alkaloids: The four possible C_{22}, C_{25}-solanidan-3-ones have been prepared. A new method with commercial potential has been developed for the conversion of solasodine to pregnadienolone *via* the pyridine hydrochloride isomerization. Solasodine and tomatidine have been converted *via* the "oximino ketones" to the respective bisnorcholenic and bisnorcholanic acid 16 $\longrightarrow$ 22 lactones, of possible value as aldosterone antagonists (Drs. Sato and Ikekawa).

Steroid analysis: A method has been developed for rapid and accurate plating of radioactive steroid fractions. A fully automatic steroid analyzer has been constructed and is undergoing final testing. Major points: Preadjustment for any desired ratio of elution solvents; automatic determination of the adrenal corticoids by U.V. absorption and reducing power; recording of results on a strip chart (Drs. Johnson and Heftmann).

Adrenal cortical hormones in rat adrenal tumor tissue: While in the normal rat adrenals corticosterone and aldosterone are the end products of metabolism, tumor tissue showed a greatly decreased ability for 11-hydroxylation of added precursors (e.g. progesterone). This, in turn, leads to an accumulation of 11-deoxycorticosterone. The latter has not been isolated from rat adrenals heretofore (Drs. Johnson and Heftmann).

Steroid analysis by gas chromatography: The steroidal alkaloids solasodine and tomatidine and a number of their derivatives have been chromatographed in microgram quantities, on a column of Chromosorb W, 80–100 mesh containing 0.75 percent SE–30. The response to the finest steric differences makes possible the detection of a number of stereoisomers in a complex mixture (Drs. Sato and Ikekawa).

IR spectroscopic studies: Additional spectra have been converted to proper units for Lorentzian analysis. A Rudolph Recording Spectropolarimeter acquired recently has been put in operating condition to produce usable optical

rotatory dispersion data. Both Model-21 spectrophotometers have been equipped with auxiliary recorders for presentation of spectra on N.B.S. punch cards (Mr. H. K. Miller and Mrs. A. W. Wright).

Biogenesis of plant sterols: $^{14}C_2$-acetate or $^{14}C_2$-mevalonate were injected into tomato fruits. Stigmasterol was isolated ($\Delta^{5,22}$-stigmasta-dien-3β-ol) in pure form (and its radioactivity determined) together with some other active apparently sterol-like components. The slime mold Dictyostelium discoideum has been grown on a medium containing ^{14}C-mevalonate or $^{14}C_2$ acetate. The activity of stigmastenol (Δ^{22}-stigmasten-3β-ol), isolated in pure form, was determined (Drs. Heftmann, Bennett, and Johnson).

Biogenesis of steroid sapogenins: Homogenized tubers of Dioscorea floribunda were incubated with radioactive acetate or mevalonate. While the acetate was incorporated in a low but constant rate into diosgenin, no trace of activity could be detected in the diosgenin from the mevalonic acid incubates. Several other radioactive products have been isolated but not yet structurally elucidated. There is an appreciable difference of biosynthetic activity in the various parts of the tuber; the highest synthetic rate is found in the portion around the tuber (Drs. Heftmann and Bennett).

Cooperative work: In cooperation with the Section on Physiology, LPD, NIAID (Dr. von Brand), the Insect Physiology Lab., ARS, Dept. of Agric. (Dr. S. J. Louloudes), and the Section on Metabolism, LCPM, NHI (Dr. Y. Avigan), complete analysis and characterization of a number of steroids (sterols) from the extract of helminths, and insects (reared under artificial condition) have been carried out. Desmosterol has been isolated (rat liver) and characterized in the "cholesterol-biosynthesis" inhibited by MER–29. ^{14}C-labeled desmosterol, -methostenol and -lanosterol have been and are being synthesized in connection with the above biosynthetic work.

Analytical Services

Approximately 10,000 determinations were made of which two-thirds were for carbon, hydrogen and nitrogen, the remaining third for halides, sulfur, phosphorus, functional groups, selenium, various metals, optical rotations, weight losses, etc. These services were utilized by approximately 130 research scientists at the NIH. A limited number of analyses were performed for other government agencies. Elemental analysis for fluorine was made available during the year. A study is being made of the direct determination of oxygen in organic compounds and it is hoped that this analysis will be available by the first part of next year (Mr. McCann, Miss Parisius, Mrs. Peake, Mr. Baer, Mrs. Wong).

Laboratory of Pathology & Histochemistry

Altitude Effects

Drs. Benjamin Highman and Paul D. Altland found that dogs exposed to simulated high altitude show a transient hyperglycemia, and a sharp rise (lasting 3–7 days) in serum glutamic-oxalacetic transaminase (SGO-T), serum glutamic-pyruvic transaminse (SGP-T), serum lactic dehydrogenase (SLD) and serum alkaline phosphatase (SAk-P). The rise in SGO-T, SGP-T and SLD is diminished by the adrenergic blocking agent phenoxybenzamine and the hyperglycemia by the ganglionic blocking agent chlorisondamine. These findings support the view that altitude hypoxia produces hyperglycemia by release of endogenous catechol amines and elevates serum enzymes by altering cellular permeability. Drs. Altland and Highman have found that cholesterol-fed rabbits, exposed to 16,000 feet, up to 17 weeks, show much more severe pulmonary atheroclerosis, less severe lesions in the descending and abdominal aorta, and a much higher incidence of marked calcium deposits in both aortic and pulmonary atherosclerotic lesions; hypoxia and pulmonary hypertension are considered major contributing factors. Drs. F. Smith, Altland, and Highman find that rats acclimatized to altitude and injected with sheep erythrocytes show significantly increased (over ground level) antisheep erythrocyte hemolysin, formed during the primary immune response.

Cytogenetic Studies

Dr. J. H. Tijo in continuing collaborative study with several investigators on the chromosomal complement of patients with leukemia and with congenital anomalies, i.e. ovarian dysgenesis,

Turner's syndrome has revealed karyotype abnormalities in a number of such patients.

Collaboration with NCI an investigation of the chromosomal constitution of a series of transplantable thyroid tumor lines, in an attempt to relate properties of these lines with their chromosomal pattern, has revealed a constant chromosomal defect in the stem line of two nonfunctional anaplastic lines and on the stem line of an only moderately de-differentiated line. Also in collaboration with NCI, Dr. Tijo has developed a simplified method for the study of chromosomes of bone marrow cells without prior *in vitro* growth which has been used with good results on rat, mouse, hamster and man.

Degenerative Joint Disease and Human Rheumatism

Dr. Leon Sokoloff extended studies of the pathogenesis of degenerative joint disease and of the descriptive pathology of human rheumatism. A comprehensive analysis of the genetics of osteoarthritis in mice is nearing completion. The joint disease is transmitted as a recessive characteristic; more than one gene appears to be involved; there is no sex linkage. Studies, made in collaboration with Dr. R. S. Yamamoto, disclose that there are significant genetic differences in plasma lipids, hepatoma-formation and several other lesions in the same animals, but that there is no correlation between these findings and the joint disease. Comparative pathology of degenerative joint disease continues to be investigated. A study on vertebral coalescence in the giant dinosaur *Diplodocus longus* has been completed in collaboration with Dr. B. S. Blumberg. This is surmised to represent a traumatic osteoarthritis.

A paper on the pathogenesis of ochronotic arthropathy, written with Drs. William O'Brien and W. G. Banfield, has been accepted for publication. The data suggests that the critical problem in the development of the joint disease, as distinguished from the biochemical defect, is affinity of articular tissues for the homogentisic acid or its pigment derivatives.

Germ-Free Animals—Nutritional Deficiencies

Using germ-free mice Dr. David Beaver found that injected progesterone was quite capable of producing the typical vaginal neutrophilic exudate seen in certain phases of the estrous cycle; bacterial infection is not required.

In another study by Dr. David Beaver, the A-deficient, germ-free rat, similar to the conventional animal, exhibits widespread focal keratinizing metaplasia of mucous membranes. Germ-free animals, in addition, show degeneration or necrosis of the liver, kidney, heart, and adrenal gland. The germ-free state and the absence of infection modify the pathogenesis and histological appearance of the lesions of A deficiency, but do not prolong life. The latter fact suggests that in Vitamin A deficiency there is a significant metabolic disturbance.

Dr. L. L. Ashburn in cooperation with the Walter Reed Army Institute of Research, has shown that a low protein, choline deficient diet leads to liver cirrhosis in germ-free rats. In fact, the development of the lesion appears to progress faster than in conventional animals. These findings are being further studied as is the mechanism whereby certain antibiotics appear to delay onset and progress of the cirrhosis. Drs. Beaver and Ashburn have found that the lungs of these rats and those of germ-free rats on a pantothenic acid deficient diet show prominent lipid deposits visible on gross examination as few to abundant white spots of 1 to 3 mm size. These nodules are formed of xanthoma cells containing triglycerides and lesser amounts of anisotropic material. Although the lesions are seen occasionally in other circumstances, germ-free state and the specific abnormal diet are necessary for its maximum expression.

Goldthioglucose Obesity

Radioautography of tissues submitted to activation analysis permits localization of gold in mice made obese by goldthioglucose. This study by Dr. George Brecher shows that areas of acute necrosis seen at 12 hours correspond to gold localization. Later, minute scars contain the bulk of the gold. In other experiments it was shown that glucose cannot effectively compete with goldthioglucose in localization in the hypothalamic feeding center, as is implied in Mayer's widely accepted glucostatic theory.

Hemoglobin

The study of hemoglobin by Dr. Harvey Itano and Miss Elizabeth Robinson has continued to

furnish new information on the structure, genetic control, biosynthesis, and evolution of a protein molecule. The ability of the subunits of canine hemoglobin to combine with the subunits of both normal and abnormal human hemoglobins suggests retention of the characteristic tertiary structure in spite of changes in primary structure. Studies on the inheritance of the two types of polypeptide chains of hemoglobin have yielded the first example of a protein synthesized under the combined control of two nonadjacent genetic loci. The chains are apparently synthesized independently in identical pairs and a doubly heterozygous red cell synthesizes four different chain-pairs which combine to form four different hemoglobins.

In continued studies of abnormal human hemoglobin-I (Hb-I), Dr. Makio Murayama has found that the genetic alteration takes place in the alpha chain (N-terminal val. leu....) of the molecule. In contrast, the chemical abnormalities of Hb-S, Hb-C, and Hb-G are known to be located in the beta chain (N-terminal val.his.leu....). The amino acid sequence studies revealed the following:

Hb-A	val.leu....ala.val.try.gly.lys....
Hb-I	val.leu....ala.val.try.gly.*asp*....

A lysine residue is replaced by aspartic acid in this genetic alteration.

Hematology

The kinetics of red cell proliferation have been further elucidated by Dr. Frederick Stohlman. Erythropoietine has been shown to act by promoting the differentiation of primitive stem cells into erythroid elements; some of these cells mature without dividing; a substantial number when severe hypoxia exists. Erythropoietine, apparently, does not directly affect the erythroid elements (i.e. pronormoblasts, etc.). Preliminary evidence suggests that these rapidly maturing cells have a shortened life span.

The effectiveness of erythropoietine in the irradiated animal has been explained by studies in which it was shown that depopulating the stem cells compartment by administering erythropoietine immediately after irradiation serves as a stimulus for earlier regeneration of the stem cell compartment. This implies that one mechanism for regulation of the stem cell compartment is the total number of cells, whether fertile (i.e. capable of division) or infertile, and that differentiation of stem cells into erythroid elements results in an increased rate of division amongst the remaining stem cells.

Histochemistry

Studies on the localization in tissue sites of polysaccharide synthesis by Dr. Tadao Takeuchi have resulted in the development of a histochemical method for the cellular localization of enzymic activity forming polysaccharides from uridinediphosphate glucose (UDPG). This method localizes UDPG glycogen transferase activity to numerous tissue sites and its activity has been compared to that of amylophosphorylase in these areas. Further investigation has resulted in the development of a method for the histochemical localization of uridinetriphosphate-pyrophosphorylase activity in tissue sections.

A continuation of studies by Dr. George Glenner on the localization of proteolytic enzyme activity in pathologic and normal tissue has been extended, using newly synthesized chromogenic substrates to the demonstration of an esterolytic enzyme having several characteristics distinct from those previously described histochemically and to the investigation of an enzyme hydrolyzing a specific class of amide substrates.

In order more accurately to demonstrate the sites of numerous dehydrogenases in tissue section with the elimination of false positive localization a new route for the synthesis of pure ditetrazolium salts has been developed. A screening method for the demonstration of *Endamoebae* based on their glycogen content (a modified methenamine silver technique) has been applied to pathologic tissues for routine diagnostic purposes.

Dr. Samuel Spicer has partially characterized mucins in normal rodent and human tissues and in human pathological material by correlative studies with established autoradiographic and histochemical staining methods and by newly developed procedures. The methods developed for histochemical identification of mucins include combinations of conventional staining techniques, a periodic acid diamine procedure, and enzymatic digestion with sialidase. Application of all the available specific techniques reveals varieties of sulfomucins and sialomucins not known to exist

from biochemical methods. Such studies also suggest the possibility that the histochemical properties of the mucins in normal as well as pathological tissues may be unique in each histologic site. In other studies, cytosiderin and peracetic acid-aldehyde fuchsin stained (lipofuscin?) bodies observed in epithelial cells in a number of organs appear to be, in some instances at least, associated with strong acid phosphatase activity. The cytoplasmic particles are at present considered to be a possible morphologic manifestation of the catabolic removal of discarded cytoplasmic macromolecules.

Dr. David Beaver, in an extensive investigation of the rat preputial gland found that the structure varies greatly with the fixative used. In addition to lipids, the acinar cells contain numerous protein granules. A stain was devised which permitted the demonstration of both elements in the same section. With this and other histochemical methods the appearance (function) of the gland was evaluated in relation to sex, age, androgenic and estrogenic stimulation, and hypophysectomy.

Human Pathology

The opportunities offered to this laboratory through consultative and diagnostic studies of surgical and autopsy specimens from the Indian Health Program of the Public Health Service have continued to stimulate interest in aspects of geographic and environmental pathology. Direct contacts with these Hospitals during the year promises to improve greatly the quality of the material for study. Problems related to sarcoidosis, to dietary hemosiderosis, to diabetes, and to atherosclerosis and its complications among the Indians are of particular interest.

Attempts by Drs. Gert Laqueur and Harry Marsh to isolate in culture the organism responsible for pneumocystis carinii pneumonitis from animal and man have been continued throughout the year. All experiments failed, using culture media for fungi and protozoa and specialized bacteriologic media under aerobic and anaerobic conditions with varying acidity, viscosity and nutrients. However, recent attempts, with the collaboration of Mr. Charles Zierdt of the microbiology division of the Clinical Pathology Laboratory, have produced the first promising results utilizing highly specialized procedures. It has been possible to obtain the same organism from several animals in culture. The organisms tinctorially and morphologically resemble closely those seen in the human and animal disease; introduction into germ-free animals is expected to establish their pathogenicity.

Immunology

Dr. Emily Emmart, using fluorescent antibody and other techniques has shown that: (1) streptococcal hyaluronidase is localized in certain tissues of infected mice after *in vivo* elaboration of the enzyme; (2) glyceraldehyde-3-phosphate dehydrogenase is present in the I-band (mitochondria) of the wing and leg muscle of the roach (Periplaneta americana); (3) myosin is localized in the myofibrils (A-band) of the conduction fibers of the beef heart.

In collaboration with Dr. Marion Webster immunochemical studies on the antibodies to human kallikrein preparations in successive stages of purification have been carried out. The rise in gamma globulin has been followed by paper electrophoresis on sera of immunized rabbits. Antibody has been determined by precipitin reactions *in vitro*, in agar and by antibody inhibition of the vasodilator action of kallikrein in the dog.

Dr. Edwin Lerner, with the collaboration with Drs. Kurt Bloch and J. J. Bunim has shown that rabbits immunized repeatedly with their own fractionated gamma globulins failed to show positive reactions for rheumatoid factor, or for anti-human antibody. This is in contradistinction to the results of Milgrom, Witebsky, *et al*, and may conceivably be due to the rigorous precautions taken to exclude contamination by human, other animal, or other rabbit proteins. Drs. McMaster, Lerner and Exum have shown that experimental allergic thyroiditis has been produced in inbred guinea pigs and has been shown to correlate with the presence of delayed hypersensitivity, but not with the presence of circulating anti-thyroid antibody. The allergic thyroiditis was the earliest ever produced experimentally, and the most severe at the time intervals studied. The experimental conditions excluded direct trauma to the thyroid, sensitized reaction to thyroid, and immunologic interference by heterologous antigenic serum or tissue proteins.

Renal Structure and Function

The functions of the straight and convoluted segments of the rat proximal tubule which differ distinctly with respect to their handling of phenol or clorphenol red, were investigated by Dr. James Longley in respect to a number of other phenolsulfonephthalein dyes. Several dyes were found to behave similarly to the ones previously studied. Others were found to be accumulated by the convoluted segment only and still others to show no apparent accumulation at all. No correlation between the physical or chemical characterisitcs of the dyes studied and their handling by the kidney has yet been established.

In addition to the research projects abstracted above, certain scientists in other laboratories receive advice from members of our staff; particularly in pathologic anatomy. Our histopathological preparation unit also took part in this cooperative effort by cutting and staining about 2,000 tissue sections this year for ten investigators not in laboratories of NIAMD.

Laboratory of Pharmacology and Toxicology

Spermidine and Spermine

A striking development in the studies on the polyamines has been the observation that these amines, even in very low concentrations, have a marked stabilizing effect on protoplasts, mitochondria and bacteriophage T5. The mechanism of this effect is not clear, but may be related to the binding of these amines to nucleic acids and phospholipids.

Further work has also been carried on the enzymes and intermediates concerned in the biosynthesis of spermidine. Thiomethyladenosine has been isolated and identified as the product of the reactions: Putrescine + decarboxylated adenosylmethionine → spermidine + thiomethyladenosine.

Under certain conditions much of the spermidine of *E. coli* is present as a glutathione-containing derivative. A tentative structure has been presented for this derivative (Drs. H. Tabor, C. W. Tabor, S. M. Rosenthal, G. Jamieson and T. Viswanatha).

Histidine and Related Compounds

1. The reduction of unsubstituted imidazole and its derivatives has been achieved for the first time. This has been accomplished by carrying out the catalytic hydrogenation of these compounds in acetic anhydride. Furthermore, by this method new imidazoline and imidazolidine compounds have been prepared.

2. Imidazoleacetic acid ribotide is formed by the following enzymatic reaction: Imidazoleacetic acid + pyrophosphorylribosephosphate (PPRP) + ATP → imidazoleacetic acid ribotide + ADP + orthophosphate + pyrophosphate.

Hydrolysis of imidazoleacetic acid ribotide results in imidazoleacetic acid riboside, which we have previously shown to be present in the urine after histamine and imidazoleacetic acid administration.

3. The reaction histidine → urocanic acid + NH_3 proceeds via an ammonia-enzyme intermediate.

4. The following reaction has been demonstrated in an enzyme purified from *E. coli*: Ergothioneine → thiourocanic acid + trimethylamine (Drs. H. Tabor, G. Crowley, J. Wolff, H. Bauer, A. Peterkofsky and Miss V. Childs).

Sialic Acid.

1. A hitherto undescribed enzymatic system has been purified from mammalian liver for the biosynthesis of N-acetylneuraminic acid.

2. A sialidase has been purified from a bovine serum fraction (VI). This is the first time a sialidase has been described in mammalian tissues.

3. The enzymatic removal of sialic acid from different genetic forms of human transferrin has revealed that part of the structure of the different forms is common to all. Sialic acid is probably responsible for the differentiation between the various genetic forms of part of the transferrin molecule (with Dr. Blumbery).

4. Other studies have included the determination of the concentration of sialic acid in cerebrospinal fluids (with Dr. R. Jakoby); the demonstration that one-third of the sialic acid of cerebrospinal fluid is bound to small sized dialyzable material; a test for sialic acid-containing mucins in tissue and in tumors using a histochemical procedure (with Dr. Spicer); a study of the amount and nature of the sialic acid in a variety of nonmammalian organisms; and the demonstrations of sialic acid in an ether-soluble

lipid in sea urchin eggs (Drs. L. Warren, H. Felsenfeld, and Miss C. Spearing).

Burns and Shock

Studies have been continued on the therapy of burns in clinical cases in Peru. In a large series of adult cases oral saline was as effective as a combination of saline and plasma therapy.

The occurrence of infection in burn cases continues to be a serious problem at all ages; children under 3 years were particularly susceptible. The incidence of septicemias in these children could be decreased by large doses of plasma or gamma globulin, with some lowering of the mortality. Antibiotic therapy, on the other hand, had no effect.

In the laboratory a bacteriological study of burned mice (with Dr. E. Verder, NIAID) suggested that after burn injury, bacteria of low virulence and invasiveness are able to spread from the local area and establish generalized infections (Drs. S. M. Rosenthal, R. C. Millican, N. A. Kefalides, K. Markley, and Peruvian associates).

Mouse Leprosy

In the field of experimental leprosy better conditions for the culture of macrophages have been developed. Macrophages can now be maintained up to 100 days, permitting cultivation of *Mycobacterium leprae murium*. This represents a marked improvement over the previously existing techniques which only permitted survival of macrophages for a short time (usually less than 10 days). This is of considerable importance for leprosy studies, as this has permitted survival and growth of the *Mycobacterium leprae murium* in a given tissue culture for long periods (Dr. Y. T. Chang).

Sulfur Amino Acids

Three enzymes have been isolated from yeast that are required for the reaction:

$$\text{L(-)Methionine sulfoxide} + \text{TPNH}^{+H^{+}} \rightarrow \text{L-methionine} + \text{TPN} + H_2O.$$

In addition to this reaction two of these enzymes carry out the TPNH-catalyzed reduction of dithiols to thiols (Drs. S. Black, J. F. Thompson, and Miss Hudson).

Enzyme Activity

1. The various enzymes of the urea cycle increase with an increase in dietary protein, and decrease with a lowered dietary protein (Dr. R. T. Schimke).

2. Trypsin and chymotrypsin have been shown to contain hydroxylysine. This amino acid has not been described previously in an enzyme protein (Dr. Viswanatha with Dr. Irreverre).

3. The ability of chymotrypsin to react with diisopropylfluoridate or p-nitrophenylacetate was separated from its ability to hydrolyze peptide bonds by the differential destruction of the latter activity by treatment with N-bromosuccinimide (Dr. Viswanatha with Dr. Lawson).

4. Spectrophotometric methods have been developed for the measurement of the aldolase-dihydroxyacetone phosphate complex for kinetic studies on this enzyme after alteration by carboxypeptidase (Dr. Mehler and Miss Asnien).

Excitable Cells

Further evidence has been obtained that calcium transfer is at least one direct factor in the coupling between excitation and muscular contraction in the fibers of the "twitch," "slow" and cardiac type. Additional correlations have been established between the interactions of veratrum alkaloids with monomolecular films of stearic acid at air-water interfaces and with living membranes (Drs. A. M. Shanes, N. L. Gershfeld, S. Winegrad, S. Dikstein, and C. P. Bianchi).

Gramicidin J

During studies on the enzymatic biosynthesis of gramicidin J in extracts of *Bacillus brevis* the enzymatic formation of a dipeptide of phenylalanine and proline was observed, which may be an intermediate in gramicidin biosynthesis (Dr. K. Kurahashi).

Cholesterol Synthesis

A rabbit liver enzyme has been purified which converts phosphomevalonic acid to pyrophosphorylmevalonic acid (Dr. K. Markley and Mrs. Smallman).

Office of Mathematical Research

Work has continued along the major lines indicated in last year's report. viz.: Mathematical

formulation and analysis of neurophysiological problems (Dr. Wilfrid Rall, Mr. Ezra Shahn and Mrs. Jeanne Altmann); development of mathematical and computational methodology for assessing mathematical models based on differential equations and detailed studies of iodine metabolism in the thyroid system (Dr. Mones Berman, Mrs. Marjorie Weiss, Mr. Ezra Shahn and collaborators from other groups as noted below); general mathematical problems arising from the rate of behavior of biological systems (Dr. John Hearon). Work has been initiated in visual perception and pattern recognition (Mrs. Rosalind Marimont, NIMH, Associate Member of OMR). The work along these four lines is summarized, in the same order, below. It is not possible to give all of the results of the year's work and in each case only highlights and/or examples are included.

The solution for the external and internal field for a neuron with spherical soma and cylindrical dendrites has been obtained. Such fields have been computed numerically on the IBM–650 for a neuron with seven asymmetrically placed dendrites. In particular, fields were computed for the instants in time when the "A-spike" and "B-spike" reach their maximum potential. These detailed numerical calculations have yielded results of broad qualitative significance for the interpretation of neurophysiological recordings. The mathematical treatment of the spread of electric current between soma and branching dendritic trees has been extended to the transient case. This information allows interpretation and analysis of experiments designed to estimate the membrane time constant and elucidate the nature of synaptic potential.

The program, written for the IBM–704, originally developed for analysis of tracer kinetics has undergone major revision and consolidation and major extension in terms of generality and flexibility. In particular the program will accept input data directly in a variety of forms, take account of dependence relations among two or more parameters, take quantitative account of the information available on a given compartment or sub-system in fitting a particular compartment. The program has been adapted to non-linear systems and as a result can be made to perform the analysis of any mathematical model based on an arbitrary set of differential equations. The general program is available to any investigator. The program, and the mathematical methods which it was designed to implement, has had considerable application: Labeled glucose studies in collaboration with Dr. S. Segal, NIAMD, thyroxine studies in collaboration with Dr. H. Haddad, Washington University; a variety of problems in collaboration with Drs. S. Wollman and M. Elkind, NCI, and Drs. D. Steinberg and J. Davies, NHI.

It has been shown that if A is a matrix of order n with non-positive diagonal and non-negative off-diagonal elements then no component of a vector x, initially nonnegative and obeying $dx/dt = Ax$, can vanish at a finite time. The original intent was to demonstrate that a rate matrix with the above sign pattern guarantees positive concentrations, and conversely positive concentrations imply that sign pattern. The mathematic statement of this commonsense constraint on a real system leads to the following practical results: It follows that a necessary condition for overshoot in a linear system is that some initial concentration be below and some above the steady-state value; that, if $E_1A_{1j} < O$, every element of $-A^{-1}$ is non-negative, the smallest root is simple, and the co-factors of A have the sign of $(-1)^{n+1}$. It has been shown that a function $F(x,y)$ symmetric and homogeneous of degree zero has the property $aF/ax = aF/ay = O$ on the line $y = x$. This theorem, necessary in the error analysis of certain approximate differential equations for metabolic systems, has the consequence that class of function having an optimum which is a point of symmetry in the logarithmic plot can be defined and the class of mechanism which may underlie optimum pH, substrate-concentration, etc., can be stated.

Work has recently been initiated on a mathematical study of visual perception with special emphasis at present on color vision. The main objective is a mathematical treatment of the empirical laws of color vision designed to produce the necessary and sufficient conditions required by them. It has been shown that Grassman's laws and Abney's law are expressions of a mathematical property which has been denoted as quasi-additivity. In terms of functions: if $f(x) = f(y)$ and $f(u) = f(v)$ then $f(x+u) = f(y+v)$. In terms of the laws of color vision f will be some general transformation and x, y, u,

v may be spectral distributions or functionals of them and equality is then defined as "matching" of lights. It has been shown that if f is a one-to-one function of a linear operation Abney's law is implied. The converse of this is being studied in terms of more general spaces and the similar but more general problem of Grassman's laws is being pursued.

CLINICAL INVESTIGATIONS

The clinical and laboratory research activities of the Clinical Investigations area of N.I.A.M.D. have continued to increase during the past year. No new units have been added nor additional space acquired.

The complement of patients beds has been expanded from 65 to 70 as of March 1, 1960 and the census for the year has averaged 76 percent.

A total of 483 in-patients was admitted during the 12-month period from December 1, 1959 to November 30, 1960, an increase of 94 patients (24 percent) over the same period last year. The total patient days was 19,134, an increase of 2,192 over the preceding year. In the Admissions and Followup Department 1,585 patients were examined and studied, an increase of 65 over the past year. The average in-patient stay at the Clinical Center was 40 days.

Investigations related to the diseases studied at N.I.A.M.D. have resulted in 94 publications in scientific journals, monographs, annual reviews and medical textbooks. During the past year Dr. Joseph J. Bunim was awarded the Heberden Medal by the Heberden Society of Great Britain in recognition of his research in the rheumatic diseases. In accordance with tradition, he delivered the Heberden Oration in London. Dr. Bunim was also selected to give the Stoneburner Lecture in Richmond, Va., as guest lecturer for the Mexican Rheumatism Society in Mexico City and for the Canadian Rheumatism Association in Toronto. He delivered lectures on the rheumatic diseases at London University, Manchester University and Leiden University. Dr. Paul A. di Sant'Agnese was appointed Clinical Professor of Pediatrics at Georgetown University and Lecturer in Pediatrics at Johns Hopkins University. He was given the annual award of the Philadelphia Chapter of the National Cystic Fibrosis Research Foundation. Dr. di Sant'Agnese was guest lecturer at scientific meetings in Holland, Switzerland and Germany.

At the request of the Bureau of Medical Services of the U.S.P.H.S., members of the Clinical Investigations staff gave lectures and conducted ward rounds at the following PHS Hospitals: Baltimore, Boston, Seattle and Staten Island.

Our staff scientists have derived substantial benefit from the association of distinguished Visiting Scientists and Guest Workers who have worked at our Institute during the past year: Prof. E. G. L. Bywaters of London University, Dr. Rosalind Pitt-Rivers of the National Institute for Medical Research, England, Dr. Samuel Rose of the University of Melbourne, Australia, Dr. Serge Lissitzky of the University of Marseilles, France, Dr. P. Vilkki of the University of Turku, Finland, Dr. H. Keen of Guy's Hospital, England, Dr. Marco Andreoli of the University of Rome, Dr. T. Shiba of Osaka University, Japan and Dr. David Jackson of the University of Oregon.

Arthritis and Rheumatism Branch

Research activity in the immunological reactions in the rheumatic diseases is increasing sharply both in this country and abroad. This shift in research direction is understandable since studies conducted on the chemistry and metabolism of connective tissue over the last decade have not achieved a clear insight into the pathogenesis of the rheumatic diseases. Newer concepts and knowledge of the mechanism of antibody (both humoral and cell-bound) formation and more sophisticated immunological techniques have stimulated workers in the rheumatism field to renewed efforts in this relatively young and rapidly growing discipline.

Serological Factors in Connective Tissue Diseases

As reported last year, it was discovered in this laboratory that patients with Sjögren's syndrome (kerotoconjunctivitis sicca, xerostomia and rheumatoid arthritis or other connective tissue disease) exhibited an unusually high incidence of circulating "antibodies" or "factors" to tissue components which by and large were not organ or species specific. (These studies have been ex-

tended during 1960 and interesting results obtained). The series now includes 40 patients with Sjögren's syndrome that may be conveniently classified into four clinical groups: (1) sicca complex with rheumatoid arthritis, (2) sicca complex with scleroderma, (3) sicca complex with polymyositis or myopathy and (4) sicca complex alone. The greatest number of separate antibodies and the highest titers of these were present in the last two groups, yet the incidence of different antibodies even in the first two groups clearly exceeded that found in rheumatoid arthritis alone. The incidence of individual serological factors demonstrated in Sjögren's syndrome were: rheumatoid factor, 100 percent; antinuclear factor, 77 percent; complement fixing antibodies against tissue components, 49 percent and specific thyroglobulin antibodies, 27 percent.

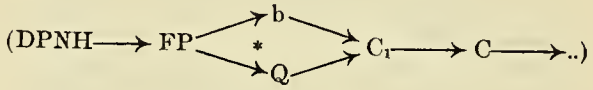
By the Ouchterlonly gel diffusion precipitin technique, precipitin lines were demonstrated between serum gamma globulin fractions from patients with Sjögren's syndrome and saline extracts from human salivary gland, liver, kidney and thyroid extracts. Attempts to isolate the active (antigenic) fraction concerned in the complement fixation reaction were made by ultracentrifugation of tissue homogenates. The antigenic component was found in the soluble (supernatant) constituent and not in the sedimented nuclei, mitochondria or microsomes. The serum constituent involved in this reaction was found in the gamma globulin fraction following ammonium sulfate and electrophoretic separation.

Of special clinical interest was the observation of the concurrence of Hashimoto's thyroiditis, arteritis, myopathy, hyposthenuria and neuropathy in some cases in this series.

The results of these studies suggest that an altered or deranged immunological mechanism may be involved in the pathogenesis of Sjögren's syndrome which is frequently associated with rheumatoid arthritis or some other connective tissue disease (scleroderma, systemic lupus erythematosus or polyarteritis nodosa).

Action of Steroids on Enzyme Systems

Inhibition of DPNH-Cytochrome C Reductase. As described in the 1959 Annual Report, low concentration of hormonally active steroids and diethylstilbestrol were shown to inhibit DPNH-cytochrome c reductase between the flavoprotein and cytochrome b (or Coenzyme Q), and this inhibition could be competitively overcome by α-tocopherol and various other lipids. Furthermore, an alternate route of electron transport was identified from the flavoprotein to cytochrome c. As a future step in localizing the effect of steroids on this sequence (as described in earlier reports) the reduction of internal coenzyme Q was studied in beef heart mitochondria. As expected, steroid inhibition of Co Q reduction was observed, thus confirming the site of inhibition to be just beyond the flavoprotein level.*



Additional work has been done in characterizing the basic effect, and particularly in identifying possible physiologic sequelae. It has been shown that soluble preparations of cytochrome c reductase from microsomes and mitochondria are affected in a manner similar to the particulate preparations. Some specificity of the lipid reversal of the steroid effect is suggested since α-tocopherol does not diminish the degree of inhibition of DPNH oxidation by amytal (or chlorpromazine) both of which block the electron transport chain at approximately the same level. More intensive study of beef pituitary and human placental enzyme preparations has not revealed any striking differences in response from the rat tissues examined.

Decarboxylation of Pyruvic Acid. The DPN-dependent oxidation of pyruvic acid to acetyl coenzyme A and CO_2 is the metabolic step which permits the entry of glucose carbon into the Krebs cycle, and its rate might be important in the regulation of carbohydrate metabolism. Steroid hormones have been shown to inhibit this reaction *in vitro;* and the elevated blood levels of pyruvic acid in patients with Cushing's disease and upon steroid administration to normals [9] suggest some such action of these hormones *in vivo.*

Since the oxidation of DNPH to DPN by the DPNH-oxidase reaction is strongly inhibited by various steroid hormones, it was suspected that

the steroid inhibition of pyruvate oxidation might be due to curtailment of DPN arising from DPNH oxidation.

Pyruvate oxidation in homogenates of rat tissue was stimulated by the addition of cytochrome c and/or liver microsomes, which specifically stimulate DPNH oxidation. This showed that the rate of pyridine nucleotide oxidation could control that of pyruvate. Alpha-tocopherol, which prevented steroid inhibition of the DPNH oxidase, also overcame the steroid suppression of the pyruvate reaction. When DPNH oxidation was impeded by other inhibitors of electron transport, such as amytal or antimycin A, there was a corresponding depression of the oxidation of pyruvate, and α-tocopherol could not overcome this effect.

These findings illustrate that steroids can regulate the oxidative decarboxylation of pyruvic acid, by virtue of their ability to inhibit oxidation of DPNH. The possibility is being investigated that a direct mechanism of inhibition also exists.

It has been established by other investigators that steroid hormone administration can result in a five-fold increase in GPT activity in the rat liver. In order to examine the possibility that this induction could result from the accumulation of substrate pyruvate after steroid administration, a group of six rats was given injections of pyruvate over a 2-week period. Comparison of the liver GPT activities with six controls revealed no significant differences. The question of whether the pyruvate was actually increased at the cellular level by this procedure has not been established.

Inhibition of Glutamic Dehydrogenase (GDH) Reaction. In studying the possible sequelae of the steroid inhibition of DPNH oxidation, we were led to examine the effects of steroids on certain DPN requiring dehydrogenases. The oxidation of glutamate to α-ketoglutarate (αKG) and ammonia by DPN (or TPN) the GDH reaction was of particular interest because of the importance of glutamic acid in amino acid synthesis and breakdown, it seemed reasonable in a system which contains both glutamic dehydrogenase and DPNH-cytochrome c reductase, the inhibition of the latter reaction by steroids could depress the oxidation of glutamate when DPN was rate-limiting. In accordance with this, 5×10^{-5} M progesterone caused a 2.7-fold increase in the rate of DPNH accumulation in a system consisting of DPN, gluamate and disrupted liver particles.

When this phenomenon was examined in greater detail, however, it was found that besides their effect on DPNH-cytochrome c reductase, some steroids could inhibit glutamic dehydrogenase directly. This was shown with soluble extracts prepared by freezing and thawing liver mitochondria in distilled water. Subsequently, the reaction has been studied in considerable detail, using the crystalline enzyme prepared from beef liver. When the activity is assessed either by reduction of α-ketoglutarate or oxidation of glutamic acid, using either di- or triphosphopyridine nucleotide as co-factor, various steroids (and diethylstilbestrol (DES)) were able to produce significant inhibition.

In addition to detailed kinetic appraisal of the steroid-enzyme interaction, evidence has been obtained that the steroids induce a reversible change in the structure of the enzyme.

Diethylstilbestrol, estradiol, progesterone and testosterone (in that order), were the most effective compounds studied, while the corticoids were relatively ineffective either alone or in combination with other steroids. Diethylstilbestrol produced significant inhibition in concentrations as low as 10^{-7} M. Of considerable interest was the fact that adenosinediphosphate (ADP) (and to a much lesser extent adenosinetriphosphate (ATP) and adenosinemonophosphate (AMP)) prevented the steroid inhibition, particularly since 10 times as much ADP was necessary to reverse the inhibition observed when the reductive reaction was studied.

A study of steroid concentration effects, both in rat liver mitochondria and in the crystalline beef liver enzyme, has yielded complex data perhaps best explained on the basis of multiple binding sites for the steroid.

The steroid inhibition has been extensively examined using varying concentrations of the different substrates (glutamate, DPN, TPN, DPNH, TPNH, α-ketoglutarate, NH_3) and no clear-cut competitive relationships have been established. However, very high (Ca 10^{-2} M) concentrations of DPN, but not TPN, will prevent the steroid effect. Conversely, high concentrations of α-keto-

glutarate, DPNH, and TPNH, enhance the inhibition. The complicated kinetics of the reaction in the presence of steroid suggest that at least one form of the enzyme-inhibitor complex may retain some catalytic activity.

Some information has been obtained concerning the binding site of the steroid, suggesting that a sulfhydryl group may be involved. Parachloromercuribenzoate (PCMB), in concentrations insufficient to inhibit the enzyme, was shown to reduce the degree of steroid inhibition. Appropriately, DES was also shown to retard the formation of enzyme-PCMB complex, as measured by following the optical density of the complex at 250 mμ. On the other hand, the steroids were shown to prevent I_2 inactivation of the enzyme.

In addition to the beef and rat liver enzymes, GDH has also been prepared from chicken liver, rat testis, rat kidney, rat heart, S.typhrimurium and Neurospora crasa. Only the latter two failed to show steroid inhibition.

Thyroxine and 1,10-phanthroline, both inhibitors of the GDH reactions, were additive with steroid.

Since it had been shown by Frieden that various inhibitors of GDH could dissociate the enzyme into inactive subunits, the effects of steroids and DES on the sedimentation properties of the enzyme were examined. It was observed that DES, estradiol, and progesterone could promote a dissociation of the crystalline enzyme into two forms appearing in the ultracentrifuge as two peaks of 12–14s and 24–26s. The effectiveness of the three compounds as inhibitors of the reaction was well correlated with the degree of splitting observed. ADP, which prevented the inhibition of the reaction, also prevented the steroid dissociation of the enzyme.

Further evidence for competition between PCMB and steroid was obtained in that the PCMB-inactivated enzyme could not be split by steroid.

The intimate mechanism by which certain steroids can dissociate such a protein aggregate into subunits has not been determined. Although GDH is rich in sulfur, it is apparently not held together by S–S bonds since prolonged incubation of the enzyme with the reducing agent, thioglycollate, did not result in any decrease in activity. (In fact, thioglycollate protected GDH against loss in activity from prolonged incubation and prevented steroid inhibition.) It is perhaps revealing that GDH could also be dissociated with the detergent, sodium dodecylsulfate, although it was somewhat less effective than the steroids. A possible mechanism, then, is that of hydrophobic bond disruption.

The interesting findings of Struck and Sizer that GDH can catalyze the oxidation of amino acids other than glutamate (albeit at considerably slower rates) prompted an examination of the effects of steroids when these are used as substrates. It has been observed that steroids produce varying effects, depending on the particular substrate used. Of greatest interest was the reversible reductive amination of pyruvate to alanine which can be catalyzed by GDH at a rate 1/200 that of glutamate. That the same protein is responsible for the two activities was proved by DEAE cellulose column chromatography, heat inactivation, alcohol fractionation, and ultracentrifugation. Evidence indicates that the pyruvate-alanine interconversion is catalyzed chiefly by the dissociated form of the GDH molecule which is inactive toward glutamate. Thus the pyruvate-alanine reaction is stimulated by steroid hormones, DNPH, and 10,phonarthroline, all of which dissociate GDH into subunits and thus inhibit glutamate oxidation. On the other hand, the alanine pyruvate activity was inhibited by those things such as high concentration of DPN and TPN and ADP which cause association of enzyme and stimulate glutamate oxidation.

Thus, not only does the state of aggregation of the GDH molecule determine its catalytic behavior as Frieden has shown, but also its substrate specificity. Therefore, steroid hormones in dissociating glutamic dehydrogenase into subunits, *impede* its function in interconverting *glutamate* and *α-ketoglutarate*, while *facilitating* its function in catalyzing the *alanine dehydrogenase* reaction.

The present findings hopefully may lead to a better understanding of the mechanisms of hormone action. Although the physiological impact of such an effect on the glutamic dehydrogenase reaction cannot be appraised at the present time, these findings present an interesting model system of steroid action in which both the *catalytic behavior* and *substrate specificity* of an enzyme are changed as a result of a steroid-induced al-

teration in the structure of the enzyme molecule. In addition, the finding that a single enzyme may catalyze *different* reactions, depending on its state of aggregation, presents a model mechanism by which both diversification of enzyme function and development of new genetic characteristics could occur.

Purine Metabolism in Gout

The cause of the hyperuricemia of gout, whether increased production or diminished excretion of uric acid, has been studied further. The extent to which an excessive production of uric acid contributes to the hyperuricemia of gout has been evaluated by administering glycine-1-C^{14}, a metabolic precursor of uric acid, along with uric acid-N^{15} to normal and gouty subjects and determining the recovery of isotope in urinary uric acid. The data thereby obtained provide two independent parameters of uric acid synthesis: the extent to which glycine is incorporated into uric acid and the size of the body urate pool and its turnover. In addition, the fraction of the daily uric acid production that is disposed of by routes other than renal excretion (uricolysis and deposition in tophi) is indicated by the percent of the administered uric acid-N^{15} which is recovered in the urinary uric acid. Nine of 16 gouty patients showed an incorporation of glycine-1-C^{14} into urinary uric acid ranging from only slightly above normal to several times normal. In two of the seven remaining patients an abnormally large extrarenal disposal of uric acid masked an increased glycine incorporation. There remained five gouty subjects in whom no evidence of an increased uric acid production could be found by either glycine incorporation data or urate turnover values.

The extent to which an impaired renal handling of uric acid contributed to the hyperuricemia of this group of gouty subjects was investigated by determining the urate/inulin clearance ratio. For comparison clearance values were obtained in nongouty subjects before and after uric acid production was increased by feeding ribose nucleic acid (RNA). The group of five gouty subjects with a normal uric acid production showed a lower urate/inulin clearance value than was obtained with normal subjects who were receiving RNA. Five of the six patients who produced excessively large amounts of uric acid showed urate/inulin clearance ratios in the same range shown by nongouty subjects whose uric acid production had been increased by feeding RNA.

It would seem unlikely that a single inborn metabolic defect characteristic of all gouty patients would produce in some individuals an excessive uric acid production and in others no increase in uric acid production but instead a diminished ability of the kidney to dispose of uric acid. We are led then to the view now being explored that clinical gout may be the result of a variety of basic metabolic or physiological disturbances which have in common the induction of a hyperuricemia.

Aromatic Amino Acids

Objectives in studying patients with metabolic diseases associated with the metabolism of the aromatic amino acids, alcaptonuria, phenylketonuria, tyrosinosis and albinism, have been several: (1) to determine the exact nature of the metabolic defect in these conditions; (2) to study the hereditary pattern of these diseases and, if possible, to develop tests which will detect the heterozygous state in relatives carrying the trait; (3) to study the formation and deposition of the pigment derived from homogentisic acid and to determine how it produces the pathological changes in the connective tissues, particularly the joints (ochronotic arthritis); (4) to study the cause of ochronotic arthritis, nearly always associated with alcaptonuria; and (5) to attempt various means of treatment of these metabolic diseases.

Ascorbic Acid in Tyrosine Metabolism. Our studies have continued on the detailed mechanism by which vitamin C maintains normal tyrosine metabolism through protecting p-hydroxyphenylpyruvic acid oxidase from inhibition by its substrate. Several analogues of ascorbic acid, such as D-isoascorbic acid, have also been found to protect this enzyme from inhibition in scorbutic guinea pigs *in vivo*. In addition, a dye, 2,6-dichlorophenolindophenol, and folic acid also protect the enzyme in scorbutic guinea pigs, and the action of the latter two compounds has been studied in more detail. The dye has been found to have no anti-scorbutic effects in vitamin C-deficient animals except for its ability to maintain

tyrosine metabolism. Therefore, there is a clear distinction between the effect of vitamin C in tyrosine metabolism and the effects of the vitamin in the other aspects of scurvy. Folic acid was found not to protect p-hydroxyphenylpyruvic acid oxidase *in vitro* and we have concluded that its beneficial effect *in vivo* are through some indirect mechanism.

Structural analogues of p-hydroxyphenylpyruvate are being tested to find out the structural requirements for inhibition. In addition, a kinetic analysis of substrate-induced inhibition and that by other analogues has been made. Phenylpyruvate has been found to be even more potent an inhibitor than the substrate when preincubated with p-sydroxyphenylpyruvic acid oxidase and the inhibition is of the non-competitive type under these circumstances. Various other dyes and redox systems are being tested in place of 2,6-dichlorophenolindophenol to determine whether it is a structural or redox property of the dye which is the important component in its effectiveness in protecting the enzyme. Recent results indicate that it is the structure and/or the ease of its reduction, rather than the E_o value of the redox system which is important. The compounds active like the dye also have in common the ability to undergo a one electron change in oxidation, suggesting that a free radical may participate in the oxidation of the substrate by p-hydroxyphenylpyruvic acid oxidase.

New Toxic Effect of Prolonged Corticosteroid Therapy—Posterior Subcapsular Cataract

During the past year we have observed and reported a heretofore unknown adverse effect of prolonged corticosteroid therapy in high dosage, namely posterior subcapsular cataracts. Although many of the patients observed to have such cataracts had been receiving dexamethasone, a large number of others had never received this specific analogue, but had received both the naturally occurring and the older synthetic corticosteroid preparations. Seventeen of 44 rheumatoid arthritis patients having received corticosteroid therapy for periods of one year or longer were found to have this ocular lesion. Nineteen nonsteroid treated rheumatoid patients were examined and no such lesions were found. The appearance of the posterior subcapsular cataract was found to be associated with moderate or high dose therapy (equivalent to 10 mgm. or more of prednisone daily) for a period of one year or longer. More recently, an additional 35 corticosteroid-treated patients have been examined, and 8 of these individuals were found to possess this lenticular opacity. Among these 8 were two young boys, ages 8 and 12. The gradual improvement with decrease in size of opacity in two patients in whom it was possible to reduce or completely discontinue therapy has suggested, in some individuals at least, that this lesion may be reversible. Lenses have been obtained from one patient at autopsy and from another at surgery, although the microscopic findings are not available at the time of this report. The correlation between steroid administration and the appearance of posterior subcapsular cataracts is highly significant ($0.01 > P > 0.001$). Other potential factors of etiologic significance of cataract formation were carefully assessed, including gold therapy, salicylate therapy, X-ray exposure, and estrogen therapy, as well as calcium intake. No significant correlation was shown between these factors and the appearance of cataracts. Scientists in the Ophthalmology Branch of the NINDB cooperated in these studies.

New Antirheumatic Drugs

6-Alpha Fluorotriamcinolone. A second metabolic study involving this compound administered in dosages of 20 mgm. has now been completed by Dr. G. Donald Whedon and his group. One year ago metabolic studies of this compound were carried out with a patient with rheumatoid arthritis. The data revealed the maintenance of a positive calcium balance during the administration of 20 mgm. of this steroid daily. The second metabolic study completed during the year 1960 was performed upon a normal control patient, again at a dosage of 20 mgm. daily. In this instance a slightly negative calcium balance was induced. This compound, although only ½ to ⅓ as potent as dexamethasone in anti-inflammatory activity deserves further investigation in regard to its effect on calcium metabolism.

Hydroxychloroquine. In cooperation with the American Rheumatism Association's Working Committee on Cooperative Clinics, the NIH-

Georgetown University Rheumatology Service is participating as a member of the committee in therapeutic trials of hydroxychloroquine in rheumatoid arthritis. The entire enterprise embraces the acivity of 10 arthritis clinics located in various University centers throughout the country. The first clinical trial of hydroxychloroquine began in February of 1960 and was completed in July of the same year. Each patient in the trial was observed over a 3-month period. The trial was conducted as a double-blind study on a total of 76 patients. The parameters of arthritis activity included the evaluation of joint tenderness, grip strength, ability to walk and the erythrocyte sedimentation rate. A statistically significant difference was demonstrated between patients receiving the compound and those receiving placebo. Differences between the two groups of patients were observable as early as one month after the institution of therapy. No significant side effects were observed during the 3-month period. It is anticipated that another trial will be instituted within the next 3 months.

An Anti-Metabolic Agent. A new evaluation of an antimetabolic agent has been undertaken in patients with psoriatic arthropathy. A double-blind study has been carefully planned. To date four, of a planned series of 40 patients, have been included in the trial. Each patient will be observed over a period of 60–80 days on therapy. During this period the patients will be on a standard diet. Careful observations of joint inflammation as well as skin involvement will be made. The study is being conducted in cooperation with Dr. Eugene Van Scott and Dr. Arthur Eisen of the National Cancer Institute.

Gastroenterology

In keeping with the objective of studying the biochemistry and morphology of the small intestine of man, the gastroenterology group has set up a large number of chemical tests and other procedures for the study of intestinal absorption. These have been applied in the clinical projects described below. A laboratory study of bile pigment metabolism has also been started and an enzyme not previously isolated, has been partially purified.

Effects of Radiation and Folic Acid Antimetabolites on Intestinal Absorption

The small bowel mucosa is extremely sensitive to radiation injury. Animal studies have shown that exposure to radiation can produce not only marked morphological changes, but also alterations in absorptive functions. To explore these observations in man, a collaborative study has been initiated with investigators in NCI. Patients who are to receive radiation as cancer chemotherapy are evaluated before and after radiation exposure by assessments of intestinal absorptive capacities and by small bowel mucosal biopsy. Three patients have been studied so far and a much larger series is contemplated. The information obtained in this study will be important not only in relation to human intestinal physiology, but also in relation to the effects of radiation on man.

Similarly, animal studies have shown that interference with folic acid metabolism can produce morphological and functional changes in the small intestine. Again in collaboration with NCI, a study is in progress to evaluate intestinal function before and after patients with psoriasis receive methotrexate therapy.

Malabsorption and Osteoporosis

A long-standing project in NIAMD has been devoted to studies of osteoporosis. In collaboration with the investigators working on this problem, a preliminary survey has been made to determine whether osteoporotic patients might be suffering from clinically unsuspected steatorrhea, a condition that might cause a drain on their dietary calcium. Of five patients studied, four proved to have significant steatorrhea. Studies by the gastroenterology group suggested that the steatorrhea was not attributable to disease of the small intestine. Future studies will be directed, therefore, at the possibilities that pancreatic or biliary disease might explain the steatorrhea. The problem of how the steatorrhea may be implicated in the osteoporosis will also be investigated.

Juvenile and Adult Celiac Disease

Evidence of recent years from other laboratories suggests that celiac disease of children and nontropical sprue of adults are attributable to a single underlying disorder, that the disorder is

hereditary, and that it predisposes an affected individual to an injurious action of such proteins as wheat gluten. This theory is under evaluation by the gastroenterology group. Patients are admitted for extensive screening to determine whether they are susceptible to the toxic action of wheat protein. When a positive diagnosis is made, as many of the patient's blood relations as are available are admitted to the Clinical Center in order to study the genetics of celiac disease. Previous studies of the genetics of celiac disease have relied only on medical histories of relatives of affected individuals. In the present study careful chemical evaluations of intestinal absorption are carried out and intestinal biopsies are taken. Six members of a single family have been studied in this way. Additional families will be added to the series as they are referred.

Additional use is made of the biopsy material obtained in this project. Together with Dr. Samuel Spicer, NIAMD, the specimens are subjected to histochemical studies of their mucoproteins. Information about the mucoproteins of the intestinal mucosa of normal man is extremely scanty and even less is known about disease states. Tissues from 20 patients have been obtained during the past year and are now under study.

Protein Metabolism in Malabsorption States

Malabsorption of various etiologies is often associated with low levels of serum proteins. It has been assumed that the deficiency of these proteins is due to impairment of absorption of the protein building blocks and consequent suppression of protein synthesis. Together with Dr. Thomas Waldmann, NCI, patients with malabsorption on the gastroenterology service are being studied to characterize their protein metabolism. Four patients of a clinically unusual type, have been studied so far. In some there was evidence of impaired protein biosynthesis, but in others the protein deficiency appeared attributable to loss of protein by leakage from the gastrointestinal tract. A large series of such cases will be studied as appropriate patients become available for study.

Mammalian Metabolism of Bile Pigments

Bile pigments, derived from the heme of hemoglobin, are processed in the liver, excreted via the bile into the small intestine and eliminated in the feces. A portion of the intestinal bile pigments is reabsorbed, returned to the liver and reexcreted. The enzymatically catalyzed reactions that occur during this enterohepatic circulation of bile pigments have not been studied in detail. Such a study has been initiated in this laboratory and the first enzyme in this important pathway of metabolism has been partially purified and many of its properties have been determined. After studies of this enzyme are complete, the next one in the sequence will be explored.

Clinical Endocrinology Branch

Carbohydrate Metabolism

Glucose. It has been known for some time that mammalian liver contains enzymes capable of effecting oxidation of the first carbon atom of glucose via a pathway known as the hexose monophosphate shunt (HMP). The quantitative significance of this pathway in the intact human has not, however, been determined. Measurement of $C^{14}O_2$ and blood glucose C^{14} after the administration of $C_1{}^{14}$ glucose and $C_6{}^{14}$ glucose have been made in normal human subjects. It was found that data for the kinetics of carbon labeled bicarbonate excretion were also necessary for this formulation. In collaboration with Dr. M. Berman of the Biomathematics panel, a satisfactory analysis of the rather complex excretory patterns was obtained. The results show that in normal man about 10 percent of the total glucose is oxidized via the shunt pathway. Preliminary data indicate that this fraction is decreased in hyperthyroidism. The functional importance of the shunt pathway which cannot be evaluated from enzyme content of isolated tissues can now be determined in various diseases.

Glucose metabolism has also been studied in isolated tissues. In the isolated rat diaphragm it was shown that the transport of 3-deoxyglucose into the cell is unaffected by insulin, and does not furthermore inhibit glucose transport nor interfere with glucose oxidation. A rather extensive series of studies have been conducted on the metabolism of glucose in isolated endocrine tissues; in particular, evaluation of the extent of the shunt pathway utilizing carbon one and carbon six labeled glucose has been performed.

It has been shown that in all endocrine tissues studied a very active hexose monophosphate pathway is present. The tissues studied include the adrenal, ovary, testis, anterior pituitary, parathyroid and thyroid. The thyroid has been particularly carefully studied. It has been previously reported from this section that thyroid stimulating hormone (TSH) from the pituitary *in vitro* caused marked stimulation of the oxidation of glucose-1-C^{14} and to a lesser extent the oxidation of glucose labeled 6-C^{14}. This stimulation is manifest 5 minutes after the addition of TSH and is effective at a concentration of 3×10^{-11}M. The mechanism of this effect has been investigated and it appears likely that it is due to an increased synthesis of triphosphopyridine nucleotide from diphosphopyridine nucleotide, since TSH causes a rise in TPN and a concomitant fall in DPN, although the levels of TPNH and DPNH are not significantly changed. It has not yet been possible to demonstrate these effects in cell free systems. Other agents have also been found which effect glucose metabolism in thyroid slices. Quite low concentrations of acetylcholine stimulate both C–1 and C–6 labeled glucose oxidation and increase glucose uptake. Atropine inhibits the acetylcholine effect without altering the TSH effect. There is also partial inhibition of the acetylcholine stimulation by large amounts of iodide. Epinephrine and serotonin have also been shown to stimulate glucose oxidation by thyroid slices. This effect can be observed in cell free systems fortified with TPNH. TSH has also been shown to stimulate the oxidation of labeled pyruvate and acetate by thyroid slices *in vitro*.

Pancreatic islet cell tissue has been studied in islet cell tumors removed from individuals and in the islet cell tissue of certain fish in which this tissue is present as a discrete tissue uncontaminated by pancreatic exocrine glandular tissue. In all these specimens of islet tissue an active hexose monophosphate pathway was demonstrated. In the tumors it was shown that alloxan did not influence glucose oxidation although in one case leucine and orinase appeared to stimulate oxidation. The oxidation of glucose by islet tissue is extremely rapid, exceeding, on a weight basis, that observed in either liver or heart.

The oxidation of glucose by ovary and testis was unaffected by the trophic hormones, FSH, and LH, although in both ovary and testis an active shunt pathway was obtained. The presence of a shunt pathway was also demonstrated in the anterior pituitary and it was shown that serotonin and catechol amines stimulated the formation of carbon dioxide from one labeled glucose. The glucose metabolism in adrenal tissue was studied and an active shunt pathway was demonstrated. Interestingly enough neither ACTH nor cyclic 3′5′ AMP had an effect on oxidation of glucose.

Metabolism of glucose by the mammary gland has also been studied. Prolactin, growth hormone and ACTH have all been shown to be active in stimulating the oxidation of carbon labeled glucose by slices of rat lactating mammary glands. The same hormones have also been found to increase the glucose uptake in mammary gland. Glands obtained at the end of lactation were found to be extremely responsive to these hormones and as little as 5 μg were effective.

Insulin. Studies on the metabolism of insulin have been continued and it has been demonstrated that in the isolated perfused rat liver insulin prevents the release of potassium into the perfusate. During control perfusions in the course of 60 minutes, approximately 7½ percent of the liver potassium was released into the perfusate, with a proportional loss of water. The addition of insulin to the perfusing medium prevented both the potassium and the water loss. There appeared to be no significant effect of insulin on the net hepatic release of glucose. As was noted in last year's report, the cooling of the perfused liver inhibits the metabolism of insulin but does not interfere with binding of insulin by liver cells. To study further this phenomenon a fluorescent derivative of insulin has been prepared by the reaction of fluorescein isothiocyanate with crystalline insulin. A preparation so obtained has been shown to retain 60 percent of its hypoglycemic activity. Preliminary experiments have shown that this derivative is strongly bound to leucocytes and not to red cells. It will shortly be tested in the liver perfusion system. Work has continued on assay of biological materials for insulinlike activity and several malignant tumors have been assayed. These tumors have

been associated with hypoglycemia and in two of three studied, an extract was obtained which increased glucose uptake in the isolated diaphragm. In one extract stimulation of glycogen deposition which was partially blocked with plasma containing insulin antibodies was noted. This rather bizarre apparent synthesis of insulin by nonpancreatic carcinomatous tissue is under further study.

Galactose. Further studies have continued on the metabolism of carbon labeled galactose in individuals having the disease galactosemia. In collaboration with Dr. Topper, it has been found that menthol stimulates galactose oxidation in these subjects but not in normals. Hemolysates from galactosemic individuals failed to oxidize galactose and cannot be stimulated but hemolysates from individuals bearing the galactosemia trait show marked increase in galactose oxidation when pyruvate is added to the medium. This suggests that in the presence of even a small amount of transferase, stimulation of the 4-epimerase (as with added pyruvate) accelerates the overall oxidation of galactose. Interestingly enough the accumulation of galactose-1-phosphate in leucocytes does not appear to alter the pathway of glucose metabolism in these cells. Galactose metabolism has also been studied in the rat and it has been shown that in the experimentally hyperthyroid rats there is an accelerated disposal of galactose. Rats fed a 30 percent galactose diet have been shown to have an amino aciduria quite similar to that seen in individuals with congenital galactosemia. The main amino acids excreted are taurine, alanine, aspartic and glutamic acid. Further studies have continued on the oxidation of galactose by human leucocytes and it has been shown that leucocytes from galactosemic individuals are virtually unable to oxidize galactose whereas leucocytes from individuals apparently harboring the galactosemia gene metabolize only approximately 50 percent of the galactose that normal individuals do. Thus it seems that individuals bearing this recessive trait can be identified by study of galactose metabolism in isolated leucocytes. In collaboration with Dr. Krooth of the National Cancer Institute, skin and bone marrow cells have been grown in tissue culture for up to 6 months. Galactosemic cells showed almost no growth when the sole carbohydrate source was galactose, whereas normal cells grew as well with galactose as with glucose. Galactosemic cells grown in glucose grew reasonably well but after some months of growth were still unable to oxidize galactose to carbon dioxide. Further studies of carbohydrate metabolism on leucocytes from normal individuals, patients with glycogen storage disease and Hurler's syndrome have been performed. It has been shown that normal white cells have appreciable amounts of phosphorylase and glycogen but leucocytes from individuals with glycogen storage disease had unusually low levels of phosphorylase although the glycogen content was normal. Children with Hurler's syndrome, on the other hand, had unusually low glycogen levels in their leucocytes. Further studies on these individuals are now under way to determine the defect in this syndrome.

Biochemistry of the Thyroid

Iodide Transport. The iodide concentrating mechanisms of thyroid, mouse submaxillary, rat mammary, and rat thyroid tumor tissue slices has been studied. The tissue/medium ratio of I^{131} (T/M) is greater for thyroid tissue than for all other tissues and in all four tissues is reduced to half the control value at from 1–3 x 10^{-5} M iodide concentration. The difference in the T/M (I^-) appeared to be a function of the capacity of the system rather than the affinity for iodide ion. In all four tissues iodide concentration was a function of the external potassium concentration with half maximal stimulation attained at approximately 10^{-3}M added potassium. The glycosides, ouabain and scilliroside depress the T/M ratio in all four tissues which depression can be partially reversed by the addition of potassium, rubidium, or cesium ions. Preliminary results show that there is a minimum size requirement in salivary and thyroid tissues for monovalent anion accumulation and ions of somewhat greater partial molar ionic volume than iodide such as pertechnetate and perrhenate show greater affinity constants for thyroid tissue than iodide. There is also a potassium requirement for the concentration of these ions and the inhibitor effects suggests that their accumulation occurs by a process similar to iodide concentration.

Phospholipid Meetabolism. Phospholipid metabolism using P^{32} has been studied in thyroid slices and homogenates. It has been shown that in thyroid slices P^{32} uptake is stimulated by TSH and inhibited by digitalis glycosides. The inhibition by digitalis glycosides is largely overcome by increasing the potassium concentration in the medium. These effects are very similar to those seen with iodide concentration by the thyroid suggesting that there may be some relationship between phospholipid synthesis and iodide transport. The main phospholipid synthesized in response to TSH appears to be phosphatidyl inositol. Preliminary experiments have been performed attempting to extract a phospholipid from the thyroid which in *in vitro* systems will concentrate iodide. After extraction and chromatography on silica gel a lecithin like phospholipid has been obtained which will cause a small amount of iodide concentration under the following circumstances: Iodide is normally distributed between water and chloroform to the extent of several thousand times in favor of water. If this phospholipid extracted from the thyroid is present in the chloroform phase approximately equal partition between the two solvents is obtained. Chromatography of the radioactive iodine in the chloroform phase shows it to behave as iodide. Cell free systems of thyroid gland incorporate almost no phosphate into phospholipids in spite of supplementation with a variety of cofactors. However an homogenate system can be obtained which will synthesize phosphatidic acid from P^{32} labeled alpha-glycerol phosphate. This step, however, does not appear to be effected by TSH, by potassium, or by digitalis glycosides.

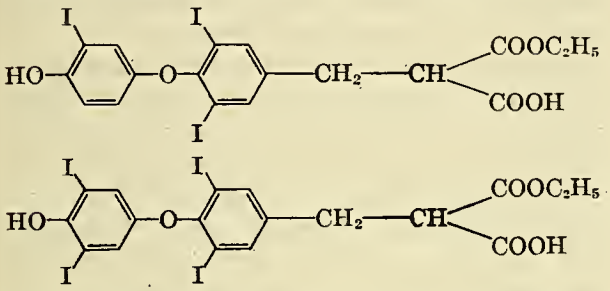
Iodoproteins. The iodoproteins of the thyroid, particularly thyroglobulin, have been the object of a rather extensive study in the past year. Thyroglobulin has been chromatographed on diethylaminoethylcellulose. It has been shown that thyroglobulin apparently homogenous with respect to sedimentation in the ultracentrifuge can be separated into three separate components on this ion exchange column. Preliminary studies have failed to disclose the chemical basis for the heterogeneity which is now under study. The iodination of thyroglobulin *in vitro* has been studied and it has been shown that in water at pH 9 only relatively small amounts of iodine are incorporated into tyrosine. In 8 Molar urea, however, or in detergent almost all the tyrosyl groups appear to be equally reactive toward iodine, suggesting that a substantial proportion of the tyrosine residues are either buried in the protein or bonded in some way as to inhibit iodination at pH 9. This correlates reasonably well with the titration of thyroglobulin which shows that a substantial proportion of the tyrosine groups in thyroglobulin have an abnormal pK being almost 1½ units more alkaline than the pK of free tyrosine. Recently a third type of tyrosyl group constituting about 15 percent of the total has been discovered. These tyrosyl groups do not begin to ionize until the pH of about 12½ is reached and they then ionize at a rate which may be followed spectrophotometrically. Concomitant with these changes there are seen changes in the molecular size and a fairly symmetrical boundary in the ultracentrifuge occurs which has a sedimentation constant of about 6. This is the minimum size so far observed in aqueous media for this protein. In collaboration with Dr. R. F. Steiner of the Naval Medical Research Institute, thyroglobulin has been studied with the polarization of fluorescence technique. In this case, conjugation with dimethylaminoaphthalene sulfonyl chloride has been used.

The effects of treatment with urea, guanidine or detergent are more profound than those resulting from thermal or alkaline denaturation. In each case, the transition is gradual. In no instance was any evidence of a cooperative process obtained. In detergents, urea and guanidine the transition is essentially reversible. The loosening of the macromolectular structure by the latter reagents renders the disulfide bridges susceptible to the action of reducing agents. However, even after reduction considerable internal rigidity remains.

More recently studies have been undertaken on the structure of thyroglobulin as revealed by its antigenic properties. It was found that a fully hydrolyzed preparation of thyroglobulin retained the ability to precipitate rabbit antibodies to whole thyroglobulin as determined in gel diffusion. This was true when trypsin, chymotrypsin or pepsin was used for hydroylsis. The hydrolysate contained a mixture of components with an average sedimentation constant of 3 to 4. On prolonged hydroylsis even smaller fragments are obtained which are dialyzable and which have

the capacity, *albeit* minimal to precipitate rabbit antibodies and are capable of inhibiting the precipitation of native thyroglobulin with antiserum. These active components have been fractionated using sephadex and dialysis and it now appears that some of them have a molecular weight below 10,000.

Thyroxine and Iodotyrosine Synthesis. In order to study the structural features of the thyroxine molecular necessary for its biological activity a variety of thyroxine analogues have been synthesized. These include the following:



An interesting synthesis of thyroxine which results in a rather high yield has been studied. In this synthesis diiodotyrosine and its keto analogue, 3,5-diiodo-4-hydroxyphenylpyruvic acid (HPPA) are used. A number of experiments were carried out to determine the structural features of HPPA required in this synthesis. The requirement for a three carbon atom side chain and for a free keto group seem to be so far absolute. Furthermore, the acylamino acid in which HPPA is peptide linked to glycine is also not effective. Incubation in the presence of ninhydrin in order to form the 3,5-diiodo-4-hydroxyphenylacetaldehyde *in situ* inhibited the synthesis of thyroxine. Studies with both radioactive diiodotyrosine and radioactive HPPA have shown, at least in preliminary form, that one molecule of HPPA reacts with one molecule of DIT. Because of the yield and for other reasons, it had been suspected that perhaps HPPA might act only as a catalyst. It now seems to actually be incorporated into the thyroxine. In the process of this study, it was necessary to investigate the synthetic methods for the preparation of keto acid peptides. A variety of methods used for peptide syntheses were investigated and all except the reaction of HPPA with benzyl carbamate seemed to require blocking of the enolic hydroxyl by some sort of protective group.

Because of uncertainties concerning the quanitation of relative amounts of iodinated tyrosines and thyronines in the thyroid and blood, reinvestigation of this problem was undertaken using a method of isotopic equilibrium. This method was validated with the finding that calculated and analyzed iodine values for thyroid iodine and for blood iodine agreed relatively closely. In rats on a relatively generous iodine diet, the molar ratios of MIT, DIT, T_4 and T_3 in the thyroid were, respectively, 9, 15, 5, and 1. In the serum, it was shown that thyroxine accounted for approximately 95 percent of the serum iodine and triiodothyronine for approximately 4 percent. Kinetic studies of metabolism of these amino acids and their volume of distribution coupled with the higher potency of triiodothyronine suggests that in the rat almost half of the metabolic activity of the thyroidal secretions is accounted for by triiodothyronine. For the analysis of iodothyronines high voltage electrophoresis has been investigated and several organic solvents, formamide-glycine, pyridine-aqueous ammonia, and dimethylformamide-aqueous ammonia have been found to be very satisfactory for separation of iodotyrosines and iodothyronines. Also a method was developed for the detection of iodinated materials on paper chromatograms or electrophoretic runs in which the cerate arsenite reaction was used and alpha-phenanthroline ferrous sulfate was employed as an indicator. This is highly sensitive and reasonably specific.

Hormone Transport in Blood. Studies of thyroxine binding proteins have continued. An explanation for the ability of a variety of drugs to lower the serum PBI has been obtained. It has been shown that salicylate and dinitrophenol will competitively inhibit thyroxine binding to the prealbumin moiety of human plasma. Two additional drugs, tetrachlorothyronine and diphenylhydantoin have been shown to compete with thyroxine for binding sites on the thyroxine binding alpho globulin. All four of these drugs cause a lowering of the serum PBI apparently by interfering with thyroxine binding so that the concentration of free thyroxine is elevated, hence its degradation accelerated and a new steady state in which the normal level of free thyroxine is reached whereby the total thyroxine is lowered. Studies have been continued in col-

laboration with Dr. Blumberg on two-dimensional paper and starch gel electrophoresis of thyroxine binding proteins and serum. The identity of band 1 as seen in starch gel with pre-albumin as demonstrated in electrophoresis in ammonium carbonate buffer has been established. The identity of the thyroxine binding globulin with band 4 in starch gel also seems fairly clear. Work has progressed on the isolation of a corticosteroid binding protein from serum. Chromatography on DEAE and Ca phosphate gel have reslted in over 100 fold purification. This binding protein runs just behind albumin on starch gel electrophoresis.

Effect of Thyroxine on Isolated Enzyme Systems. Earlier work on inhibition of dehydrogenases by thyroxine has been extended. The native molecule of glutamic dehydrogenase (GDH) has a sedimentation rate of −26S. Thyroxine causes dissociation into units of −14S and inhibition of enzymatic activity. Other halogenated phenols cause dissociation and inhibition in about the same ratio. ADP and 5′AMP reverse the thyroxine induced inhibition and cause reassociation of GDH. The kinetics of inhibition of thyroxine and TPN, DPN and ADP favor binding of thyroxine at the second site on the enzyme. Of considerable interest is the retention of action by the thyroxine treated (and hence dissociated) enzyme with norvaline as a substrate. Furthermore, the dissociated enzymes action on norvaline is inhibited by ADP.

Metabolic Diseases Branch

Mineral Metabolism

Dietary Calcium in Osteoporosis. Metabolic studies from this Branch are continuing to produce pertinent evidence of the important relationship of dietary calcium intake to the pathogenesis and possible therapy of post-menopausal and senile osteoporosis, a disorder estimated to affect approximately one-fourth of all women over the age of 50 years. As the result of this work, the concept of altered bone metabolism in osteoporosis is being expanded toward recognition of a dual set of influences, hormonal and nutritional, and the idea of a complex etiology is gradually being more widely appreciated.

Prior metabolic balance studies by this Branch have demonstrated the highly significant positive relationship between the level of calcium intake in the diet and calcium storage in patients with this disease. Radioisotopic studies with calcium-45 in revealing a normal rate of deposition of calcium in new bone formation in osteoporosis, have pointed to bone resorption as an important mechanism by which demineralization develops in this disease, a mechanism previously accorded little significance. In illuminating the importance of resorption, the studies have indicated the inadequacy of the long-held previous concept of the pathogenesis of osteoporosis solely as impaired osteoid matrix formation resulting from gonadal-corticoid hormonal imbalance.

Recent studies by this Branch continue progressively to indicate the complex nature of the pathogenesis of osteoporosis, suggesting particularly at present different processes by which intestinal absorption and utilization of calcium may be impaired. A small group of patients with osteoporosis has been found which do not store calcium well even when the intake of this mineral is raised to very high levels. The initial common finding is steatorrhea at a subclinical level. Collaborative studies are in progress with the Clinical Gastroenterology group of this Institute in an effort to characterize the steatorrhea in these patients, whether of intestinal mucosal origin or pancreatic, and investigation is being made of the interrelationships of fat and mineral intestinal absorption. Preliminary observations suggest in some of these patients an abnormality of sweat electrolyte excretion similar to that seen in the heterozygous carrier state of cystic fibrosis.

Hormonal and Nutritional Influences on Bone Metabolism. Balance and radioisotopic studies of calcium accumulation and turnover are continuing in patients with a variety of bone diseases under the influence of a number of nutritional and hormonal factors. Recent refinement of isotopic techniques is now making it possible to evaluate isotope excretion curves for 30 to 40 days after administration of a tracer dose, so that it is anticipated that these extended data will provide significant information on rates of bone resorption as well as on currently calculatable rates of "bone formation." Utilization or tolerance tests of the handling of intravenously administered citrate and calcium are also being

employed for analysis of mineral dynamics. Calcium tolerance studies thus far suggest two sites of action of corticosteroids in the metabolism of calcium, one at the level of bone and the other involving renal excretion; possible action at the intestinal mucosa cannot be detected by this technique.

A 66-day metabolic balance study on a normal control volunteer was conducted to examine the metabolic effects of a new synthetic 6-alpha-fluorinated corticosteroid. Previous study on this project of a patient with active rheumatoid arthritis had demonstrated that administration of this compound resulted in (a) absence of significant sodium and water retention or of potassium loss, (b) nitrogen loss as anticipated, (c) slight decrease in urinary calcium, and (d) after brief initial fecal loss of calcium, progressive calcium retention. The normal subject showed similar results with respect to nitrogen and electrolytes but a consistent increase in fecal calcium. These results, after comparison with similar studies by this laboratory with other corticosteroids and other patients, both normal and with rheumatoid arthritis, suggest that a different mode of action of the corticosteroids on calcium metabolism exists in patients with rheumatoid arthritis as compared with normal subjects. Whether this different action has its principal locus in bone or in intestinal mucosa is not yet clear.

Human Total Energy Metabolism

Obesity. The Metabolic Chamber group is putting greatest effort into studies of obesity because of importance of the latter as a major health problem, a problem susceptible in part to attack by precise measurements of total energy expenditure.

The principal question under study, which if settled we believe would constitute a significant contribution to the very complex matter of human obesity, is to establish or disprove the existence of a "species" of obese human beings which under conditions of normal household activity can maintain their body weight at caloric intake levels (less than 1,300 calories/day) which would be inadequate for most other individuals; this state has been termed "hypophagic obesity." Prior studies by Olson and others have not included body composition measurements (such as fat-free, water-free body mass, sometimes called "metabolically active tissue") to provide an appropriate standard of reference for comparison of subjects nor accurate measurement of energy expenditure (precision of Metabolic Chamber for continuous 24 hour determination ± 100 Kilocalories or less), nor has caloric balance been followed for periods long enough to be certain that weight maintenance on a low caloric intake did not represent merely fluid retention, disguising the loss of body fat which would provide extra calories. Studies being conducted for the Chamber are rectifying these deficiencies and include close observation of extent of physical activity and measurement of a variety of biochemical and metabolic indices. Active collaboration with Dr. Robert Olson, professor of biochemistry, University of Pittsburgh School of Public Health, is planned for the winter of 1961 when he will begin to make available to us certain patients he has characterized as having "hypophagic obesity."

Current concepts of "metabolic" obesity include the possibility that there is a group of individuals genetically predisposed to obesity by way of abnormal rates of fat synthesis or mobilization, perhaps intensified by physical inactivity. Another possibility, it seems to us, is that an originally normal individual first develops excess body fat through overeating; it then becomes difficult for him to lose body fat because obesity may initiate a group of influences (physical, physiologic and endocrine-metabolic) which promote the maintenance of positive caloric balance; evidences for a number of these influences are accumulating in studies of excessively obese subjects. The most provocative (preliminary) finding along these lines is the pattern of urinary corticosteroid excretion which resembles a functional Cushingoid state; an abnormal pituitary-adrenal cortical influence in obesity would tend to retard fat mobilization and utilization.

Current studies of obesity have been directed primarily toward study of the relative influence of enforced energy expenditure by exercise on appetite and on rate of weight loss during caloric restriction, and also study of work capacity (cardio-pulmonary function during exercise) in the obese subject. Studies thus far on four obese women and on four women of normal weight have indicated the following principal findings

among several noted: 1) Exercise clearly contributed to the daily negative caloric balance on intakes of 650 to 1,000 kilocalories per day, as demonstrated by 24 hour measurements of energy expenditure in the Metabolic Chamber; and 2) Energy expenditure at standard treadmill rates and slopes decreased progressively as body weight was lost by obese subjects; when obese subjects were re-loaded with a canvas vest containing lead shot to a previous natural weight, energy expenditure per unit weight transported was usually less, indicating inefficient transport of obesity tissue as compared to inert weight.

Energy Metabolism in the Cold; Relation to Body Fat. A paper is in preparation on a study of the energy metabolic response to 50°F. exposure involving 8 male and 1 female subjects ranging in body fat content from 15 to 45 percent. In summary of the principal findings, a specific formula was derived (with the aid of Dr. J. Z. Hearon, Office of Mathematical Research, NIAMD), for the inverse relationship between percent body fat and resting heat production (energy expenditure response) per unit of body surface area. It was demonstrated clearly that when obese subjects are exposed to cold, their relatively small metabolic response results in a net saving of energy as compared to the energy balance situation in a lean individual; this represents a modest but definite influence acting toward maintenance of positive caloric balance in the obese. Body insulation was found to be directly related to the amount of body fat, but not completely accounted for by body fat. Wide individual differences in metabolic response were noted among subjects matched in age, previous cold exposure and body fat content. The subjective reaction to cold was unrelated to the metabolic response; in other words, well insulated obese individuals displaying less metabolic response in calories expended than thin subjects either might or might not display as intense a subjective discomfort in the cold. Metabolic response in the cold was periodic, occurring in cycles having periods of 7 and 18 minutes; the 18 minute peaks were associated with frank muscular shivering. Characterization of the metabolic response to cold with the precision indicated would not have been possible without instrumentation of the sort built into the NIAMD Metabolic Chamber.

Blood Diseases

Initial Stages of Blood Coagulation. During the past year, these studies have involved chiefly evaluation of the metabolism of antihemophilic factor (AHF) and the role of AHF in thromboplastin formation. It is well known that most clotting factors are synthesized in the liver because various factors become deficient at different stages of hepatic dysfunction. However, AHF never becomes depressed with liver disease (or even following hepatectomy of experimental animals), and moreover is not depressed by any other disease process. Therefore it has not been possible to induce AHF deficiency to assess regeneration rates in animals capable of forming this substance.

Opportunity to study acquired AHF deficiency was afforded by the finding in three patients of an unusual coagulation abnormality which proved to be complete deficiency of AHF due to an abnormal component in the gamma globulin fraction of plasma which specifically inactivates this factor. The patients studied were two elderly females, both of whom had elevated gamma globulin levels, and a middle aged man who had a normal gamma globulin level. In each case there was no underlying disease and no apparent reason for development of the abnormal gamma globulin. Although the physical chemical properties of the gamma globulin are those of a 7S antibody and its combination with AHF is stoichiometric, it does not fix complement with or precipitate AHF, and therefore cannot be classified as yet as a true antibody. Of particular interest was the finding that the anti-AHF in as little as 25 ml. of a patient's plasma would produce a marked AHF deficiency in a normal human being, as well as in laboratory animals. This has permitted for the first time an evaluation of regeneration rate of AHF in normal individuals and animals and provides a means of determining the site of synthesis of AHF.

Another aspect of the work has been experimental treatment for the acquired disease. Both plasmapharesis and exchange transfusions have been used, the latter approach having produced a remarkable clinical remission. Methods of as-

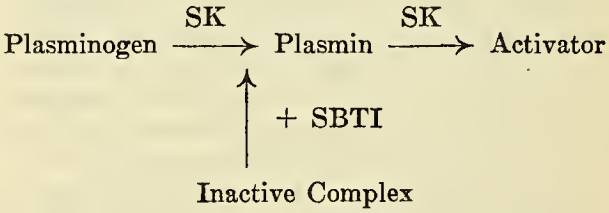
saying effects of the inhibitor during initial stages of coagulation have been developed and are being used to determine the step in which AHF is involved in formation of thromboplastin.

Effect of Divalent Ion Chelators on Blood Coagulation. These studies have shown that calcium not only is an integral part of AHF and Factor V, but also is strongly bound to fibrinogen. Some years ago it was found that EDTA interfered with the coagulant properties of fibrinogen, and it has generally been assumed that chelation of ionic calcium was responsible for this effect. Our recent studies employing highly purified fibrinogen indicate that fibrinogen contains 5 to 10 atoms of firmly-bound calcium per molecule, and that EDTA forms a complex with the calcium attached to fibrinogen, rather than effecting a reversal of the calcium-fibrinogen complex. Studies which are being pursued further with C^{14} labeled EDTA promise to provide information concerning the molecular structure of fibrinogen and the active sites involved in its polymerization.

In evaluating the dissociation of Ca-AHF and Ca-Factor V complexes, it became necessary to determine the equilibrium concentration of Ca^{++} in mixtures containing Mg^{++}, protein capable of binding Mg and Ca, and the chelator, citrate, oxalate or EDTA. Dr. J. Z. Hearon, Office of Mathematical Research, NIAMD, has derived formulae for calculating equilibrium concentrations of the divalent cations in these complex mixtures. This has permitted a precise comparison of the conditions which reverse calcium clotting factor complexes, as well as the minimum amount of free calcium required for various coagulation reactions. Although these studies were undertaken with respect to the coagulation system, evaluation of the concentrations of free ions in complex mixtures has bearing on numerous physiologic problems involving divalent cations.

Factor VII. These studies have been continued chiefly with respect to developing a better form of therapy for the congenital deficiency state and determining the survival of the factor in the circulation. We have used a concentrate which permits administering a therapeutic dose of Factor VII in a 2.5 ml. volume instead of the 250 ml. of plasma previously required. Recent observations suggest that the concentrate is adequately absorbed following subcutaneous or intramuscular administration and that a simply administered prophylactic dose may be entirely effective. Ability to provide large amounts of the material in a single small injection has permitted separation of the rate of exchange with extra vascular spaces from the true biological life.

Proteolytic Enzyme Therapy for Intravascular Thrombosis. The euglobulin fraction of human plasma contains a precursor substance called plasminogen, which on the addition of streptokinase (SK), a substance in the culture filtrate of streptococci, develops proteolytic activity. SK produces a second activity in human plasminogen called "activator" activity, which is capable of provoking proteolytic activity in plasma of animals which do not contain activator. In every step of the purification of human plasminogen, both the precursor of the proteolytic enzyme, plasmin, and the precursor of the "activator" activity are concentrated equally. It has been concluded from most kinetic analyses in the literature that plasminogen is a mixture of two substances and that streptokinase first activates the "activator", then the "activator" produces the proteolytic enzyme, plasmin. Our studies of the kinetics of this system indicate that plasminogen is a single substance which, under the influence of SK, forms two consecutive derivatives, the first being the active enzyme, plasmin, and the second an altered form of plasmin which is capable of provoking proteolytic activity in animal plasma. The steps in the reaction can be differentiated by specific inhibition of the intermediate derivative with soy bean trypsin inhibitor (SBTI):



A New Immunologic Disease. A thrombocytopenic state which develops in the absence of any apparent underlying disorder represents the relatively common syndrome of idiopathic thrombocytopenic purpura (ITP). Similarities between

this syndrome and thrombocytopenic states which can be produced experimentally with antiplatelet antibodies suggest that ITP is caused by an immunologic process. However, up to now neither a definite antibody nor a naturally occurring antigen has been implicated in ITP. Studies done in the past year concerned differentiation of a specific immunologic type of purpura which heretofore was indistinguishable from ITP. The pathogenesis of the disease proved to involve mismatch of an inherited platelet antigen and subsequent development of a complement-fixing antiplatelet isoantibody which was capable of producing severe thrombocytopenia in the sensitized individual. Complete and immediate cure was produced by exchange transfusion.

The antigen and antibody involved have been purified and extensively characterized, the nature of the immuno reactions have been defined precisely, and the genetic control of the platelet antigen has been fully characterized.

The following are some of the most interesting entirely new facts in the field of immuno-hematology which were derived from this study: An allelic pair of autosomal genes in human beings determines three platelet genotypes which can be distinguished by quantitative immunologic studies as phenotypes. This is the first time blood cell phenotypes corresponding to the genotypes have been differentiated. An antigen on platelets of 98 percent of the local population is lacking on platelets of 2 percent. Thus mismatch of the antigen occurs in approximately 2 percent of all transfusions. The antigen fortunately is only weakly antigenic. Even if an antibody forms, it is unable to react with the recipient's platelets unless some free antigen remains in the circulation to coat the recipient's platelets and permit attachment of antibody. This is the first time that transfer of a free cellular antigen has been implicated in a disease of sensitivity. Exchange transfusion produced a cure primarily by effecting removal of free antigen rather than removal of antibody.

The most sensitive technique for measuring the immunoreactions involved is quantitative complement fixation; for other techniques generally applied in immuno-hematology such as platelet agglutination, Coombs serum procedures, and staining with fluoresceine-tagged antibodies were an order of magnitude less sensitive or completely useless. Just as in the case of drug antibodies which we have studied, the present antibody was found to produce thrombocytopenia *in vivo* in man and animals at concentrations which are not detectable by the most sensitive techniques available. Immunoreactions of the type delineated could account for a number of diseases considered to be "autoimmune" processes only because no foreign antigen has been identified as yet.

A New Purpuric Disease. In 1955 Gardner and Diamond described an unusual form of autosensitivity in which the patient's own red cells produced large painful ecchymoses when extravasated into the skin. We found that these lesions, which were produced by as little as 6 micrograms of red cell stroma, could be precisely duplicated by intradermal injections of as little as 1 microgram of histamine or by injection of any agent which released skin histamine (such as basic amines or trypsin). The conclusions were that ecchymoses were mediated by histamine, released as the result of an antigen-antibody reaction occurring intradermally. Since then we have had opportunity to study five other patients with a similar disorder but with varying sensitivity both to histamine and to red cell stroma. As a result we have defined a spectrum of symptomology which these patients may present, and have thereby shed some light on the nature of obscure fixed tissue antibodies and their effects on vascular permeability.

Antibody Reactions in Anemia and Purpura. Following our finding that the complex reactions which take place between quinidine, antibody, platelets, and complement in quinidine thrombocytopenic purpura are the same as the reactions which take place between stibophen, antibody, red cells, and complement in stibophen hemolytic anemia, we have continued work with the rare antibody induced by stibophen in an attempt to resolve the question of antigen specificity, measure the valence of antibody, and determine the number of sites for antibody attachment per cell. These studies have required development and modification of methods for measuring extremely low concentrations of catechols by spectrophotometric, spectrophotofluorometric, and isotopic labeling techniques combined with immuno-electrophoresis. Progress so far indicates that the

first step of the over-all reaction which results in an antibody-drug-cell-complement complex is the attachment of drug to antibody. This is an important finding, for if the first step of complex formation is combination of antibody with drug, rather than cell with drug, the implications are that the cell-drug complex is not the antigen but that the antibody-drug complex may be nonspecifically adsorbed on cell membranes, just as other non-antibody plasma proteins are adsorbed. Explicit information concerning this type of immunoreaction and its effects on cells is essential in order to provide a basis for interpreting the obscure, apparently nonspecific reactions between cellular components and gamma globulin in diseases such as rheumatoid arthritis, Sjögren's syndrome, and lupus erythematosus.

Pediatric Metabolism Branch

In the first year of operation, the Pediatric Metabolism Branch has devoted most of its efforts to the investigation of cystic fibrosis of the pancreas: 51 patients with this disorder have been studied. Other diseases leading to malabsorption in children and disorders due to glycogen storage have also been the object of study.

Cystic Fibrosis of the Pancreas

Principal aim of the investigations being conducted on cystic fibrosis is to elucidate and, if possible, uncover the basic defect in mucopolysaccharide metabolism presumably responsible for the generalized disturbance of exocrine secretions which gives rise to the clinical manifestations of this disease.

Mucus Structure and Mucopolysaccharide Metabolism

Evidence was obtained in previous studies of the occurrence in duodenal fluid of patients with cystic fibrosis of mucopolysaccharide compounds characterized by abnormal chemical structure and physico-chemical behavior. These mucoprotein fractions have an increased fucose and decreased sialic acid content affecting their solubility in organic solvents. If this obtains in mucous secretions throughout the body, it would be possible for a change in the physico-chemical environment of body fluids to cause irreversible precipitation of mucoproteins in structures such as the pancreas, liver, and bronchi, thus initiating the chain of clinical events and leading to many of the symptoms manifested by patients with this disease.

In collaboration with Dr. Dische, Department of Biochemistry, Columbia University, further investigations along these lines have been actively pursued in duodenal juice, urine, and other secretions of patients with cystic fibrosis. Work is proceeding on isolation and further analysis of these mucoprotein components by fractionation with organic solvents, continuous-flow electrophoresis, and chromatography. Factors which may influence the solubility, physical characteristics, susceptibility to precipitation and denaturation of these abnormal mucoid components are being tested and preliminary findings have been obtained which may be of major importance in explaining the genesis of the disease.

Electrolyte and Genetic Studies

In cystic fibrosis there is a unique abnormality of sweat, saliva and tears leading to marked increase in the sodium chloride content of these secretions. Evidence has been obtained in previous studies and in the course of present investigations that varying genetic endowment may affect not only the levels of electrolytes in these secretions, but also their response to certain types of stress (e.g., dietary salt restriction, administration of steroids, environmental heat). The electrolyte behavior is quite different in this recessive disorder in patients with all of the manifestations of cystic fibrosis (homozygotes) and those with incompletely manifested disease (heterozygotes). In addition, from these observations there is reason to believe that cases occur in which the gene is only partially expressed. Such investigations are being pursued by means of metabolic studies of sodium chloride metabolism and measurement of various parameters of adrenal function. It is hoped that this approach will lead to further clarification of the genetic transmission and basic mechanisms involved in cystic fibrosis.

Determination of electrolyte levels in sweat, the so-called "sweat test," is the cornerstone of diagnosis in patients with all of the manifestations of cystic fibrosis. However, many variable factors are involved in these determinations (e.g.: rate sweating) impairing the usefulness of this

test in family studies and in patients who have only some, but not all, of the manifestations of the disease. Attempts are being made to perfect the methods used so as to eliminate possible errors and enhance the value of this assay in diagnostic and genetic studies.

The possible relation of cystic fibrosis in children to many chronic pulmonary and common gastrointestinal disorders in adults is one of the more challenging leads at the present time. It is planned to pursue these investigations actively and a beginning has been made in this direction. In addition, further definition of other conditions in the pediatric age group leading to chronic lung involvement (e.g., bronchial asthma and others) is being attempted.

Intestinal Absorption

Evidence has been obtained in previous investigations by means of intestinal fat and fatty acids labeled with I^{131} that pancreatic insufficiency may not be the only factor involved in the intestinal malabsorption in cystic fibrosis. These researches are being pursued with the hope of further elucidation of the mechanisms involved.

Fat and nitrogen balance studies are also being conducted to define the impaired metabolism and defective development and nutrition present in fibrocystic patients with only partial pancreatic deficiency. In addition, it is planned to investigate the effects of varying dosages and different kinds of pancreatic extracts on the intestinal absorption of patients with pancreatic achylia.

Pulmonary Involvement

The pulmonary involvement dominates the clinical picture and determines the fate of patients with cystic fibrosis. Therapeutic trials of antibiotic drugs, of enzymes and physical methods of treatment of this complication are being actively pursued. Preliminary results have been encouraging.

Intestinal Malabsorption in Children

Many of the techniques perfected for the study of cystic fibrosis can be applied to further investigation of other diseases leading to intestinal insufficiency in children. The recently developed fat absorption tests by means of fats tagged with I^{131}, the oral small intestinal biopsy, and the recognition of wheat and rye gluten as a noxious factor in the diet of many pediatric patients with malabsorption offer new tools for investigation of this very confused field. Several children with a variety of conditions leading to this clinical manifestation have been studied in the past year.

Glycogen Storage Disease

Congenital and usually familial errors of carbohydrate metabolism lead to a group of disorders characterized by accumulation of glycogen in various tissues and organs leading to enlargement and dysfunction of the structure involved, and frequently to death.

In recent times by the application of chemical techniques several different diseases have been defined, each one characterized by the absence of an enzyme necessary for completion of the carbohydrate cycle. Much further definition is necessary as to the metabolic consequences and clinical manifestations of these conditions. This represents a very dynamic field and other syndromes will undoubtedly be recognized as investigations are pursued.

A beginning has been made in this direction and several patients with disorders of glycogen storage have been studied.

NATIONAL INSTITUTE OF MENTAL HEALTH

BASIC RESEARCH

Introduction[1]

This is my fourth and final annual report. Since each of the preceding reports undertook a discussion of general questions and since the questions themselves are interrelated, it may be helpful to introduce this last report by a résumé of major points considered.

I

The first report, written in 1957, depicts the urgency for research which will enable men to understand how to substitute a just system of law for the present international anarchy. The most staggering problems facing man are no longer those of food, clothing, shelter, power and transportation, although these are not yet equably distributed; instead, the problems he knows least how to solve and which cause him the greatest tension are those relating to the perception of actions and of shibboleths, the translation of ideas, the momentum of traditional concepts, the adhesive behavior of groups, and the communication of ideals and goals. Men of all nations are in need, promptly, to possess vastly improved means for comprehending and coping with a myriad of problems relating to perception, memory and emotion. Men need urgently to learn how to become more constructively adaptive as interdependent individuals within a completely comprehensive, planetary level of social integration.

Several research domains relating to brain and behavior are pertinent to the solution of the social and political problems that now seem so overwhelming. We need urgently to know how to increase man's capabilities for adaptation to an environment wherein his greatest threat arises from other men. Man's aspirations for rational action, and his definitions of what is rational, depend in large measure upon insights which can be gained only through an improved understanding of the fundamental processes of brain and behavior. Ignorance of the limits and potentialities of these processes acts as a handicap in present attempts to solve our most pressing social and political problems and leaves man's adaptive behavior almost exclusively subject to brutish prescientific impulse.

Defense and diplomacy cannot be primarily devoted to the advancement of concepts underlying interpersonal and social integration: they must proceed on the basis of whatever is the contemporary level of understanding of human relations, no matter how inadequate this may seem in the context of their problems. Defense and diplomacy are, nevertheless, providing a precious gap of time within which the essential political and social growth and adaptation must take place. It is what we accomplish during this gap of time by way of advancing knowledge and encouraging constructive social adaptation that will count effectively. Progress in the discovery and communication of more rational ways to improve human relations will itself act as a relaxant to threatening tensions. Progress will be increasingly rapid as we deliberately cultivate the best opportunities for thoughtful and creative research enterprise in these fields. Our view is that the brain-behavior system is an evolutionary product which is playing its role in human development just as do teeth and claws: yet from this great transactional organ we can expect far more constructive and creative potentialities than from teeth and claws. The brain-behavior system, in

[1] Written by Robert Livingston, M.D.
As in previous annual reports, the laboratory chiefs have provided comprehensive statements of research progress achieved during the year. I am attempting here to continue an exploration of questions relating to broader horizons of the mental and neurological sciences. In the immediacy and seeming urgency of our daily existence, we tend to put these questions aside as if they were not prime to our purpose. Yet, I believe that they are truly pertinent to our ultimate best contributions to science and to our fellow man. These annual reports ought to be considered as fragments in a continuing insight-seeking discourse rather than as pretensions of discovered truth.

our view, is a very incompletely exploited instrument for survival.

A further theme of the 1957 report discusses the essential role of creativity—the *sine qua non* for conceptual progress in any field. No new level of understanding can be achieved without: (i) a substantial mastery of the field of intended accomplishment; (ii) an internal dedication and discipline devoted to a program of thought and action which has to surpass the contemporary limits of conception; (iii) a capacity for nimble imaginative interplay of ideas and images; (iv) a confident degree of intellectual nonconformity; and (v) a capacity to communicate effectively the newly acquired conceptual formulations. It is obvious that these criteria place special demands not only upon individuals who aspire to be scientifically creative, but also upon institutions which aspire to recruit and retain creative scientists.

II

The second in this series of Annual Reports, that for 1958, substantiates that all branches of science, and indeed all learning, depend in their ultimate formulations upon concepts relating to human sensory, mnemonic, judgmental and effectual mechanisms. Our present concepts relating to these functions are not fixed and immutable but are changing rapidly. As insight is extended we shall be able to improve the operational definitions of reality relating to all branches of learning, those concerned with the rest of the universe as well as with ourselves. Our profoundest concern as scientists is with the intellectual content of our disciplines and with the creative processes essential to achieving substantial progress, as contrasted to fashionable satisfaction, throughout these fields. All administrative considerations should be devoted to the development, encouragement and exercise of these essentially intellectual faculties.

A further point of the 1958 report is that the idea of the separateness and incommensurability of mind and body is not an automatically self-understood feature of human existence. Such a division is not presupposed by other thoughtful cultures. Our contemporary dualistic assumption is inherited directly from Greek scholars who first entertained this idea at a particular moment in history, around 400 B.C. No notion of the separateness of mind and body appears before that time. However, western civilization has sustained the idea for such a long time that it has become deeply imbedded in our language and habits of thought. We escape from the idea only by circumlocutions and we seldom really abandon it. I believe that this assumption is not only unnecessary but that it acts as an obstacle to our search for a more adequate understanding and to our efforts in the prevention and cure of mental and neurological disorders. This handicap is also to be regretted because dualism fosters professional, intellectual and conceptual isolation among scientists who are trying to understand whole man.

III

The 1959 annual report concerns relations between science and society. Science as a human value, the place of science in the evolution of human values, the contribution of science in rationalizing and liberalizing society; the requirement in science that professional responsibility should pervade all scientific activity as well as the relations between science and society; the requirement for evaluating scientists and for evaluating science as a body of knowledge, and for making decisions which commit a large proportion of society's wealth to scientific enterprise; the problems generated by the cultural interface between science and Government, and the administrative consequences of these considerations, are deliberated.

The balance of the third report describes biological and social evolution as these proceed toward greater degrees of obligate cooperation and interdependence. Each step is achieved at the expense of more arbitrary modes of behavior which operate at lower levels of biological and social integration. This loss is counterbalanced by substantial gains in freedom and power for self-determination enjoyed by successively higher levels of organizational integration. The progressive enlargement of freedom within the more highly integrated systems can best be appreciated by scanning biological and social evolution

over a longer time scale than is usually considered for the next essential step, that is, the establishment of a just and adaptive system for international integration. From these considerations follow certain implications relating to the comportment of the individual, family community, institution, nation and international organization in the direction of providing a scheme for social integration on a planetary scale.

IV

In this final annual report my purpose is to examine some of the biological limitations and opportunities which underlie human capabilities for the social and cultural adaptations essential to survival. First, it is necessary to examine three challenging problems.

> "* * * *we are here as on a darkling plain*
> *Swept with confused alarms of struggle and flight,*
> *Where ignorant armies clash by night.*"
> Matthew Arnold

> "*War is the chief stupidity of man.*"
> Charles Erskine Scott Wood

The Technological Challenge

In what significant ways has technology altered individual and national powers for action? In what ways does this automatically narrow the focus of responsibility for perceptions and judgments conditioning the utilization of national powers?

Within recent years it has become possible to pulverize the largest cities with only two or three packages of explosive. All other cities can be destroyed with a single package. There are many simple as well as sophisticated ways of delivering these packages. The number of packages available is said to be greater than the number of civic targets. Policies of threat and retaliation among the most powerful nations are roughly balanced, in an uneasy equilibrium, through the competitive development and deployment of instruments for massive extermination. For the first time mankind as a whole is vulnerable. The world is now a decade and a half deep into a gigantic arms race. History warns us that no previous arms race has ever ended peaceably.

Packages of modern explosive can be dispatched on the initiative of only a few men. They can be sent off from a variety of locations and delivered to any civilized target on the globe within minutes. Since surprise assault might blunt or disorganize counterattack, each antagonist is obliged to maintain himself in readiness for momentary massive retaliation. Within the last half century, the interval for all-out commitment relative to national security has shrunken from months to minutes. Deliberate judgment and widespread consultation must take place well in advance of the critical moment and must be based on forecasted probabilities and contingencies which are impossible to anticipate completely.

Several non-atomic powers are busily preparing themselves for their own independent national capability for massive extermination. There will be several more members and members-by-alliance in the "nuclear club" within 5 years. Just what extremity of desperation in any given nation will trip off an international struggle involving massive extermination is nowhere publicly defined. In several countries, national purposes and national desperation are rather vaingloriously and personally defined. A large number of existing and potential triggers are capable of frustrating national purposes and of aggravating desperation. These triggers may suddenly reinforce one another in an uncontrollable "avalanche effect." These triggers are not under the confident control of any individual, any nation, or any existing combination of nations.

National constraints against the use of weapons for massive extermination may be moral or constitutional in character or based on fear of retaliation. They may also be based on the realization that many untried and possibly more successful ways to further national purposes exist and might be explored. There are no enforceable constraints operating at the international level. Until constitutional machinery is adopted which will ensure an acceptable system for international justice, each nation is left to its own dreadful devices. Even if all of the present international crises were settled, other alternative and emergent problems would become equivalently critical. A quick riffle through the chronicles of history will illustrate that men have to

face a continuing challenge of getting along with one another and of sharing the world's resources.

Although men bemoan the cost of weapons for massive extermination, the cost itself does not provide notable constraint: weapons for massive extermination yield an order of magnitude *less expensive* destruction than any previous military techniques. The costs seem extraordinarily high only because the world is preparing the means for many orders of magnitude more destruction than was ever before contemplated. If general destruction is man's principal objective, he is now pursuing full tilt the most efficient and economical courses by which to achieve that end.

Communication of threatening events and polarization of national attitudes is rapid and world-wide. Unfortunately, it is commonplace now to suppose that charges and insults must be answered practically instantly and in the same vein; any other reaction is apt to be misconstrued at home and abroad.

All of this has vastly changed the nature and potentialities of individuals and nations and is altering the whole fabric of human relations. It has placed the fate of all mankind and the disposal of all the monuments of human achievement at the disposal of a relatively few persons scattered around the world who have in their control practically unlimited powers for destructive action. The situation in which these men must operate on behalf of the national complexes they represent is dynamic and worsening. Most importantly, the individuals concerned are themselves committed in percepts, passions and judgments according to their own life experience which, of course, has largely been dedicated to a leadership role in their own idiosyncratic society. We have not yet comprehended, much less begun to treat rationally, the vast implications stemming from this technological revolution.

The Social Challenge

Unbelievable force has come into man's possession just as he has begun to find out that distortions deeply affecting perceptions, feelings and judgments are normally and inevitably incorporated as part of the baggage of all adults. World-wide communication has become practically instantaneous just as man has begun to appreciate the biological crudeness and inadequacy of the transactions of language. The world is becoming overcrowded just as man is beginning to appreciate the social value of the individual and beginning to develop a primitive insight into constructive human relations. Man has become proficient at polarizing mass attitudes of what is right and what is wrong, but is scarcely as far along in fundamental ethical reasoning (if, indeed, as far) as some of the ancient Greeks. There is as yet too little cognizance of the range of assumptions and criteria upon which every system of rectitude is based, and too little patience with the requirements for cross-cultural understanding.

Man still faces the same two old choices if he would avoid war: either he must discover and establish institutions of government that will substitute a universality of values, purposes and means for the present conflicting systems, or he must provide institutions that will admit and protect differences of values, purposes and means, but yet guarantee agreement in those actions essential to maintain order and at the same time accommodate change. As Walter Millis has declared of a world without war: "it would still be true, in the sense in which Jefferson presumably meant it, that 'the tree of liberty' would have to be 'watered by the blood of martyrs.' Men would still die for principle or passion; but they would not die as the helpless, systemic victims of a world order based upon highly organized and armed national sovereignties. Deprived of the easy simplicities and illusory securities of the war system, statesmanship would meet more, not less, difficult problems than those it must now confront; and it would take brains, illuminated by vision in the leaders and education in their followers to surmount them * * * A world from which "organized war has been excluded would not be an easy one, and it would raise threats to various groups, economic interests, ideals and convictions which may well seem greater than the (still almost unimaginable) threat presented to all by a continuance of the war system itself. But it would be a viable world * * * its attainment, while immeasurably difficult, does not seem to be impossible."

We have come only a little way in the required direction at the individual, national and international levels. For instance, the entire budget, from all member nations, for operations of the United Nations, the only organization having an

internationally endorsed charter for arranging the settlement of international disputes, just equals the size of the budget for cleaning the streets in New York City. The cost of the United Nations, prorated over the world population, is less than two cents annually. The yearly cost for armaments alone, not including espionage, propaganda and conventional diplomacy, prorated in the same way, is more than three thousand times greater than this, and uses up nearly half of the total product of human and other resources on a world scale.

1. *The need for purposeful aspirations on a world scale.* The most critical weakness of human society today is its lack of concerted purpose to achieve more comprehensive social and political integration. It is widely acknowledged that men of all nations are thrown together perforce of modern technology, but the consequences of this fact are not sufficiently taken into account in men's actions. It is as though we really believed that if we stood upon our traditions, the present state of the world would revert to some more comfortable epoch of the past. Such faith is folly.

Every human shares certain fundamental interests and objectives with all others: that it is better to be alive than dead, fed than starved, free than enslaved. There are other shared interests, but none is more fundamental and none is in greater present jeopardy. Existing social systems are not arranged so as to safeguard even these rudimentary needs on a universal scale. It is rare for any existing system to advocate their fulfillment for anyone who does not belong to the same system. No individual can safeguard fulfillment of these needs for himself, nor for any other. No social system can safeguard them for its own people, nor for any other—although we continue to extend our faith in this possibility. The chief problem is to establish a *new* system that is sufficient to satisfy the needs for survival, sustenance and self-realization for all mankind. Of course, people will not then be satisfied, for even though alive, fed and free, they will still have problems enough; there will remain plenty of difficulties to challenge man's capacities for further social progress.

2. *The need for purposeful actions on a world scale.* The dimensions of social need and the arena of struggle for power and wealth are world-wide in scale. Perforce of these facts, creative thinking of ways to satisfy fundamental human needs and at the same time to arrange for the just settlement of disputes must be conducted on a global scale. There is now no way to safeguard our lives or our welfare without achieving such safeguards for everyone. We need a fresh analysis of the role of all institutions bearing directly or indirectly on the requirement for a world government that supports universal maximum individual self-realization. Whether traditional ideas and traditional institutions can be helpful should not be presupposed prior to a thorough, objective and disinterested analysis of the requirement. What was adequate for less extended needs and aspirations will probably not now suffice. Paralleling this, we need research to enlarge man's grasp of the processes of human relations. Analysis, research and the required revision of institutions should be carried out with the deliberative participation of as many as possible of those concerned, that is, with the widest possible world base. This is essential in order to enable the consideration of this problem among the world's most creative thinkers, of which no single nation has a monopoly; moreover, world-wide participation will ensure insight into the requirements and means for modifying all pertinent institutions in the desired direction.

No nation or confederation of nations, acting unilaterally, can put world government into effect. This cannot be accomplished either by suasion or coercion because any unilateral endeavor giving evidence of the likelihood of success would undoubtedly provoke a full scale war before it had gotten very far. Thus, the only alternative to the present uneasy situation seems to be to invite the broadest world base of creative social thinkers to undertake research and deliberations leading to the development of plans for a more inclusive community of mankind. This undertaking of research and discussion may not yield ideas upon which agreement can be established in the near future; but, if this objective is purposefully undertaken, sound and satisfying ideas are ultimately achievable.

We have substantial reason to hope for progress in this enterprise through application of the methods of science. Social evolution in the past has come about through far slower and more haphazard processes. When research and discussion

dedicated to this goal get underway, this fact by itself will encourage world leaders and mankind as a whole to be more tentative, to tolerate greater ambiguities and to exercise more patience concerning international relations. The findings of such research and the results of world-based deliberations as to its application can be submitted for national recognition or rejection; popular referenda might be appropriate for the determination of final acceptability of the evolved plans.

The world prospect will be improved by the encouragement and development of a world-view in these undertakings; not a view to conquering the world but to making it safer and, in other basic ways, better for all mankind. Naturally, attitudes and impulses which during the last four centuries have worked to create strong nation-states have established a prominent place in men's thinking and ways of life. Our United States' national experience of nearly two centuries and our individual strivings to improve national greatness, national security and national welfare are dominant features of our own existence. These attitudes and impulses should not be discarded in assuming a world-view. A world-view and world-government appear to be the only possible ways for securing enduring national greatness, national security and national welfare. Greatness, security and welfare cannot be sustained indefinitely on a merely national scale. As a nation, we are beginning to recognize this and to put it into increasing practice in our foreign and defense policies, foreign aid, military aid and mutual security programs. Something new has already been added to nationalism, something which obviously accommodates continuing patriotic devotion to nationality. We are learning to be more rational about more complex and more inclusive human transactions, to perceive the consequences of our actions in a wider context and in a longer view. Growth in freedom and opportunity for self-realization is known to increase with each more inclusive level of social integration and will increase even more when our identifications are extended to include all mankind.

3. *Urgency of action.* Most leaders in the powerful nations appear to comprehend that precipitation of all out war amounts to mutual murder and suicide: at present, a metastable military stalemate exists. This metastable stalemate, however, can be easily upset by desperate individuals and groups, even among the least powerful nations. They may conceive they have nothing to lose and perhaps something to gain, or they may simply behave irrationally. Therefore, time is our most precious commodity. We need to proceed in all deliberate haste toward the goal of world-wide social integration. What kind of time schedule might be estimated for this task? The necessary research and discussion need time; too urgent a desire to arrive quickly at an agreed upon plan may lead to inadequate improvisation and to the blunting of creative efforts which might otherwise provide a far more desirable solution. The social and attitudinal changes required are probably equivalent in revolutionary dimension to those which have taken place around the world during the last fifteen years. Perhaps fifteen years may be accepted as a *minimum* time if we begin immediately in a purposeful and dedicated way. On the other hand, we cannot afford to wait until the beginning of the next century before we belong in a true sense to one world.

4. *The "idealistic" course is the only realistic course.* Mankind can look back with justifiable pride on a number of now widely accepted revolutions in attitude and behavior which grew out of enlarged respect for human needs viewed increasingly objectively. We can expect traditional forms of thinking to reject and probably actively oppose research and discussion dedicated to the establishment of a system for the integration of mankind as a whole. Such pursuits may be thought of as "visionary," "idealistic" and "unrealistic." Yet, if any lesson can be clearly drawn from recent history, it is this: that many ideas labeled only recently as visionary, idealistic and unrealistic have already become the only tenable, practical and realistic ones. Much of the sinew and solvent of the most powerful nations of the world has been expanded in the process of learning this lesson.

Ideas, not things, rule mankind. Traditional ideas and consistent forms of behavior preserve continuity. They are reassuring to our immediate associates and bring us such comfort as attaches to the familiar. Yet, in the long run, this comfort may be illusory. For centuries, religious and racial tolerance were considered by all right-minded people to be contrary to any reasonable

conception of morality. Epileptics and the insane were beaten and brutally incarcerated. The object of such treatment was to punish and drive out evil spirits possessing these individuals. All right-thinking people were convinced that this was in the best interests of both the victims and society. The use of lightning-rods was vehemently condemned as "an impious attempt to defeat the will of God" and as a means of "helping criminals to escape." Vaccination and even anesthesia were abhorred on moral grounds as contrary to nature.

It may bring us comfort that the only period of history we have to face is our own. Yet it remains to be seen whether we can meet the main requirement of our day, namely, to establish some kind of world order under a just system of law. It is now high time that we assume the obligation that our conscious awareness and our conscience thrust upon us. It remains to be seen whether we can generate the sense of confidence and purpose necessary to meet this social challenge. If we make our best effort and yet fail in this attempt, we cannot lose anything not already lost.

The Individual Challenge

1. *The problem of individual commitment.* A newborn babe can grow up to achieve a practically complete behavioral adaptation to any culture regardless of his ancestral background. Physiognomic differences scarcely interfere with this adaptation. A baby is readily accepted because he is commitable, yet uncommitted. Within a few years, however, a child's potentialities for adaptation to another culture become progressively limited. By the time he reaches manhood, indistinguishable behavioral adaptation is virtually impossible. Commitment involves internal polarization with respect to all aspects of life, likes and dislikes, even including things which are presumably detached from personal involvement. The presence or absence, nearness or distance of mountains, flatlands, rivers, and the sea come to loom importantly along with personal associations in commitment and is notable in the commitment of "home-sickness."

Once the process of commitment has begun, in reference to language, customs and habitat, everything that is consistent with the initial pattern is easily and congenially adopted. Whatever is contrary to that pattern may be overlooked (not even sensed), may be sensed but dismissed as meaningless, may be met with surprise and incredulity, or may be subjectively distorted and inappropriately acted upon, as if it actually conformed to the familiar pattern. The recognition of discordance with previous experience is characteristically associated with emotional excitement—possibly anticipating the potentialities for threat and opportunity which accompany every novel experience. Adaptation to new patterns of language, customs and habitat is a slow and demanding process associated with many inappropriate responses.

Educational processes and the methods of science legitimize and encourage the systematic examination and objectification of percepts and ideas that may have only the most tenuous credentials. The foundations of intellectual growth depend upon this cultural legitimization. Education is properly called "the servant of all our purposes" (John W. Gardner). Education and discovery are associated with the intellectually satisfying response of "perspective dissolving surprise" (William Gorman). Gradually, unfamiliar patterns become accepted and ultimately incorporated into an automatically functioning system of percepts, judgments and actions. Common sense is the residue of prejudices acquired during education (Albert Einstein). Our capacity to perceive, accept and accommodate novelty slows downs with advancing years—as though we become increasingly impounded by our commitment. The important thing for us to comprehend is that the process of becoming committed is an indispensable and central part of our biological heritage, that it virtually ordains throughout all our development, recognition and reaction to all aspects of life.

Our biological endowment thus equips us to adapt so as to behave "successfully" in a given environment. The very factors which establish an easy and automatic behavior in our accustomed environment will operate to our disadvantage when we are exposed to a differing environment. This is especially compelling if we are unable to recognize the extent and significance of the novelty and deal with it as if it were familiar. Adaptation to experience carries with it a dominating expectation that the environment will be enduringly consistent. In a later section

we will examine the extent to which all our sensations and ideas are dependent—not upon the external events we are contemplating but upon the purposeful direction our lives have taken as a result of our individual idiosyncratic experience. An incorporated pattern does not give way easily to conversion, even when the necessity for a different adaptation is fully accepted intellectually and is reckoned to be life saving. For example, obeying the simple but contrary rule of turning your head to the right instead of to the left in order to avoid oncoming traffic turns out to be no trivial adjustment. Taken from the point of view of the need mankind now dimly discerns, to comprehend the percepts and judgments and to anticipate the actions of people from entirely different cultures, the problem of idiosyncratic commitment can be seen to be interfering in a pervasive way with rational international behavior.

2. *The problem of conformity.* Any kind of behavioral adaptation, any kind of individual or social progress requires nonconformity: nonconformity with one's own past adaptive, experience-guided behavior or nonconformity with the adaptive, experience-guided behavior of the group. The whole history of intellectual progress consists of steps taken which liberate us from purely reflexive behavior, from the limitations of immediate and imperious perception, judgment and response. The "inertia" of men's minds is reflected in the intellectual turmoil which usually attends a shift from one level of understanding to the next: that the world is round, that the earth revolves around the sun, that blood flows through our vessels "as it were, in a circle," that men belong in biological continuity with the rest of the animal kingdom, and that something as small as germs can fell a strong man. Most of these intellectual conversions took decades and sometimes centuries. Each step, small or large, demands a break with the consistency of previous thought. Each contributor to intellectual progress must be, by definition, a nonconformist. As Ben Shahn wrote, "Without nonconformity we would have had no Bill of Rights nor Magna Carta, no public education system, no nation upon this continent, no continent, no science at all, no philosophy, and considerably fewer religions. All this is pretty obvious. But it seems to be less obvious that to create anything at all in any field, and especially anything of outstanding worth, requires nonconformity, or a want of satisfaction with things as they are. The creative person—the nonconformist—may be in profound disagreement with the present way of things, or he may simply wish to add his views, to render a personal account of matters * * *

"Yet, when it comes to the matter of just what kind of nonconformity shall be encouraged, liberality of view recedes. There seems to be no exact place where nonconformity can be fitted in: it must not be admitted into the university curriculum—that would produce chaos. In politics it is certainly inadvisable—at least for the time being. It cannot be practiced in journalism * * * In science—least of all, alas!" Shahn goes on to conclude that "The degree of nonconformity present—and tolerated—in a society might be looked upon as a symptom of its state of health."

Saltatory advancement of social concepts—the introduction of ways of thinking that change the entire character and direction of social progress, that lead to the acceptance of a substantially more fundamental ideal, that creatively reformulate the nature of a social problem or its solution, that cut short years of social strife—are not likely to occur except where circumstances are especially favorable for nonconformist social thinking. The concept that "all men are created equal" was a product of such circumstances. Improvement in social theory, increase in grasp of social problems and their solution depend upon advances of this sort—intellectually creative advances—and upon their institutionalization. As Hastings Rashdall said: "Ideals become great historic forces by embodying themselves in institutions."

3. *The power of purpose.* It is not an accident that the words *curiosity* and *cure* come from the same origin. They imply being full of care, taking pains; having a desire to learn about, to comprehend; possessing empathy for and, thus, striving fully to understand the object of contemplation. Our guide for human progress, individually and collectively, grows out of our humane interests, understood objectively, in the long range and in the largest sense, humane interests made as consciously self-aware as possible. It is not likely that man can attain what he does not strive for. And it is impossible for

him to seek what he conceives as unattainable. Thus far, human purpose has predominantly sought provincial advantage. This has been gradually enlarged from individual to family, to community, to nation, without there being unendurable losses along the way. Provincial advantage is now possible only on a world-scale.

By far the most important influence on social progress is consciously directed purpose. Men need to instill in themselves a sense of great purpose and high resolve to gain a more comprehensive level of social integration. The phrase "that all men are created equal" once played a role of great importance in the establishment of our nation. "That all men are created equal" became the reiterated definition of purpose of the Federal Government in the campaign utterances of Abraham Lincoln. This phrase became Lincoln's principal personal and administrative theme and his main means for mobilizing what ultimately saved the Union. At that time, there was little the United States could do, nor was there so much at stake for the rest of mankind, regarding the global inclusiveness of that phrase. "That all men are created equal" has, to a regrettable degree, remained rhetorical even within our own borders since then. But there is every indication that the Founding Fathers took this principle seriously; they purposefully established institutions of government dedicated to its recognition and fulfillment. It is our turn now to support and improve such institutions and to extend their application as widely as possible.

Education is a servant of our purposes, and so is science. Both seek the truth. Both debunk authority. They teach that the individual, community, nation and the universe are always in the process of *becoming*, and that no part of these great transactions can be made to stand still. There is a powerful anti-inertial effect on society contributed by education and science. Cultural integrity and the safeguards formerly dependent upon the preservation of traditional patterns of human relations can now be guaranteed through the broadening experience of education combined with the creative contributions of science. They emphasize the intrinsically creative aspects of man's individual life and his ineluctable capacity to develop increasing degrees of freedom for himself and his fellow men.

Education and science are not automatically liberalizing. They can be encouraged to seek truths of differential value favoring one individual, one company or one nation. New knowledge can be deliberately fostered and exploited to yield powerfully disadvantageous consequences to other groups. In such efforts, education and science quickly outstrip any other system to achieve unilateral advantage. But the same endeavor can be extended to seek truths of advantage to mankind as a whole. It is reasonable to anticipate that science and education can outperform any other mode of approach toward this goal. The community of teachers and scientists has been international in character since long before nationalism began to be a force in human affairs. Educators and scientists are professionally trained to be adaptive; they are professionally dedicated to the discovery of larger and simpler patterns of transaction and organization.

Mainly, it is to facilitate the establishment of worthier and more humane purposes that I urge the fostering of the broadest horizons in the labors of educators and scientists. Mankind has amply illustrated that it can always retreat to more primitive perceptions, judgments and actions. When research, encouraged in this way, adds new concepts to the traditional ones, men will at least have an enlarged freedom of choice and probably they will also enjoy greatly increased powers for constructive action. The rate at which meaningful new knowledge is appearing in the biological, sociological and psychological sciences, and the nature of the new information provide confidence that substantial progress can be made in the direction of helping all men become more constructively adaptive as members of interdependent social systems.

The social sciences have been comparatively slow to develop partly because the subject matter is vastly more complicated than anything dealt with in biology or physics, and partly because there has always been a strong *tabu* against investigating social processes. The social sciences are now in transition from empirical to theoretical bases. By analogy from other branches of science it is known that this kind of transition increases tremendously the scope and power of such studies. The capacity of the social sciences to provide insight and mechanisms for internationalization of the control of social be-

havior will ultimately dwarf the practical contributions of any other domain of science. The most impressive utilitarian value of science will attach to science when it becomes effective in the shaping of constructive human behavior. This contribution will have an equally important impact upon the mainstream of human intellectual activity, the philosophy of thought, vitality of ideas, and the generation of security as well as welfare for all mankind. The question is not whether such achievements are feasible but whether they can be instituted within the gap of time that may be available.

The Natural Foundations for Human Adaptability

What may be the bases for our commitment to earlier developed views of reality, patterns of ideation and judgment, and modes of behavior? How and to what extent may these interfere with re-adaptability? How compelling is previous experience in shaping and limiting what we perceive? How compelling is previous experience in our interpretation and reaction to novelty? To what extent do we remain blind to the unconscious influences of previous experience? What can be done in a positive way toward gaining a more adequate insight into both the limits and opportunities of human adaptability?

In this section I shall attempt to be more explicit concerning the internal processes by means of which man adapts to a given environment and by means of which he must re-adapt to previously unexperienced patterns. For illustrations, I have selected a number of observations for brief outline: many of these are old and well substantiated findings of research; some are drawn from commonplace experiences; a few are new and must be admitted only tentatively. What I seek is a working conceptualization of man as an adaptative social being.

1. *Developmental considerations.* The presence of developing muscle cells in the body of the vertebrate embryo induces the specialization and maturation of motor cells in brain stem and spinal cord. Motor nerves send connections to these muscle cells and induce muscular contractions before the sensory nerves have even gained access to the periphery and well before they have developed the capacity to transmit impulses. Therefore, in the first stages of life, responses to sensory stimulation are secondary, not primary, for action.

From the beginning, the embryo acts in an integrated fashion. Its first movements are mass actions. Later, more specialized and particularized actions arise through the individuation of motor patterns derived out of a background of mass actions. As development progresses, mass actions are gradually held in check until, in the mature organism, they provide mainly the tonal and postural adjustments stabilizing the platform upon which an individuated act is carried out. During the process of generating new patterns of activity, the nervous system continues to behave in an integrated fashion; newly integrated patterns are built up from older integrated patterns. The nervous system preserves a fundamental continuity of self-integrity during all adaptation.

Nerves serving particular muscles seem to develop a unique specification, an endowment received according to the particular peripheral relations they establish. The specifications are different if deliberate transpositions are made in the peripheral motor organization. Something of the same sort occurs in the specification of second and higher order central sensory units, as we shall see later. These constitute the most elementary phenomena of commitment.

2. *The origins of action.* For each incoming or outgoing neuron there are at least five thousand central neurons whose activities are confined to the brain and spinal cord. The total number of these central neurons is estimated at a staggering ten billions units. The nervous system used to be thought of as being activated primarily through the influx of outside stimuli. However, when direct observation of individual neuronal units became possible, it was discovered that some cells in each sense organ maintain a degree of "steady state" activity even in the absence of identifiable stimuli. Moreover, a substantial proportion of neurons in the brain and spinal cord show "spontaneous" activity from early embryonic stages onward, even during sleep, hibernation and deep anesthesia. "Spontaneous" activity and "steady state" activity are probably functionally equivalent; neither requires excitation from outside the neuron itself, although the rates of activity can be modified by

outside influences. Spontaneously active units are found within all regions of the central nervous system, even in aggregates of neurons separated from the nervous system. Various ganglionic masses throughout the brain interact with neighboring and even remote parts. The nervous system is thus made up of vast aggregates of neurons each of which exhibits its own activity, yet all of the aggregates are bound together into mutually interdependent transactional functions constituting an integrated whole.

It is evident that the nervous system is active as well as reactive. It is built for action. Even the earliest sensory messages enter upon a performance-oriented transaction. In respect to sensory messages, the central neurons govern: (i) the degree to which any incoming signal can invade and alter the central transactional system, and (ii) the degree to which such influence may be reflected during any subsequent motor performance. It is obvious that no sensory signal enters upon a *tabula rasa.* Neuronal messages entering the central nervous system can achieve direct and indirect relations with a very large number of mutually interdependent aggregations of neurons already organized into an integrated system.

3. *Genetically determined and undetermined behavior.* It is obvious that some sensory stimuli contribute quite directly to motor performance. Through genetically inscribed pathways, certain sensory signals can induce stereotyped reflex responses. Nonetheless, even these imperious reflex responses are clearly "conditioned" by ongoing activity in nearby and even remote parts of the central nervous system. This feature supplies the principal diagnostic value to clinical reflex response testing.

Through more elaborate but still genetically ordained paths, the sensory system can play a "releasing" and guiding role in what has been referred to as "instinctual" behavior. Although reflex behavior is relatively fixed and is not profoundly changed by maturation and learning, instinctual behavior may await certain stages of nervous system or endocrine maturation and may be subject to some modification according to experience and learning. Beyond reflexive and instinctual systems, however, all of the rest of the central nervous system seems to be highly modifiable. Thus, reflex pathways are the least modifiable, most direct, and shortest-circuited; paths relating to instinctual behavior are longer-circuited and more modifiable; the remainder of the central nervous system organization is relatively long-circuited and uncommitted.

Our problem relates chiefly to this long-circuited, most modifiable compartment: *What evidence is there that this system is not committed at birth? What evidence is there that this system can be committed to neuronal patterns relating to experience and that, following such commitment, the system is relatively less free for re-commitment?*

It is reasonable to suppose that because of the vast numbers of neurons involved and because of the tenuousness of many of the mutually interdependent relations, extremely subtle changes in time and tide of activity in only a few units may have an enormous effect on the outcome of any given initial state. It is important to remember that such a system can only progress from one stage to the next, from an uncommitted, integrated performance to a committed but still integrated performance. In each successive state, the organism is active and acting, even if that action is simply to hold more overt action in abeyance. The organism can never revert to its original uncommitted state but must instead progress from one committed state to the next and so on. Presumably this progression will be retarded according to whatever in the next pattern of adaptation may be incongruous or in conflict with the presently ongoing integrated system.

Pertinent to these organismic considerations is Whitehead's solution of the apparent conflict between concepts of permanence and becoming, and between determinism and free will. The organism becomes something else on the basis of its given ("permanent") endowment; direction of "free will" is based upon what has been determined up to the present: direction is always from somewhere, from something.

4. *The processes of neuronal commitment.*

(a) *Congenital absence of sense.* Persons born totally blind never know what vision is like. They have no visual memories or visual dreams. They do not "see stars" or anything else "visual" if bumped on the head. Individuals who become blind after having some experience with sight, that is, after the first few years of childhood, usually retain for the rest of their lives powerful

memories of visual patterns. They may have vivid impressions of color and light elicitable by memory or dreaming. Kimio Eto, one of the best koto players in Japan, describes his visual experiences as follows: "I was very fortunate; when I went blind, I was old enough to know color quite vividly. As I play, certain notes and melodies bring back certain colors, in a perfect form. Somehow, I feel that my world must be one of the most beautiful."

Persons with congenital cataracts who can see only diffused light during childhood do not have good vision immediately after the cataracts have been removed, even if they have satisfactory corrective lenses. It takes such individuals a long time to learn to employ visual signals independently of their other sense modalities in the identification of simple geometrical figures. It takes them even longer to develop any confidence of action in circumstances of visual dependence. It is doubtful whether they ever do see "normally" if the cataract removal has been delayed beyond childhood. (A roughly parallel account is given of chimpanzees raised in complete darkness for some months. The animal studies control for some of the ambiguities obtaining in patients with congenital cataracts, e.g., that there might have been other congenital defects affecting vision, or interference by virtue of the surgery or post-operatively inadequate optical accommodation.)

We are led by these facts to surmise: (i) in the absence of access to the outside world through a particular sensory modality, the otherwise appropriate brain pathways do not organize central representation of that aspect of the world, (ii) sensory experiences in early childhood may be lastingly committing with respect to central representation, and (iii) the absence of such childhood experiences may be limiting with respect to the later fitness of that same system for central representation.

(b) *Phantom sensation.* When a limb is congenitally missing or is amputated at birth or shortly thereafter, there is no evidence of phantom sensation referable to the missing part. Nevertheless, when a limb is amputated anytime after early childhood, the individual uniformly has a phantom which usually persists for the duration of his life. Phantoms may be associated with the loss of fingers, toes, penis, breasts, ears or nose, but the most vivid and enduring phantoms follow major limb amputations. Conscious awareness of phantom parts relate to those to which conscious attention is ordinarily directed; thus, the phantom fingers, hand, and wrist may be vividly experienced, whereas the forearm and upper arm may be foreshortened and only vaguely experienced. The phantom can usually be described in detail as to its exact position in space, in relation to the rest of the subject's body, its motility, and its participation in "voluntary" movements. There may be detailed sensations relating to temperature sense, muscle tension, joint position, nail bed, skin tension, tickle, whether hairs are erected or not, etc. Normally, phantoms can be caused to change position, to "move at the free will" of the subject. The subjective feeling of "thumbing your nose" with a phantom is said to be exactly equivalent to that of the normal extremity, just as simple but not as risky.

We are led further to surmise: (i) the specification of "sign" given to second and higher order sensory units by virtue of sensory experience is integrated into patterns, (ii) these patterns involve sensory and motor integration, (iii) these central patterns continue to remain coordinated even in the absence of the peripheral organ (the limb) and the peripheral motor nerve endings and effectors and the sensory end-organs which are nominally considered to be responsible for the origination and organization of limb sensation. Peripherally assigned functions have been taken over, as it were, by second and higher order motor and sensory neurons which continue to function in accordance with their experienced commitments. This means that muscle, tendon and skin receptors activated in concert while experiencing active and passive movements of the previously intact limb have somehow conferred "pattern integrity" as well as "sign" upon higher order sensory and motor units relating to that limb. This "pattern integrity" and "sign" never appear without abundant explicit personal experiences. Yet commitment of childhood is adequate for an indefinite duration of the phantom, even in the absence of any further impulses from the missing extremity. Despite intellectual and visual evidence to the contrary, the phantom continues to survive in conscious awareness. The previously experienced integrated patterns re-

main inscribed and integrated according to previous commitment.

It might be interjected that the phantom represents nothing more than "memory," but this begs the question: Where does this "memory" reside? The phantom (or "memory") can be modified by cooling the amputation stump, by injecting procaine or alcohol into the amputated nerve trunks and the local spinal sympathetic ganglia, and by spinal anesthesia. Furthermore, phantoms following transection of the spinal cord are specifically and characteristically different from phantoms following limb amputation. These facts combine to suggest that the phantom as a "memory" not only originates initially from ascending impulses but may, after the amputation is performed, continue to have its "roots" in parts close to the original input stages.

(c) *Conditioning, learning, and the process of becoming.* The natural history of developing perceptual, motor, cognitive and social skills is being studied in various branches of psychology, anthropology and sociology. Considerable is now known about conditioning and other forms of learning, including social imitation, and learning dependent on the symbolic processes of language and mathematics. Learning in any form appears to depend upon: (i) confrontation of the subject by an unfamiliar configuration; (ii) devotion by the subject of attention to that configuration; (iii) existence in the subject of appropriate motivation or drive; (iv) development of a new central neuronal configuration yielding ideas or action relating to the unfamiliar configuration; and (v) feedback to the subject of information respecting success or failure, appropriateness or inappropriateness, of his ideas or actions. Failure of any one of these five essentials precludes learning anything at all.

It is also obvious that the subject must make use of an internalized neuronal scheme of the universe in which he and his body parts are represented in organized ways which can be variously articulated with systems representing objects other than himself and with systems representing time and space. Very little of this arrangement appears to be genetically determined, as we have observed. History teaches us that men have held very different notions about themselves and about objects around them. Certainly, in the natural history of the individual, such a linked succession of different concepts of self and beyond self takes place.

Early psychological studies emphasized the high degree of presumptive correspondence between physical objects and the subjective report of these objects as perceived, and the high degree of correspondence among reports from different individuals observing the same object. This tended to stress the impartiality and consistency of the perceived world. Naturally, the first test objects were selected as being relatively unambiguous. Naturally, the subjects were required to "pay attention" to the object and were given certain other stabilizing instructions. The subjects were advertently and inadvertently highly motivated to provide what might be considered "normal" subjective reactions. It was quickly recognized that "inattentiveness" or "fatigue" or "suggestion" have remarkable effects upon the perception.

Certain objects turned out to be interpreted as having "size constancy" and other attributes which depend upon the subject making certain sophisticated assumptions concerning both space and object, both of which acts of assumption are based on previous and not on the immediate experiential conditions. Later it became fashionable deliberately to influence perception by inducing in subjects certain prescribed expectations. Both past experiences and induced expectations are profoundly influential on percepts, judgments, and behavior. Still more recently, it was demonstrated that an individual may be converted in his perception or judgment of even simple and familiar geometrical configurations by confronting him with contrary ("incorrect") but concerted views of other subjects. The interesting thing is that, under such conditions, objects come to appear to the subject as if they actually do correspond to the socially adopted view. Other investigators pointed out that children are more reliable witnesses than adults for certain kinds of objective reality. This is assumed to be because children lack the persuasive commitments incorporated into the perceptual apparatus of the adult as a result of his longer experience.

From infancy, through puberty, adulthood, "parentage" and finally old age, we are really a succession of different persons. We have different appetites, perceptions, emotions, activities and different capacities for conscious awareness

and responsible behavior. At different ages, we obviously have different schemes for ourselves and for the rest of the universe. Not only do we change rapidly, but the world changes rapidly around us. We tend to adjust ourselves to these changes through a combination of adapting ourselves to the circumstances and adapting circumstances to our changing needs. In the process, we tend to underestimate the true extent of the alteration of our internal and external state. For instance, as adults, we would have a difficult time converting our body image back to a prepuberty stage even though we have had abundant experience with that former personal body scheme. We have since that period become something else. We are continuously in the process of becoming.

On a shorter time scale we are continually changing in ways that profoundly affect our perceptions, judgments and actions. For instance, when we are satiated, it is difficult for us to feel an adequate compassion and urgency for those who are hungry. For this reason, a good restaurant does not permit cooks and waiters to eat until after serving the customers. When we are food deprived for 24 hours or more, our attention is swiftly attracted and held by food odors, our judgment is biased respecting the value of, say, an onion, and our actions are both consciously and unconsciously dedicated to food procurement. Although such tides of appetite and satiety are generally short run, if a man is chronically starved or cold or penurious, he learns to take advantage of features of his environment not attended to by others and he may develop a life-long commitment relating to highly specialized perceptions, judgments and actions.

The educational, social, political, military and economic changes which have revolutionized the pattern of lives within this generation are eloquent testimony to change and to the range of human capabilities for adaptation to change. We can appreciate from the experience gained in these years the powerful influence of conscious and rational dedication to successful adaptation. We can likewise appreciate the stimulating as well as disruptive potentialities imposed by change. Yet, characteristically, we do not project into our views of the future a degree and rate of change equivalent to what we have gone through in the past. This limitation of projective imagination tends to influence us to underestimate our powers for conscious dedication and rational election in the direction of successful accommodation among the various alternatives we are confronting.

(d) *Sensory deprivation.* When normal subjects are placed in circumstances of sensory deprivation (really, a reduction or monotony of sensory input instead of true deprivation), they characteristically develop certain sensory, judgmental and motor defects. This is true whether the deprivation is induced by confinement in a light-proof, sound-proof cubicle or submergence in a constant temperature pool of water. After some hours or at most 2 or 3 days of confinement, subjects begin to lose their normal ability to control their thought processes. They cannot sustain a line of thinking or bring to mind familiar ideas. Soon thereafter hallucinations may appear, beginning with visualized geometrical patterns and going on to include auditory and somaesthetic impressions. Hallucinations thereafter become more elaborate and intrusive, and eventually may involve elaborate perceptions of bodily movement, voices and moving scenes. The subject's ability to respond to his own or to outside commands, to discriminate test objects, to think, and even to perform simple motor skills such as handwriting and walking, becomes disturbed. Recovery may take several hours or even days.

One is left with an impression from such studies that contact with "reality" may be more dependent upon experience than we generally suppose. We presumably are normally encountering frequent and automatic validations and cross-validations of our internal circuits through continually varying perceptual, judgmental and action experiences. This process may be contributing to the maintenance of "normalcy." When this process is substantially reduced as in sensory deprivation, "spontaneous" patterns of activity in the central nervous system may begin to emerge from controls depending upon continuing perceptual, judgmental and behavioral experience. Such experiments have suggestive implications as to the nature of hallucinations experienced by lonely explorers, prisoners, truck drivers on long and monotonous runs, febrile and

isolated patients, and perhaps also frank mental disorder.

Pure-bred Scotty dog puppies have been separated so that half of a litter could be raised in an experience-limiting environment and the other half in private homes. Over a period of months the experience-limited animals developed a persisting hyperactivity and an incapacity to learn simple discriminations and motor performances which were quickly comprehended by their siblings. The effects of upbringing in such circumstances appear to be long lasting.

We are led to conclude that the relationship between experience and the internal neuronal processes governing perception, ideation and action is one of great interdependence. Varied experience may be indispensable in fostering and assuring continuity of a capacity to maintain contact with and to deal appropriately with a changing world.

(e) *Sensory stimulation.* It is a commonplace experience to alight from long, noisy airplane flight and thereafter for some hours to experience a continuing "noise in the head." Similarly, hair blowing on scalp or forearms for some hours may be followed by persisting sensations imitative of the "blowing hair" experience. Disembarking from a rough ocean crossing may be followed by continuing or recurrent sensations of motion of the now stable environment, as if it were a moving ship at sea. The percept may be associated with a wide-based, "rollicking" gait said to characterize the sailor ashore. The French refer to this illusion of motion as *mal de dèbarquement.* If the seas have become gradually rougher during the crossing, the first occasion for vomiting from sea sickness may be experienced on solid land.

One is led to interpret this as a phenomenon of temporary functional commitment. It underlines the reality of ongoing central activity as a controlling force in perception and action, and illustrates once more an experience-imposed lack of correspondence between percept and outside reality.

(f) *Related internal processes.* Recently developed techniques have been introduced which make possible crude observations in experimental animals of internal changes accompanying adaptive behavior. Only the most primitive and preliminary insights have been gained thus far, but the domain is vast and promising. A little is now known which can provide internal (neuronal) linkage patterns complementary to the external (behavioral) linkage patterns established by the social sciences. Obviously the nervous system provides whatever mechanisms we have not only for physiological coordination within ourselves but also for our comportment in the world around us including our management of social relations.

It is now possible to delineate certain generalized changes of functional activity within the brain which are associated with alterations between sleep and wakefulness. The mechanisms of "arousal" and "attention" and the more particularized "orienting reflex" are understood in a preliminary way. Activation of certain brain loci induces animals to behave as if they were being "rewarded"; activation of other regions has a "punishing" effect; activation of still other parts of the brain is neutral with respect to such reinforcements. Some parts of the brain are clearly associated with appetite and other parts, physiologically linked with these appetitive centers, are clearly related to emotion. Both the appetitive and emotion systems seem to be bound up in what we mean by the terms "motivation" and "drive." A wide variety of conditioning experiments has revealed some degree of modifiability of at least certain of these central circuits. Central responses to standard sensory signals, for example, are notably different depending on whether or not the test stimuli are associated with other "significant" (rewarding or punishing) stimuli.

5. *Effects of commitment.* This section is based largely upon experiments conducted by the late Adelbert Ames, Jr. Although his work does not yet have a widespread influence in contemporary thought, this is undoubtedly relateable to the difficulty imposed upon the communication of insight into transactional mechanisms by means of languages built for the expression of linear cause and effect of relationships. Ames resorted principally to demonstrations to convey the meaning of his work. Ames's demonstrations, unfortunately, take quite as deliberate and conscientious study as do scientific papers. They are frequently mistakenly treated as if they were simple tricks and no more sophisticated than vulgar parlor magic. Nonetheless, the hypo-

theses and interpretations which were the reason for Ames's development of these demonstrations are among the most profound and far-reaching ideas concerning man's relations with the universe including himself and his fellow man. John Dewey wrote that Ames's work "is by far the most important work done in the psychological-philosophical field during this century—I am tempted to say the *only* really important work."

Ames's experiments deal with the perception of distance, objects, and motion, and with the sequential significances of objects in motion in relation to the observer and his capacity to anticipate the future course of such objects. He first demonstrates that the content of perceptual awareness includes striking contributions on the part of the observer. Such contributions are not obtainable from the objective reality of the present, but are based upon assumed familiarities which, in turn, depend upon the observer's experience with cues now recurrent in the present situation. Physiological events as well as object-originated cues are compounded in our perceptual awareness. Ames sought demonstrations of what we "subjectively" contribute to our perceptions of "something out there." Our past experiences contribute weighted information on what we have most usually experienced at times when we had similar sensory cues. Brightness, size, parallax, convergence and disparity are visual cues which contribute to assumptions about location and movement of objects. Characteristics such as hardness, malleability, resiliency, combustibility, etc. are assumed on the basis of more or less similar visual, auditory and somesthetic cues.

"We cannot reach an understanding of what the environmental situation does contribute to visual awareness if we think of the environment as an immediate, instantaneous existence indepedently apart from environmental situations earlier experienced by the observer or independent or environmental situations we might experience later." In encountering noncorrespondence between what we assume to be the reality of a given situation and our prediction of its consequences, we feel insecure as a function of the intensity of the discrepancy of cues. "Fundamentally, our feelings of security or insecurity as to subjective significances arise from processes below the level of awareness * * * These sensed feelings may be the original value judgments."

Ames's demonstrations show that we make instantaneous and complex adjustments to perceived cues in the direction of assuring correspondence between previous experiences and the present conscious awareness. For example, if you put on aniseikonic glasses and observe the room in which you are now sitting, you will instantaneously and automatically correct for the distortion imposed by the lenses in such a way as to maintain your percept of the room according to your previous experience with architectural symmetry. Through use of different lenses, different problems can be imposed upon your capacities to make the present percept conform to your previous experience. One pair of lenses, for example, can project on your retinae cues which correspond to the area of the ceiling being twice that of the floor, the walls sloping out with appropriate changes in the angles relating walls to each other and to the floor and ceiling. Other lenses can be made to cause a 30° slope of the entire room. Nevertheless, almost all adults fail to see anything discrepant about their percept of the room which is given "normal" room configurations. You may feel only mild unsureness about this complexly readjusted percept. After further experience with aniseikonic glasses it is likely that you may become able to perceive the room according to the veridical visual cues actually being presented to your sense receptors, that is, as distorted. The basis for your previous instantaneous and automatic adjustment of the incoming cues to fit your past experience lies with you, the observer, and not with the cues. A beginning schoolchild, having less experiential commitment with the usual architectual symmetry of rooms, will far more quickly perceive the distortion. Presumably an adult savage would similarly more quickly perceive the room correctly, as distorted.

If aniseikonic glasses which project an apparent slope to the environment are employed out-of-doors, the tilt may be recognized while looking at fields and rolling hills. But if the scene contains a lake, the tilt is automatically rejected in conscious awareness presumably because sloping water violates previous experience. If, instead of using aniseikonic lenses, a room is ac-

tually distorted, you will perceive the room as normally symmetrical. But then individuals in the room are perceived as distorted in size according to experience dominated assumptions which you make about the symmetry of the room.

Expectations as to future consequences ("sequential significances") have their impact on actions. If you are asked to bounce a rubber ball, a steel ball, or a golf ball, you will make appropriately different movements in order to catch the ball off the bounce. These movements are based upon previous experience with similar objects. If a ball of putty is substituted, you will not expect it to bounce. But if the ball of putty is made of a silicone compound known as "silly putty," malleable although it is, it will bounce much more briskly than a rubber ball. In order to perceive the nature of objective reality and to predict its "sequential significance" and the influence of your actions on the succeeding sequence of events, we automatically make a host of assumptions and predictions. The skill of an accomplished athlete depends on his ability to make accurately weighted assumptions on the basis of his extensive past experience, weighted particularly according to experiences most closely resembling the present.

Demonstrations and experiments of this kind indicate that an observer develops conscious awareness only of those aspects of his surroundings which "carry significances" to him. These significances sustain, extend, or vary the satisfaction of his purposes as these are carried out in action. "An observer's experiences have compounded into a 'subconscious' recognition that the significance of his environment is not disclosed by his perception of what and where 'objects' are but by the conseqquences of his purposeful behavior guided by his prehensions that he knows his perceptions of objects are only symbolic referents. * * * Learning occurs only in relation to events of which the learner has already had experience and only under conditions of noncorrespondence between his prehensions of sequential events and the sequences occurring." In other words, we admit to conscious awareness that which has meaning to us in relation to our purposes. We learn from those things admitted to conscious awareness which do not correspond to our basic assumptions. *Overcoming a noncorrespondence by inappropriate construction of past assumptions does not eventuate in learning new characteristics of the environment but in a lost opportunity for learning, and in a subsidence of the internally generated urge to establish correspondence.*

6. *Conversion of commitments.* About the turn of the century, G. M. Stratton, in a series of brilliant experiments, demonstrated that it is possible to induce rather complete conversions and reversals of committed patterns. Stratton's experiments have been widely replicated and extended since then but their mysteries are by no means plumbed. In brief, by means of lenses, it is possible to project on the retina an inversion of the visual field, or a reversal of left and right sides of the field, or a combination of both up-down and left-right reversals in the same pair of glasses. When you first wear such glasses there is considerable uneasiness and conflict, but after a matter of days of continuing experience, "correspondence" is restored! An individual can thereafter ride a bicycle, fence, play golf and perform other visually dependent activities with ease and skill.

In the process of getting used to an upside-down world, you begin by making elaborate conscious thoughts concerning the course and improvement of movements: you "know intellectually" that it is necessary to move your hand downward in order to reach a nearby object which your conscious awareness labels as above your line of vision, but your previous commitments dictate otherwise. Making the movement in the right direction requires deliberation at first; many inappropriate actions take place. Later, entirely appropriate movements are made smoothly and automatically. Still later, and after a further period of experience, you discover that there is no further conflict of cues: the perceived world is restored to its traditional "right-side-upness." Learning to adjust to a left-right reversal is more difficult, requiring severalfold longer experience. Learning to adjust to the combined up-down and left-right reversals is yet more difficult.

In these experiments all identifications of the outside world attaching to "thereness" have to be specifically relearned in all visual patterns despite the non-correspondence of this requirement with past visual, somesthetic and motor experiences. When the glasses are removed a

similar (but distinctly shorter) period of readjustment to the normal configuration is required. That is, soon after the glasses are removed, you cannot perform simple acts like riding a bicycle because of the new "noncorrespondences" between incoming cues and the assumptions built up during experiences obtaining while wearing the reversing lenses. The right-side-up world briefly appears "upside-down" once more.

The general principle involved here has been extended with roughly analogous results in relation to motion and color as well as form cues in vision, auditory localization reversals, transplantation of tendons and muscles, ect. Apparently man can accommodate certain kinds of conversion of long standing and elaborately inscribed commitments. This is not possible for the salamander, however. Visual field reversals in the salamander are inappropriately responded to in an enduring fashion. Presumably, somewhere between lower and higher vertebrates a certain degree of freedom for functional reorganization has been acquired.

7. *Interpretive formulations relating to these observations.* In this section we intend being deliberately speculative. To some extent we are discussing "a bag of cats and dogs." Nevertheless, the following tentative suppositions commend themselves for consideration:

(a) Not much of the nervous system is indubitably and inevitably committed in the beginning, not even the motor cells of the "final common path." The motor and sensory units become most firmly committed by virtue of the particular attachments they assume. The second and higher order units associated with them are less imperiously committed. Even circuits devoted to instinctual behavior are subject to some influence through experience. Neuronal mechanisms still more remote from direct input and output relations may be still more modifiable by experience.

(b) The commitment of motor and sensory units seems to be quite firmly established early in life. The commitment of second and higher order sensory and motor units appears to take place on the basis of the "sign" committed by particular muscle and sensory end-organs and also on the basis of the "pattern integrity" built up through coordinated sensory and motor experiences. Body image formation apparently occurs early in life and may last indefinitely.

(c) Other kinds of commitment take place in a more plastic, alterable form. The neuronal circuitry of this latter variety is still mostly a mystery and calls for further analysis. Apparently it is an endowment of higher evolutionary forms. Somehow experiences in perception, judgment and action are laid down in unconsciously operating patterns which contribute in a continuing way to conscious awareness and behavior. Past experiences are weighted according to the familiarity of apparent recurrences in the present and are closely bound up with one's aims and purposes. It is impossible to disentangle which parts of any ongoing perceptual experience are based on "objective reality" and how much is contributed from an unconscious synthesis of our present sensory cues, our previous experiences and our purposes. As Ames has established, we cannot be aware of the relative proportions of the working triad: outside object, conceptual content, and purposes, excepting through painstaking experimental isolation of the three interdependent components. This is the main business of science. But the pragmatic solution of "what works" and the universality of experience, which are so useful in most other areas of science and technology, are difficult to apply to human relations because of the requirement to obtain a full consciousness and consideration of the attendant assumptions which in turn relate to idiosyncratic individual and cultural experiences. The homogeneity of the "stuff" of contemplation of the physical sciences is wanting here.

(d) Internal processes of commitment occlude from our perception those things which are lacking in significance for us. This makes it difficult to perceive changes in our environment excepting in accordance with the extent of whatever familiarities attach to an otherwise novel situation; novel aspects of the environment need to be painstakingly apprehended, through familiarizing experience with the non-correspondences obtaining between our assumptions and forecasts on the one hand and what later happens. Learning depends entirely on our facing up to the noncorrespondences between our environment and our upbringing to that moment. Failure to reckon with noncorrespondences precludes our

learning anything or achieving diversified growth or departing from the dead center of our present limited existence. Failure to deal with noncorrespondences limits our grasp of nature and reduces our predictive capabilities upon which survival and well-being depend.

(e) Internal processes tend automatically to dispose of noncorrespondences through a process of internal misprehension of the non-familiar. It is as though the nervous system, having to work upon learning, lazily precludes acceptance of non-correspondences for as long as it can—that is, until failure of behavioral achievements, assignable to failure to find a mechanism for correspondence, enlarges our purposes to correct that defect. Misprehension results in an instantaneous and comprehensive oversight or systematic misinterpretation of all of the incongruent, noncorresponding cues. This failure is likely to occur whenever the incoming incongruent cues are weak or are dominated by familiar corresponding cues. This is especially likely to occur in a social context among persons having essentially the same background of experiences. There is a powerful tendency toward conformity in perceptions, judgments and actions resulting from the socially integrative purposes of being considered "normal" and of considering oneself a "normal." This tendency for social conformity powerfully reinforces our internal experience-endowed assumptions and forecasts.

(f) It is not only difficult to perceive noncorrespondences, but learning is entirely dependent upon our recognition of such noncorrespondence (consciously or unconsciously) within a framework of a purposive desire for survival and growth.

(g) It is evident that we are not only continually weighting assumptions according to our experiences and purposes but evidently we depend upon a continuing normalizing influence of sensory messages in order to maintain functional control over the relations between outside stimuli and our conscious awareness. Thus, sensory deprivation is followed by a relaxation of correspondence between "objective reality" and conscious awareness and, also, by a relaxation of the internal controls by which we make use of our conscious awareness.

(h) To continue the process of becoming, to increase our comprehension of the characteristics of ourselves and the physical and social universe around us, to learn, to create and to achieve anything new requires: (i) exposure to conditions leading to a noncorrespondence between our projections based on past experiences, purpose and evolving events; (ii) recognition by us of the non-correspondence (this implies that significance is attached to the non-correspondence with respect to our purposes); (iii) devotion of attention to those characteristics which specify the non-correspondence (this usually involves making predictions and producing ideas and actions which may be more or less appropriate); (iv) development in us of a new body of weighted assumptions now competent to correspond to the formerly non-correspondent features of the situation; and (v) feed-back of information respecting the appropriateness and inappropriateness of our newly adjusted predictions and actions. These requirements include specific insights fundamental to learning.

(i) The nervous system is built for action. This action is always expressive of a dynamic flux of purposes generated within the organism. The nervous system is integrated in the function of its parts and as a whole. Recurrent patterns of stimuli impinging from the outside may be represented and re-represented in central patterns, but always in conservative relationship to previously established ongoing patterns and in conformity to presently obtaining purposes.

Implications Relating to National Government and Diplomacy

Here, we intend to consider national survivability, adaptability and growth in the light of implications arising from our consideration of individual existences. The first and most obvious recommendation contributed by the analogy is the need to establish and identify integrated national purposes. The purposes of a nation, like those of an individual, are manifold and always in flux. Some are short-term and others long-range. National purposes demand purposeful dedication and responsibility among the nation's citizens and allies, public identification of individual and group accomplishments in the light of national purposes, and some means for the expression of national satisfaction in the

development and exercise of national capabilities. A wise nation, like a wise individual, has worthy goals, defined in terms of long-range potentialities. The ultimate achievement of any nation depends upon the creation of institutions which embody national ideals and upon the degree of aspiration and self-discipline exercised by its servants and its citizens. The pursuit of national excellence, like the pursuit of individual excellence, is an ennobling and liberating as well as disciplining enterprise.

A nation, like a man wishing to be understood, identifies and displays its purposes so as to aid others to understand and to anticipate appropriately its national actions. Narrow and negative purposes, and purposes inadequately projected, present handicaps to communication. This is especially important to bear in mind because purposes affect foreign as well as national perceptions, judgments and actions. National purposes expressed for export should be entirely congruent with those conveyed internally. As with an individual, the flux of national purposes contains conflicts. This may call for alternations of effort in support of conflicting short-term purposes and to keep the country moving in that general direction.

It would seem appropriate in a nation, as in an individual, to benefit by directing attention to noncorrespondences between national assumptions and predictions based on past experience and the actual sequence of events. As with an individual, discovery of such noncorrespondences is difficult. First, it is frustrated by the assumption that the behavior of other nations and national representatives will be consistent with our limited impression of their world-view, experience and purposes. Second, the discovery of noncorrespondences is frustrated by any national influence in the direction of national conformity of views. The goal in attending to noncorrespondences is to become as fully aware as possible of all characteristics of objective reality. We need not be blind, nationally: but this requires a positive policy of cultivating persons able to perceive, and encouraged to perceive, noncorrespondences between our national percepts, ideas and actions and the objective events of history. There is a positive national need for persons who understand the previous experiences and purposes of other nations so thoroughly that they are able to make better predictions than those based on our own national sets of assumptions and purposes. It takes positive national purpose to be receptive and responsive to evidences of noncorrespondence.

A third requirement is for abundant and spirited activity on a national scale; activity which acknowledges the inevitability of change; activity which recognizes the processes of *becoming* on a national scale. Our nation should have the zest to utilize the potentialities offered by change rather than to suffer passively from the limitations imposed by change. National activity should correspond with the evolution and achievement of national purposes, to the perception of "national self" and the "national self" in relation to the rest of the world. National activity should reflect the evolution and adjustment of national perspectives, concepts and ideals. National activity should be balanced by rational insight into the fact that it is impossible to succeed one hundred percent of the time, that it may be more deadly to be afraid of making mistakes than to make them. There needs to be a national willingness to act tentatively, to utilize what turns out to be inappropriate action as a key to growth, to be internally and externally candid about misdirected and incorrect interpretations and actions and, on occasion, to withhold action in favor of a theoretical testing out of alternatives in the search for a more desirable course of action. Such a trial of alternatives can yield insight into more dimensions than can be discovered simply by the traditional techniques of predication.

We need a national scheme of working assumptions based upon objectively evaluated past experiences projected realistically into the future, weighted according to present and projected needs of the nation. This scheme should be open-ended, plastic and modifiable. It should be as objectively oriented to reality as possible through a continuing testing against events as they take place. Such a scheme will reveal many non-correspondences but should not itself be considered infallible. The chief difficulty lies in keeping a scheme of working assumptions sufficiently dynamic and future-oriented. The only safeguard resides in having institutional mechanisms for objectifying information concerning outside relations. This is improved by maximiz-

ing international transactions. I suppose that a nation can suffer from isolation from the rest of the world in ways analogous to an individual exposed to sensory deprivation. In such circumstances, we might expect patterns based on past experience to interfere with incoming signals and with the objectification of national self-awareness.

Science can play a valuable role in relation to national security and welfare according to this scheme. First, it is the professional business of science to discover noncorrespondences. Science advances on the basis of the discovery of discrepancies between present limited concepts and the behavior of nature. The techniques of science are directed toward the objectification of these discoveries and their interpretation and application in the largest and most general sense.

Second, science characteristically builds models and makes internal trial-and-error exercises. Sometimes these are entirely mental processes; they may involve elaborate equipment. By means of experiments, scientists attempt to grasp the significant relations of natural phenomena without having to replicate nature in its entirety. Science can play an important role in the testing of alternative perceptions, judgments and activities and in the selection of techniques prior to the undertaking of expensive and committing national actions.

Third, science continually practices the development of schemes of weighted assumptions which are as far as possible generalized to include all sense-experiences, both past and future. These are open-ended schemes of science, always accessible to new evidence of non-correspondence.

Fourth, as a system of thought, science demands a high degree of intellectual freedom, is not imposed by coercion or persuasion, and is not destroyed when found internally inconsistent. Paradoxically, science becomes stronger and more coherent as its limited views are made manifest. The search for Scientific Truth (which can never be realized) becomes increasingly powerful as error is discovered in lesser scientific truths. A lie in science cannot persist for it will be found out through the continuing activities of science.

Fifth, science has already outstripped all other forces affecting business, law and politics. In the near future, equally powerful influences will be realizable through biological, psychological and social insight. Just as man learned through invention and, later, through science and technology that he did not have to submit to nature "in the raw," he will soon find that in areas of behavioral barbarism and international anarchy, science can equivalently liberate man from dependence upon chance and fate. Society has much to learn as to what science is not, what it cannot do, and what are the conditions that science requires. But, in competition with what science can do, traditional modes of action are clearly inadequate. The competition of a scientific society cannot be met entirely by recourse to tradition.

Within the last few years there has been an intellectual convergence among many scientific disciplines: psychology, the social sciences, cybernetics (including techniques for operations research and systems analysis), ethology, embryology, neuroanatomy, neurophysiology, neurochemistry and pharmacology. Studies involving these disciplines have rather abruptly begun to contribute meaningfully to one another. Each discipline represents a corps of scientists striving hard to learn truths about processes which overlap the other disciplines. Each has much to contribute toward the resolution of our most urgent national needs. Reciprocally, the congeries of science need the stimulation of high national purpose.

Parallel with the convergence of research devotion among several related disciplines has been the establishment of institutions dedicated to their support as conjoined enterprises and of individuals trained in the no man's land between previously isolated disciplines. The entire atmosphere is one of rapidly changing long-range perspectives. What is confidently to be anticipated from these trends is the development of a more inclusive and coherent view of human relations. But as compared with the magnitude and acuity of the great problems facing mankind, the present effort is far too little. This is one non-correspondence which needs to be recognized on a national scale and to be followed up in purposeful action. We hope the effort will not much longer remain too little and will not be augmented too late. Time and intellectual manpower and other resources must be increasingly resolutely devoted to progress in understanding human relations and international relations in all

their dimensions: progress toward the solution of our most urgent national problems depends upon conceptual advances peculiarly susceptible to discovery through the activities of science.

General Program Considerations

Administration means ministration to. It connotes a stewardship rather than an option of power. Its accomplishments are to be measured in terms of services rendered toward the achievement of the larger objectives of the organization. There is no significance to the measurement of administrative accomplishments in any other terms. The evaluation of administration must be made according to progress the organization makes in the direction of its long-range goals and not according to the degree of conformity to administrative principles considered apart from this context of mission. Too easily we fall into the habit of following the "right procedure" at the expense of seeking the "right result."

These principles seem self-evident; but like many fundamental notions they are so general as to be readily overlooked in practice. Their neglect leads to cynicism that that administration is best which administers least. This is not true. The truth is that that administration is best which administers best. It is easier to appreciate this fact on a relative scale, through comparing one administration with another or with an ideal conception of an administration.

Administration needs continually to be strengthened and revivified in courage and ingenuity in moving the organization toward the fulfillment of its long-range objectives. Administration is not an equilibrium-seeking device. It needs to anticipate and prepare for what the organization is going to require and not simply respond to emergencies when these arise.

Specifically, this means, in the intramural research programs of the National Institutes of Health, that we must arrange our resources of personnel, time, space, and equipment so as to yield the most significant advances toward a new and more comprehensive level of understanding health and disease. We need to attract and sustain examples of the best minds in each of the cogent fields of biomedical knowledge and research. We need to insure that they will be able to recognize that this environment is a community of scholars brought together for the purpose of an essentially intellectual endeavor, that the overriding administrative as well as professional priority is for creative persons to address themselves to the solution of broadly conceived biomedical problems. If this is not obviously recognized as the highest priority goal, and if it is not clearly demonstrated in all administrative actions, we are in need of radical overhauling. Not everyone needs to be a fullblown genius to participate worthily in the accomplishment of this mission; geniuses are only too rare. *But the creative genius is the indispensable ingredient of any organization that aspires to bring forth new knowledge. Highly creative persons are likely to be attracted wherever there are the greatest opportunities for intellectual growth and development. This means that the National Institutes of Health must provide an environment clearly equal to or better than that of any other environment in the competition for creative talent. Even more appropriate than this competitive goal is the aim to achieve an ideal research and educational environment—through deliberate pioneering in the design of self-perfecting administrative patterns within which science is enabled to flourish.* This is why the Assembly of Scientists and an Academic Senate may prove invaluble in the long run. In the last few years, the National Institutes of Health have been looking outward administratively and striving to produce such facilitative devices through its extramural activities. In the course of this worthy objective, the Institutes have relatively neglected the comparative standing of their own research programs in Bethesda.

A more adequate understanding of biomedical problems cannot be achieved in the abstract; it is brought about through the consideration of materials with which the creative scientist is already familiar. Even the most gifted and energetic person must acquire and maintain a mastery in the field of his professional endeavors. This is why the educational and Sabbatical Leave programs at the National Institutes of Health are so vital. The scientist must have a keen sense of what needs to be done and command the skill to solve a given problem. He must carry out his program of thought and action at the limits of conception. And he must then follow through

by communicating his new level of understanding in a clear form.

Each significant step in the development of a new concept is bound to seem alien and eccentric. Intellectual nonconformity is an indispensable part of the creative process. Moreover, the processes of creative accomplishment require from the scientist not only a thorough understanding of the problem, internal discipline and devotion, but also the nimble exercise of combinatory play of abstract imagery. Abstract thinking in turn is much affected by the individual's attitude of identification with his working environment, his peers and his leaders all the way to the top of the organization. Truly creative contributions to science cannot be squeezed out as paste is extruded from a tube, simply by manipulating technical materials. Discipline of the creative progress must come from within the individual: coming from outside, discipline of the creative process can only be provided by example.

A few outstanding investigators effectively engaged in research and exhibiting general professional satisfaction with their working environment can go a long way toward providing the entire research establishment with a professionally magnetic aura of excellence. If for any reason, the most gifted scientists in an institution have quit working, are uncordially supported in their research activities, or are seriously thinking about leaving, that institution's professional reputation is heading for wreck and ruin. No amount of administrative hustling or environmental luxury can induce or stem a tide which actually ebbs and floods entirely on the basis of intangible professional values. This is why it is necessary that the professional values and the intellectual processes essential to research are guaranteed an enduring recognition and support by being embodied in institutional form. It is in this way that "ideals become great historic forces." Representation of professional views through an Assembly of Scientists or Academic Senate, revivification of professional skills through worthwhile educational programs and Sabbatical Leave, professional recognition through the awarding of a positive and continuing commitment of Tenure are means by which increased professional responsibilities are being placed in the hands of scientists at the National Institutes of Health. These institutional forms provide the best means for protecting administrators against outside intervention on the part of persons who may lack an understanding of the conditions essential for the success of any scientific enterprise.

The End of the Combined Basic Research Program, NIMH–NINDB

In 1951, Dr. Seymour S. Kety became the first Scientific Director of the National Institute of Mental Health. Shortly thereafter he assumed a parallel responsibility for the research program of the National Institute of Neurological Diseases and Blindness. Dr. Kety did a noble job in making initial assumptions upon which a strong, multidisciplinary program could be developed, and in carrying this design through to fulfillment by a highly successful program of recruitment for senior scientists. Because the obvious distinctions between clinical mental health and clinical neurology programs do not obtain in relation to the nonclinical studies, Dr. Kety established a combined Basic Research Program for the two Institutes.

By the autumn of 1959, when I followed Dr. Kety's example in requesting relief from administrative work, there was considerable momentum behind an administrative impulse to obtain organizational symmetry among the seven Institutes. It was therefore decided that, at the time of my relief, each Institute would appoint its own Scientific Director and each would support its own independent Basic Research Program. It was reasoned that having worked together for 10 years and having the prospect of sharing a new NIMH–NINDB laboratory for research building 4 years hence, scientists of the formerly combined Basic Research Program would be able to continue whatever worthwhile interdisciplinary collaboration had been established.

The program division was undertaken in the summer of 1960 as follows: The Laboratories of Biophysics and Neuroanatomical Sciences and the Section on Lipid Chemistry in the Laboratory of Neurochemistry were assigned to NINDB. Dr. G. Milton Shy accepted the newly created position of Associate Director for Intramural Research (Scientific Director), NINDB, in August of 1960. The Laboratory of Neurophysiology is to remain a combined laboratory, sup-

ported by both Institutes until the new laboratory research building is completed about 4 years hence. The Laboratories of Cellular Pharmacology, Psychology, Clinical Sciences, Socio-Environmental Studies, Neurobiology, The Addiction Research Center, and the Section on Physical Chemistry in the Laboratory of Neurochemistry were assigned to NIMH. Recruitment continues for an Associate Director for Intramural Research (Scientific Director), for NIMH.

I wish to take this opportunity to express to those for whom and to whom I have been responsible my sincere appreciation for the privilege of serving four exciting, enjoyable and thoroughly busy years.

Sed haec prius fuere.
(Gaius Catullus)

Laboratory of Neurochemistry[2]

As of the end of June 1960, the Basic Research Program, NIMH–NINDB *per se* was dissolved and such programs independently established within the respective Institutes. The Laboratory of Neurochemistry having been a dual-Institute operation, i.e., the Section on Lipid Chemistry under the direction of Dr. Roscoe O. Brady being the NINDB-supported facility and the Section on Physical Chemistry under the leadership of Dr. Sidney Bernhard conducting research through NIMH support, was one of several affected by this transition.

Having been acting chief of this Laboratory for over three and a half years I am impelled by my esteem to express sincere admiration and respect for each member, both past and present, of the Laboratory for individual intellectual endeavors and the high quality of research performed. Because of the scarcity of space, this Laboratory was precluded from developing beyond its initial phase and operated under extremely difficult conditions, Despite this, both sections through research accomplishments are recognized nationally and internationally in the field of medical research and have added to the prestige of their respective Institutes and that of the National Institutes of Health.

[2] Prepared by Robert B. Livingston, M.D.

Physical Chemistry

The polynucleotide complex of polyinosinic and polycytidylic acid is known from previous work in this Section to give a diffraction pattern resembling that of natural ribonucleic acid. Further investigations have shown that it is possible to produce a crystalline form of this material. X-ray investigations have demonstrated that the structure of the crystalline form is very similar to that of deoxyribonucleic acid. In addition, it has been possible to transform the noncrystalline form into the crystalline form in the fiber. This evidence indicates that the structure of the natural ribonucleic acid is, in its ordered regions, two stranded in form with hydrogen bonded base pairs similar to those in deoxyribonucleic acid. Large single crystals have been grown of the proteolytic enzymes, chymotrypsin and nagarse. An investigation of the properties of the nagarse shows that it is possible to proceed further into a detailed investigation of the molecular structure of this material.

Perhaps the single most important discovery in the work on aggregation of dyes bound to polyanions is that the structure of a polyanion determines in part the strength of dye-dye interaction so that the color of bound dye can tell us about the polyanion to which it is bound. We have collected a considerable body of data which supports the generalization that the more rigid and well ordered the polyanion, the weaker the dye-dye interaction. For example, all samples of native, well ordered deoxyribonucleic acid (DNA) examined show the same low value of this interaction, but when they are disordered by heat denaturation, the strength of the interaction increases. This observation has been developed to the point where the color of bound dye can be used to determine the degree of nativeness of DNA specimens. Applying this general principle to structural changes in synthetic nucleic acids it has been possible to confirm certain hypothesized transitions. A quantitative method of analysis in the microgram range for nucleic acids, mucopolysaccharides, and synthetic polyanions has been developed. A detailed study of the interaction between dyes bound to plant, animal and synthetic acid polysaccharides has been completed. As a result of this work it is now possible to differentiate among them as well as

to relate the observed differences in the staining properties of these polymers to the type of binding site, and their number and relative locations on the sugar moieties.

In the study of the synthesis and properties of enzyme models the testing of detailed theories of enzymic catalysis by chemical synthesis of model compounds and studies of their catalytic and chemical properties is being carried out. Ultimately, via such investigations, to synthesize a model enzyme.

A theoretical approach to the problem of sequence determination in proteins is being studied. The major block to rapid development of this field is that the experimental determination of the amino acid sequence in even a single protein is an extremely difficult and laborious affair. The choice of methods of protein fragmentation, average size of peptides, selection of peptides to be analyzed, and amount of sequence data to be obtained from each peptide as well as the method for using the data collected in reconstructing the overall sequence are all a matter of choice. We have been examining this problem from a logical-mathematical point of view in an effort to find an experimental design which will minimize experimental effort and an information processing system which will utilize experimental data optimally.

Investigations are being carried on to produce and investigate natural variants of a proteolytic enzyme. In order to examine the enzymatic mechanism and substrate specificity of enzymes, it would be extremely valuable to have available different forms of the enzyme having altered catalytic properties and specificity. By selecting for mutant forms of a bacterial enzyme, it is hoped that a large number of such variants can be produced.

Laboratory of Neurobiology

The Surgeon General, on October 18, 1960, approved the establishment of the Laboratory of Neurobiology within the Basic Research Program of the National Institute of Mental Health.

This Laboratory will conduct research on the brain using mainly neuroanatomical, neurophysiological, biophysical and behavioral techniques, in the search for an improved understanding of perception, learning, memory, judgment and other complex functions. Specifically, studies will be carried out on:

Mechanisms involved in the central control of sensory transmission;
Mechanisms underlying the prolonged effects of certain sensory stimuli;
Mechanisms of integration between sensory and motor pathways; and
Chemical and metabolic linkages related to electrophysiological mechanisms of nerve conduction.

The following joined the staff of this Laboratory:

Dr. Bo E. Gernandt, Visiting Scientist, continuing his fruitful investigations of spinal, cerebellar and brainstem mechanisms underlying posture and locomotion. Since 1958 and until joining this Laboratory, Dr. Gernandt had been affiliated with the Laboratory of Neurophysiology under the leadership of Dr. Wade H. Marshall.

Dr. Muneo Shimamura of the Department of Physiology, Hokkaido University School of Medicine, with wide experience in brainstem mechanisms controlling motor functions, currently a visiting scientist, is collaborating with Dr. Gernandt.

Dr. Arnold Starr, Research Associate within the NIH Research Associates Program, having completed a two-year residency in Neurology under the aegis of Dr. Derek E. Denny-Brown, is studying mechanisms underlying prolonged effects of sensory stimulation.

Mr. Gabriel Frommer, Commissioned Officer in PHS, a candidate for the Ph.D. from Brown University, with experience in thalamic responses to taste stimulation under the tutelage of Dr. Carl Pfaffmann, is investigating mechanisms involved in the central control of sensory transmission.

Mrs. Rosalind Marimont, Theoretical Mathematician, with twelve years' experience in the theory of higher forms of mathematics and computer logic, is studying the formal requirements for pattern recognition.

The Laboratory has been in existence for so short a time that no progress can be reported. Until additional space becomes available we will

not be able to begin work on the chemical and metabolic mechanisms relating to electrophysiology.

Laboratory of Neurophysiology

Investigators in this Laboratory are working on a great variety of fundamental problems of the nervous system. Problems range from the initiation of the nerve impulse to social behavior of the South American Squirrel Monkey (Saimiri sciureus). Material used extends from the "simple" plant cell of Nitella, which reacts with an action potential resembling a nerve cell, to the whole brain of the monkey.

The Section on Special Senses, led by Dr. Ichiji Tasaki, has continued work of fundamental importance on the analysis of the excitable membrane. The squid nerve has been exploited with most ingenious experiments. Tracer work with experiments designed and interpreted on the basis of irreversible thermodynamics was done on both animate and inanimate systems. It was found that the squid nerve membrane was far more permeable to cations than to anions. This fact was related to the fixed negative charge hypothesis originally advanced by Michaelis and Teorell (the latter was a collaborator in this research in 1959). This theory predicates a membrane containing internal negative charges which should tend to attract cations. Following up the consequences of this concept, Dr. Tasaki hypothesized that divalent cations such as Ca^{++} or Mg^{++} should abolish a prolonged action potential (upper stable state of Tasaki) and that the reaction should be endothermic.

In a most effective series of experiments, Dr. Spyropoulos, using the single node preparation of the toad nerve, produced conclusive evidence for such a theory. Delivery of a small amount of Ca^{++} ion to a node in the upper stable state immediately returned it to the lower stable state, i.e. abolished the action potential and returned the membrane to the resting state. Since the displacement of K^+ ion by Ca^{++} is an endothermic reaction, it was hypothesized that sudden heating of a node would accomplish the same effect. This was found to be true. Conversely, release of Ca^{++} ion from the negative sites in the membrane should be exothermic and a sudden pulse of cooling was found to initiate an action potential in a resting membrane. This is in impressive agreement with the theory, or at the very least, constitutes reasonable ground for further work along this line of approach. According to Dr. Tasaki's theory the membrane in the resting state is locked in because the divalent Ca^{++} ions successfully compete with K^+ ions at the negative sites in the membrane.

The movement of Na^+ and K^+ ions which accompanies and follows the action potential has been further studied with tracer techniques using the fractionator. The fractionator was first introduced by these investigations in 1959. This important tool has been further developed and now occupies a secure place in the analysis of the nerve impulse. With this device it is possible to get time distributed samples, with a time resolution as short as 25 msec., of tracer bearing wash fluid from a segment of nerve. By prolonging the nerve action potential it is theoretically possible to ask the question: Is there really a time succession of Na^+ flowing in and K^+ flowing out of a nerve during the rising and falling phases of the action potential? Results so far obtained indicate that both K^{42} and Na^{24} flow out of the axon both at beginning and end of the action potential. Further development of the fractionator toward shorter time resolution and greater sensitivity is proceeding. These experiments have been extended to a primitive plant cell (Nitella) which also exhibits an action potential.

These researches have gone far to elucidate general properties of excitable membrane and to permanently change our conceptions of the nerve impulse.

Three visiting scientists, Dr. A. Arvanitaki-Chalazonitis, N. Chalazonitis from France, and Dr. T. Oikawa from Japan collaborated in these researches at the Marine Biological Laboratory, Woods Hole, Mass.

The Section on the Spinal Cord, led by Dr. Karl Frank, has continued in important work on fundamental analysis of the nervous system. The investigation of the roles of the principal parts of the neuron, axon and axon hillock, soma and dendrites has been extended. The technique employed (described in last year's report NINDB–NP–SC–3) permits activation of a single neuron in a large population. This allows examination of the electric field around the neu-

ron. It was found that the fields for the two main features, the A and B spikes, were not coextensive and further that the B spike was followed by a positive afterpotential. Dr. W. Rall, Office of Mathematical Research, NIAMD, has been a valuable collaborator on this project. His computations of external fields of this type not only are substantial creative contributions, but are also in excellent agreement with the experimental data.

Dr. T. Oikawa, visiting scientist, has investigated a large nerve cell in the goldfish. The data strongly suggest that the antidromic impulse does not invade the soma and dendrites. No evidence was seen of separate A and B spikes common to many other types of cells.

Drs. P. G. Nelson and S. D. Erulkar have been investigating basic mechanisms of hearing in the auditory system at the collicular and geniculate level in the brain. They have observed both inhibitory and excitatory processes in the same cell depending on which ear was stimulated. One of the fascianting problems of hearing is the fact that the human can discriminate time differences of 10μsec "between the ears". Studies of this type will eventually explain how times of arrival to the two ears can be analysed to this level of precision.

Drs. Frank and Nelson have undertaken an ambitious but not unrealistic study of the fundamental mechanisms of learning. One of the hypotheses of learning involves the anatomical and functional organization of synaptic connections. In this project methods are employed which permit long time activation of certain pathways to the spinal cord neurons. After periods of time, the physiological characteristics of neurons so activated will be compared with control neurons on the opposite side of the spinal cord. The time has come for such developments and Dr. Frank has other projects under consideration in this field of study. It is particularly pertinent that this Section go into this area of research because of the broad and detailed experience acquired in the past decade.

The Section on Membrane Physiology's program is directed into the general area of the ubiquitous excitable membrane and is currently investigating muscle fibers. The surface membrane of both nerve and muscle fibers is 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 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. The work of the Section on Membrane Physiology is to investigate these intriguing fundamental speculations.

The Section on General Neurophysiology has been involved in intracellular analysis of the pyramidal cells of hippocampus, patterning of somatic sensory impulses in cortex and thalamus, work on direct electrical initiation of the cortex and correlation of slow electrical changes and pH of the cerebral cortex.

The hippocampal project has been pursued in a brilliant and energetic way by Drs. Eric Kandel and Alden Spencer for a period of over 2 years. They have worked out important constants of the pyramidal cell and secured much information on synaptic activation and reaction properties of the pyramidal cell. This work was extended to a study of convulsive seizure patterns. Drs. Kandel and Spencer have received unusual recognition for men of their age. They have been invited to famous laboratories and have also accepted invitations to present their work in a symposium to be published in "Epilepsia", and a presentation to a symposium entitled "Current Problems in Electrobiology" conducted by the American Association for the Advancement of Science and the New York Academy of Sciences.

Dr. Barbara Renkin has proceeded with analysis of sensory patterns, relating cortex and thal-

amus. This academic year she is a Fellow of the National Science Foundation and is on leave from this laboratory and is working with the distinguished neurophysiologist, Professor Granit at the Karolinska Institute in Stockholm.

Mr. Anthony Bak is developing a brilliantly designed averager for retrieving data from noise. The final model is not yet completed, so final judgment cannot be made at this time. It will be used by Drs. Frank, MacLean and others. Mr. Bak has also given valuable collaboration to several projects in the laboratory. Among these is his introduction of the cathodyne, which was crucial in Dr. Spyropoulos' project on the control of nerve impulse in single fibers by chemical and thermal manipulation.

Other work in this section is proceeding on specific relation of pH changes in cortex and spreading cortical depression of Leao. There is also a general project underway on cortical reactions involving analysis of dendritic and soma activity using implanted electrodes.

The Section on Limbic Integration and Behavior, led by Dr. Paul D. MacLean, is very busy with important contributions to the general area of brain and behavior. The squirrel monkey from South America is the principal experimental animal used by this laboratory. Further developments of techniques for employment of this monkey have been made and the stereotaxic brain atlas (project M–NP–LI–9, 1959) is approaching completion. The work of this section uniquely combines studies of neuroanatomy, neurophysiology and behavior.

Drs. Gergen and MacLean are proceeding with general physiological anatomical analysis of the hippocampus. Under chlorolose anesthesia they have found visually evoked reactions. Search for representation of other exteroceptors in this primitive part of the brain will be made.

The study of convulsive reactions in the hippocampus and effect of these reactions on other parts of the brain and, in turn, on total behavior has been continued. It is known that the hippocampal part of the limbic system is peculiarly sensitive to convulsions and convulsive like reactions. These studies of mechanisms of the total system reactions are of great importance for understanding brain and behavior.

Work on localization of genital function has been continued with the monkey. Dr. MacLean is the first investigator to systematically demonstrate and study the direct physiological and anatomical loci in the brains of animals, electrical and chemical activation of which produced specific sexual responses. This important work is proceeding and an entirely new chapter is being developed in knowledge of brain. As an important example, it has been found that electrical stimulation of the anterior thalamic group produces penile erection. This is specific for the medial part of the medial dorsal nucleus. This nuclear group is directly connected to the phylogenetically new frontal lobes. This constitutes the first proof of a specific anatomical and functional relation of the frontal lobes and a primitive function of the "old" limbic system. This mechanism probably explains some of the behavioral changes observed in patients after frontal lobotomy.

As an important corollary to all work in this section are studies of individual and social behavior of the squirrel monkey.

The senior scientists in the laboratory have received many invitations to present their work at scientific and educational meetings. This shows that the work of the laboratory is respected and is at the very least accepted as well as can be expected of current research.

Laboratory of Cellular Pharmacology

The work of the laboratory in 1960 has continued along the four main topics which were discussed in the summary report of 1959, namely: (a) mechanisms and pathways of protein biosynthesis; (b) biological methylation; (c) biological oxygenation; and (d) alkaloid biosynthesis. The protein synthesis project may be regarded as the main project of the laboratory at the present time.

The studies on the mechanisms of protein synthesis as well as the determination of the structure of proteins as specified by the sequence of amino acids in the peptide chain are generally considered one of the outstanding problems in biology today. Until recently a relationship between ribonucleic acid and protein synthesis was predicated largely on indirect grounds, but in recent years a more direct and compelling basis for postulating a strict relationship stemmed from the discovery that a special small molecular

weight RNA (S-RNA) may be directly involved in protein synthesis as an acceptor of activated amino acids and as a donor of the amino acid moiety to proteins. A concentrated, and presumably long range, effort directed at the elucidation of the chemistry, molecular configuration and biological characteristics of S-RNA appeared therefore rather attractive as an approach to the central problem of the relationship between the nucleic acids and protein synthesis. In particular, since the S-RNA exhibits marked specificity in its acceptor function towards different amino acids, it appears that elucidation of the basis for this biological specificity might represent a major advance in our understanding of a biological "coding" mechanism.

The experimental problems which need to be solved before the structure of S-RNA can be elucidated are very numerous and challenging. Nucleic acid chemistry is still in a rather primitive state and satisfactory techniques for separation of different species of nucleic acids are most rudimentary. Furthermore, specific methods of degradation which have proved of paramount importance in the determination of the amino acid sequence in proteins are almost totally lacking. Finally, techniques for separation and identification of fragments from the degradation of nucleic acids are quite inadequate. The problem, therefore, requires a long and patient search for new techniques and/or new applications of existing methods to the nucleic acid problem. No single major breakthrough may be expected, but it is anticipated that over a period of years these technical problems will be gradually overcome and allow answers and resolutions of the central question. The laboratory's efforts have been directed toward all three of the main areas outlined above, namely, towards purification of nucleic acids, toward the study of specific and nonspecific hydrolysis methods, and towards identification of nucleic acid fragments and good progress has been achieved in all fronts. (See CP 14, 16, 23, 26.)

The study on biological methylation continues to occupy the interest of the laboratory, both in the Section on Proteins and in the Section on Alkaloid Biosynthesis, and in fact, also in the Section on Cellular Regulatory Mechanisms. In the Section on Proteins, we have continued work on the enzymatic biosynthesis of methionine. The enzyme thetin-homocysteine methylpherase has been the subject of extensive study in the laboratory for a number of years. This protein has been considered to be of special interest because of its ability to readily undergo an irreversible transformation in which monomer units are united by disulfide bonds to form a continuous and defined mixture of molecular species with from one to more than 100 monomer units per molecule. Electron microscopy studies led to the suspicion that the monomer unit itself might well be made up of smaller subunits which, in fact, were revealed by treatment of the thetin-homocysteine methylpherase with relatively low concentration of organic detergents. The present evidence suggests that thetin-homocysteine methylpherase monomer indeed consists of three presumably identical subunits of molecular weight approximating 60,000. This finding is in line with recent suggestions by Kendren that the molecular weight of a single protein unit may never exceed the size of 50 to 100,000, all larger proteins being composed of subunits held together in highly specific three dimensional configuration. The biological function and significance of the enzyme thetin-homocysteine methylpherase remains the object of speculation, particularly in view of the discovery in this laboratory of another liver enzyme, betaine-homocysteine methylpherase, whose more natural substrate specificity suggests it plays a leading role in the physiological synthesis of methionine from betaine-homocysteine.

The Section on Alkaloid Biosynthesis, now in its second year of existence, has continued studies on the biosynthesis of the alkaloid N-methyltyramine, hordeine and gramine by cell free extracts of barley or millet. The methyl group of these compounds is donated by S-adenosylmethionine. Several other compounds which have been suggested in the literature as possible methyl donors are inactive in this system. These findings were extended in a series of experiments in which it was shown that barley can synthesize S-adenosylmethionine identical to that found in vertebrate even to the extent of having the same stereochemical configuration about the asymmetric sulfur and oc-carbon atoms. Together, these facts indicate that the predominate pathway of plant transmethylation does lie

through S-adenosylmethionine just as it does in vertebrates and microorganisms.

These findings illustrate once again the value of studying fundamental enzymatic mechanisms in whatever biological material is most convenient with the assurance that the facts in a given form may well apply to widely divergent species. Although transmethylation is apparently extremely important for all forms of life, the enzymatic mechanisms at work here are imperfectly understood and it is hoped that further work with botanical systems may clarify details of the process.

A matter which requires further exploration is suggested by the structural resemblance of two of the particular plant alkaloids studied to the adrenal hormones of mammals and of a third to mammalian serotonin. If the role, as yet unknown, of these compounds in plant metabolism can be elucidated, we may gain thereby an important lead to discovering the role of these neurohormones and of chemically related hallucinogenic materials.

The formation of the alkaloids now being studied is known to be under not only genetic control, but under other controls as well, so that the formation occurs in a dramatic outburst at a specified stage of ontogenesis and in restricted types of tissues. It seems not unlikely that a study of the interplay of the control mechanisms which are at work here will give insight into the important question of how enzyme formation and activity is governed in higher organisms. The genetic, environmental, tissue specific and hormonal factors cooperating in this system are undoubtedly complex but it is hoped that the relative ease of experimental control of the plant will aid considerably in work on this question.

The Section on Cellular Regulatory Mechanisms continued its fundamental studies of biological oxygenation. The reactions which have been under investigation, namely, the hydroxylation of phenylalanine and of DOPamine present interesting similarities. Both reactions require, in addition to various and specific enzyme fractions, vitamin coenzymes which play the role of electron donors in the reaction in which they participate. Although the biochemical mechanisms of both hydroxylation reactions may be similar, the vitamin cofactors which are involved in these reactions are different, namely, a pteridine compound analogous to, but not identical with, tetrahydrofolic acid functions in the phenylalanine hydroxylation while ascorbic acid plays a role in the DOPamine hydroxylation reaction system.

Earlier work from Dr. Kaufman's laboratory has established that reduced pyridine nucleotides were necessary for the enzymatic conversion of phenylalanine to tyrosine. The participation of *reduced* pyridine nucleotides in an *oxidative* reaction was an intriguing aspect of the problem which has now been answered since it has been shown that the function of the reduced pyridine nucleotide is to maintain the pteridine cofactor in its reduced tetrahydro form.

The significance of Dr. Kaufman's studies on the enzymatic conversion from phenylalanine to tyrosine to the pathogenesis of the disease of the oligophrenia phenylpyruvica is self evident since this disease is characterized by a genetically determined inability to effect this conversion. One of the difficulties which has been hampering progress in studies of the relationship between the metabolic defect and the physiopathology of the disease has been the unavailability of an experimental animal showing this disease. With the goal of finding such an animal, several long term experiments have been initiated. In one direction, it has been found by Dr. Kaufman that a genetic strain of rat (developed by Kretch) which shows poor maze performance also has lower levels of phenylalanine hydroxylase than in a control strain of animals. There are indications that some of the physical properties of this enzyme may actually differ in these two strains being less stable *in vitro* from the behaviorally inadequate animals. In another approach, Dr. Kaufman and Dr. Goodfriend have kept weanling rats on high phenylalanine diets, an environmental situation which mimics that faced by the phenylketonuric infant. After several months on this diet the animals are tested for maze performance. Preliminary results indicate that rats kept on this diet give a definitely poorer maze performance. It may prove to be possible, through either of these lines of investigation, to produce a "mentally retarded" experimental animal.

Studies on the biosynthesis of norepinephrine are of particular relevance and complement beau-

tifully the studies of Drs. Mudd and Mann in the Alkaloid Section.

Dr. Kaufman is spending a year in the Laboratory of Prof. Ernst Hadorn at the University of Zurich, exploring the general problem of cellular differentiation, particularly in relation to the regulatory role of the gene or gene product in ontogenetic development. The project on genetic and developmental studies on tryptophane pyrollase in drosophila melanogaster is a clear indication that he is making rapid progress in this endeavor and deriving from it great benefit and stimulation.

In 1960, the Laboratory enjoyed the collaboration of Dr. Claude Blanc, formerly of Marseille University; Dr. Maxine Singer, NIAMD, Dr. Theodore Goodfriend, formerly with the Endocrinology Branch, NCI; Dr. Peter Weiss, NIMH; and Dr. C. W. Clancy, University of Oregon.

Technical Development

In the calendar year 1960, the Section on Technical Development has completed approximately 350 projects in support of the Basic and Clinical Research Program, NIMH–NINDB. Some of these will be itemized later in this report. The majority of work has been in response to direct requests by the investigator, while some originated internally. Other functions and services performed by the Section will be outlined, along with a few words in regard to the future operations. Needs will be mentioned along with how they have been met or should be met.

In line with the recently emphasized policy of making "Excess the First Source of Supply," this section has worked closely with Mr. Paul Schenk of the Property Utilization Unit in an endeavor to fill as many of our needs as possible from excess materials made available from other Federal agencies. Due to the efforts of Mr. Schenk and his staff, this section has obtained many thousands of dollars worth of serviceable equipment and components. On a prorated basis, this will result in a very significant dollar saving over a period of many years. As the mechaisms for obtaining excess materials are further developed, it will become increasingly easier and quicker to garner a portion of our working needs in this manner. Money saved in this manner can be applied towards the purchase price of new equipment for our "Repairs and Calibrations" unit. In many cases, components and equipment have been reissued by this section to other laboratories and, in the case of real abundance, have been issued to laboratories in the NHI and NIAMD. An item of interest is the acquisition of five 35-mm moving frame cameras, which we feel can be adapted to the program needs. The original cost was about $1000 each. We obtained them for freight charges.

Components other than Federal Surplus continue to be available on a limited basis for issue to the various laboratories. As in the past, laboratories needing components in quantity are encouraged to furnish their own stocks. However, I believe that purchase orders for mechanical and electronic components originating in other laboratories should now be screened by this section to avoid the purchase of materials which may be on hand in this section and excess to our needs. Also, equipment of some kinds is available for loan to help forestall delays in procurement. An exception to this policy will be found in the "Repairs and Calibrations" area where our standards will not be available for loan.

Reference is made to the memorandum issued by the Director of Basic Research NIMH–NINDB on April 13, 1960, to the Laboratory Chiefs, Basic Research program in regard to the more efficient use of storerooms. Per request, this section has assumed the responsibility of clearing these areas (3N–264 and 2D–35) and creating a combination service area and live storage area facility in each room. In one room (2D–35) the facility is operative, in the other (3N–264) we are still trying to recruit a competent person for the assignment. Numerous items have been removed for remote storage in Building 9 and more will be when the Building 9 areas can be improved security-wise. Work requests have been submitted. In passing, I want to thank all the scientists for their ready cooperation.

Three persons were added to the section during the year. In October, Mr. Ross R. Snider, Supervisory Electronic Engineer came on duty in the position of section chief. During July, Mr. Leo G. Leitner, Electronic Technician, entered on duty. Mr. Snider will provide the high-level professional direction the section has lacked in recent years. Mr. Leitner is scheduled to be a part of the Repairs and Calibrations service

which we will provide in the future. Also, for the first time the satellite concept is in practice. Mr. John A. Cooley, Electronic Technician, was brought on duty in October as a member of this section but attached to the Laboratory of Psychology, NIMH, on a full-time basis. He will have as his responsibility, all the instrumentation problems of that Laboratory, less the section on Animal Behavior, as far as he is capable and equipped. We had one summer employee this year, Mr. Gary Gloyd. He was a great help in our storeroom reorganization and in the Excess Property Utilization program.

Having additional personnel this year was responsible for our being able to detach two men to the Marine Biological Laboratory, Woods Hole, Mass., during the summer. Mr. Byrne was TAD to the Section on Special Scenses, Laboratory of Neurophysiology for 8 weeks and Mr. Leitner also was TAD to Section on Special Scenses, Laboratory of Neurophysiology for 8 weeks. The two periods were partially overlapping due to the needs of the scientists at Woods Hole. This is cited as an example of the direct assistance concept described in last year's report. We were able to devote some 640 regular man-hours of this direct assistance, in a lump sum, so to speak, for the first time.

The section's man-hours are divided into various categories, as they have been in years past. Time is divided between the new and the old. Design, development, construction and consultation occupy their portion while maintenance, repair and modification occupy theirs. Much more needs to be done in the latter group. Our "Repairs and Calibrations" unit is intended to help rectify a situation which finds many meters and recording devices throughout the program badly in need of calibration.

Our filing system and library continue to be available to interested persons. Both have been methodically expanded during the year and will continue to be. The library contains books spefically on Medical and Biological Physics and Electronics as well as general texts and reference works in the physical sciences. The literature files are maintained up-to-date by virtue of being on manufacturers' and distributors' mailing lists, as well as scanning technical publications for new items and new manufacturers and/or subsidiaries.

Various instrumentation problems have either not been started or not completed due to the Section's workload. Paramount among these are modification kits to reduce 60 cycle interference and reduce artifacts in EEG recordings, and battery replacement power supplies for use with preamplifiers requiring DC filaments and plate voltages. In regard to EEG records, the goal is a machine which is not so sensitive to AC pick-up when other equipment is operated in the same immediate area. In regard to the battery replacement supplies, we would like to eliminate the considerable expense of batteries as well as reduce the inopportune breakdowns due to battery failure. Other projects that have lagged, due to the continued nonavailability of uninterrupted time, are a remote positioner for micropipette electrodes and a vibrating drive to permit penetrating single cells without moving them.

There have been two changes in the section. One is a matter of policy while one is a functional change. We have had to reduce sharply the amount of work done in our shops personally by the various investigators. With the additional people in the section and without additional space for them to work, safety and efficiency dictated this change in policy. However, we can now furnish faster service on this type of work by members of the section. The new function will be a "Repairs and Calibrations" unit, now in the embryo stage, which will, as its sole duty, engage in the preventative maintenance, repair and calibration of equipment in the Basic Research program. We will attempt to work out some mutually convenient scheduling in this regard.

Included below is a brief run-down on a few of the projects completed or partially completed this year.

Three additional slave scope assemblies are nearing completion similar to the one mentioned in last year's report. Minor design variations were called for and were incorporated. Two slave scopes are for use with a single beam oscilloscope and one for a dual-beam scope. These units permit the simultaneous viewing and photographing of an oscillographic waveform, along with various other data superimposed on cathode ray tube by mirrors.

A thermoelectric heating and cooling system was developed, making use of the properties of

the relatively new thermoelectric junction. By causing various calibrated currents to flow, it is possible to produce desired temperatures. The direction of this current flow determines whether heating or cooling takes place and the amount of current determines actual degree change.

A quadruple photocell marker system was developed for psychological experimentation. Each of the four channels are actuated by light pulses on the order of 10 milliseconds. Fast output relays are used to perform controlling functions. Regulated power is provided for amplifier stages and for the photo conductive cells.

A response timer and counter was designed and built for child development studies. Infants are presented with attractive switches which control various colors and combinations of lights. Number of responses and time per response are counted and recorded.

Numerous plexiglass chambers have been machined and assembled for various laboratories. Some provide for the flow of liquids to maintain a biological preparation at constant temperature. Others provide the facility for dissecting nerves, etc. One was built of a size and shape to restrain a live fish during experimentation.

A motor driven switch was designed and built in cooperation with Laboratory of Neurophysiology. This consists of ten magnetic reed switches, arranged in a radial pattern around a revolving permanent magnet. The switches are energized sequentially by controlled magnetic fields. Since each reed switch is sealed in a vacuum, the switches provide the high open-shut resistance ratio necessary to any sampling system which involves the charging of capacitors for extracting signal from noise, when the signals are repetitive but considerably smaller than the noise.

Several electrometer input preamplifiers have been constructed from specifications previously established. Also, a chopper-stabilized preamplifier was built and tested. This enables small DC signals to be recorded without the super-regulated power necessary if the amplifier is direct coupled throughout.

Several important areas of endeavor face us in the coming year. Among these is the dual closed circuit television monitoring system for the sterile surgery wing. All the physiological information which is gathered from the patient is then fed to the recording room. The graphic records are televised and displayed on monitor cathode ray tubes for the surgeon to refer to as the operation progresses. We also have the EEG, EKG, EMG, GSR, CO_2 and temperature recorders to install and maintain. When this system is in operation it will require the full time of at least one capable man merely for maintenance.

Other problems center around the tape recording of physiological data and the preparation of this data for computer analysis. We have made further progress in the positioning of micropipettes and the coming year should see an adequate instrument for this purpose. Our contribution to the Research program will continue to increase in all the applicable fields as the Section continues to grow in competence and responsibility.

CLINICAL INVESTIGATIONS

Introduction[3]

The reports of the laboratory and branch chiefs which summarize the studies now underway in the Clinical Investigations program demonstrate clearly the nature of the progress made during the past year. Although some projects have been brought to highly successful conclusion and others are far enough along to promise significant results, in my opinion the most important and promising development has been the increasing pervasiveness of a truly interdisciplinary atmosphere. By this I do not refer simply to such studies as the coping project in the Adult Psychiatry Branch or the schizophrenia research in the Laboratory of Clinical Science. These and many other investigations by their very nature demand the participation of a varying number of the behavioral disciplines which are strongly represented in our program. Nor do I imply, of course, that NIH has in any sense a monopoly on interest in the prosecution of interdisciplinary research. But those of us who have spent our professional lives in hospitals or in university departments are repeatedly impressed by the unique character of the Clinical Center setting.

[3] Prepared by Robert A. Cohen, M.D., Director of Clinical Investigations.

The interaction at the regular staff conferences over the eight years of our existence has had a noticeable impact. The individual social or biological scientist no longer approaches a representative problem even in his own discipline without some consideration of its broader implications. Such thinking, by taking into account a wider range of possibilities, leads to a more critical evaluation of the phenomena we are attempting to understand. As the range and depth of our studies show, this atmosphere has not had a stifling effect upon individual initiative; rather, it has promoted careful attention to methodology, precise evaluation of results, and, what is most important, a search for more powerful conceptualizations.

In previous reports it has been noted that external circumstances dictated the establishment of our several laboratories and branches almost as islands quite distinct from one another. To some degree and in some areas it is desirable that this should remain the case. But it has been interesting and stimulating to note the spontaneous appearance of several areas convergence—to name only one, the impact of the family on behavior and personality development is being studied from various points of view by each of the major research groups. The organization of Clinical Investigations was designed to foster the development of such convergences of interest in the hope that they might lead to significant advances in behavior theory.

In the last annual report I discussed some of the external and internal conditions which affect our creativity, our sense of fulfillment and satisfaction in our work, and our productivity. These are so commonly matters of concern for any program director that mention of them might have seemed almost banal. In retrospect the matters which were uppermost in my attention at the time were related to the desires of the investigators for a greater degree of participation in decision-making which led to the development of the Assembly of Scientists of NIMH–NINDB, and to the fact that each month brought attractive, and in several instances successful, offers to our staff from universities and other research centers. Some turnover of staff is desirable, of course, to keep a group from becoming too inbred, and some is to be expected in any event. But the achievement of the long-term goals we have set for Clinical Investigations do depend in part upon the continued interaction of a number of the more senior staff. Further, when valued members of its staff take appointments elsewhere, it behooves any institution to scrutinize its own practices once more to assure that everything that can be done to promote the success of its mission is being done.

My concerns of last year seemed to have had almost a prophetic quality when three of our laboratory chiefs were simultaneouslyy seriously considering offers of important university posts. Dr. John Clausen accepted the directorship of the Human Development Center at the University of California and Dr. Seymour Kety has accepted the Chair of Psychiatry at Johns Hopkins University Medical School. Although we were mindful of the fact that 51 percent of the chairmen of departments queried by the authors of the Academic Marketplace placed their own departments among the top five in the country, all this occurred at a time when we honestly felt that our own research was stronger and more solidly based than it had ever been previously, that the problems of development were largely successfully worked through, and that the next 10 years might be expected to show what this type of research organization could accomplish—a research organization which, unlike some others, had been largely shaped by its members.

In thinking back to the concerns we have had about NIH as an institution in which one might spend his professional life, I do not believe that we have identified any issues not mentioned in the 1959 report. It is generally felt that we are making progress toward a more effective delegation of decision-making in this large organization, and that we are developing reasonable methods for program evaluation. Some problems related to these areas are not solved, but in a living dynamic organization these and similar matters are the focus of continuing interest and can never be permitted to become ossified into a body of inflexible regulations.

Perhaps of all the issues mentioned in the last report, the one most pertinent to our immediate situation is the concept of the full-time research organization. What kinds of institutional supports need to be built into it to promote the total professional development of its members at various phases of their professional careers, and

at various phases in their periods of creativity? The university affords supports for research, supports for teaching, and—for the clinician—supports for practice. One may engage in any or all of these activities and feel that he is making a worthwhile contribution. The development of suitable supports for our institution is a task for the investigator and the administrator, and in my opinion the ultimate success of NIH as a research institute will be largely dependent on the results of our joint efforts.

Finally, the year has increased our debt to Miss Agnes Middleton, Mr. Stanley Hirsch, Mrs. Hazel Rea, and Dr. William C. Jenkins. Miss Middleton, Director of Psychiatric Nursing, and Mr. Hirsch, Director of Psychiatric Social Service, and their staffs have been called upon not only to carry out most efficiently all those essential services which nurses and social workers contribute in every therapeutic setting, but also to work with a wide variety of normal volunteer subjects and to assist in the development and operation of a system of recording upon which essential research findings depended. In addition to the specific contributions which they have made to the research data, all of us—patients, normal control subjects, and investigators alike—have come to rely upon them and their staffs day in and day out and perhaps to take for granted the maintenance of the optimum human environment without which our work could not have gone forward. Because of the nature of our program, which includes so many psychosocial studies, Mrs. Rea, our administrative officer, is regularly faced with problems for which no routine solution is to be found in an institution largely devoted to studying the organic aspects of disease; in addition, she has had to develop a completely new set of procedures in connection with the Clinical Neuropharmacology Research Center at Saint Elizabeths Hospital. She has brought to her duties a combination of resourcefulness, energy, and tact which has dissolved difficulties that often seemed insoluble. Dr. Jenkins, Chief of Clinical Care and for six years our representative on the Medical Board, has carried the overall responsibility for the clinical aspects of the entire service. He has interpreted research needs to Clinical Center and NIH administration, Clinical Center needs to the research group, and he has practiced preventive maintenance so effectively and yet so quietly that few of us realize how much we have all benefited directly from his efforts and indirectly from the general esteem in which he is held by professional colleagues and patients alike.

Clinical Care

Perhaps the most significant development in this area during the past year was the establishment of a nursing unit for interdisciplinary research of all the laboratories and branches of the Clinical Investigations program. A committee made up of representatives of each of the branches and laboratories, the Chief of the Psychiatric Nursing Service, the Supervisor of Social Work for NIMH, and the Administrator of the Nursing Unit—Dr. Harold Greenberg—from the Office of the Director of Clinical Investigations, consider and approve the various research projects proposed. It is hoped that this will develop into a flexible arrangement which will make possible a variety of studies, at times with mixed diagnostic categories of patients, and will enhance the possibilities of productive interdisciplinary research. Projects presently being carried out on this unit include studies of the depressive states, the aging process, and periodic catatonia. Other studies of patients with acute schizophrenia and of schizophrenia in twins, are in prospect for the immediate future. The enthusiasm and productive efforts of all the staff members involved promise well for this venture.

The designation of a separate nursing unit for interdisciplinary research for all the laboratories and branches became possible with the consolidation on to one unit of the Adult Psychiatry Branch studies of families of patients with schizophrenia, and the studies of first year college students with psychiatric illness requiring hospitalization.

For the past year two of the five nursing units allocated to the NIMH have been used for normal volunteer subjects. Ninety individuals have been admitted to our nursing units during that time. We have enjoyed the excellent services of the Office of the Director of the Clinical Center in recruiting and arranging for these people to come here from colleges, church organizations, etc. They provide us with the opportunity for carrying out a number of studies of normal in-

dividuals, as well as serving as normal controls for a number of other studies of patients. In addition, 97 normal volunteers were seen in studies requiring outpatient visits only for some 1,500 visits. These subjects were seen in studies carried out for the most part by the Laboratory of Psychology and the Child Research Branch.

Requests for psychiatric consultations by staff of the other Institutes and the Employee Health Service totaled 156 for the year ending June 30, 1960. During the past year, at the request of the National Cancer Institute, we have arranged for one of our psychiatrists, Dr. Noel Schweig, to attend a regular conference of nursing and medical staff on one of the Nursing Units of the NCI. We have also arranged for one of our psychiatric consultants, Dr. Joseph Noshpitz, to attend a regular weekly conference on problems of children with Leukemia who are hospitalized on another Nursing Unit of the NCI.

Consultation requests for psychological testing have been met primarily by Dr. Isabelle Kendig, of the Laboratory of Psychology, as part of her research interest in self-concept and body image. Dr. Kendig has recently retired and we are now in the process of recruiting a suitable individual to take responsibility for service needs for psychological testing on our own nursing units, as well as for meeting requests from other Institutes.

The special nature of the Clinical Center, in which central services are provided and administered separately from the investigative and clinical care provided by the NIMH, calls for many interchanges between staff of the various central services and NIMH staff. That there is so little friction, and that so many potential problems get worked out in an effective and cooperative manner, is a tribute to the many members of the staff of the Clinical Center with whom we closely work. An example of this working relationship, but by no means the only one, was that of the nursing staff of Nursing Unit 2–W, who during a prolonged and complex clinical study contributed greatly to the success of the research by their exceptional efforts. It was gratifying that in recognition they received an NIH–HEW Group Award for Sustained Superior Service.

Adult Psychiatry Branch

In our Section on Psychosomatic Medicine, we have been investigating some endocrine correlates of mild psychological stress. For the most part, stress studies have centered on critical, high distress, emergencylike conditions. We, too, are pursuing such studies. However, we believe that the mild stresses of ordinary living require investigation also. While endocrine responses under everyday circumstances are likely to be less striking than under emergency conditions, they may, nevertheless, have considerable long-run significance for the human organism. These studies of mild stress, including work on relief of moderate tension, have been conducted with young adult volunteers.

During the past year six groups of normal volunteer subjects have been resident on Ward 4-East for periods of time ranging from 9 to 14 weeks. All of these subjects have been here primarily to participate in psychoendocrinological studies. In general these involve the relations between endocrine function, normal variation in emotional states, and a variety of psychological stresses both induced and occurring spontaneously. A major part of this program of research involved the creation of a fairly normal and standardized living situation within the hospital environment in which a variety of stable psychological and endocrinological observations might be obtained. This is an attempt to form out of the living situation itself a research instrument that might be used for different groups of subjects at different times but be reasonably stable and consistent for comparison of data. We are studying the phenomena of adaptation to this new environment which presents many ambiguities to the entering volunteer. In addition to acting as subjects, the volunteers participate in a variety of work-projects jobs ranging from those of lab technicians to secretarial work. The subjects keep a daily diary of experiences and thoughts and a daily mood check-list. The head nurse maintains a weekly "problem seminar" directed at specific nursing problems. A well functioning system of routine urine collections has been worked out and nursing staff fills in a weekly set of psychological ratings for each subject. The categories of psychological observations on these lists have been developed with

the collaboration of the staff so that consensual validation of categories (e.g., depressed) has been maximized. Frequent discussion and reevaluation helps maintain a high level of inter-observer reliability.

The week of admission to the Clinical Center has provided us with an opportunity for analysis of behavior-hormone relations under mild stress. So far we have studied six groups of normal volunteers, comprising 72 individuals. For most of our normal volunteers, there is an initial preoccupations with NIH, some concern about possible risks, and moderate tension on the day of admission. This is usually followed by a rapid adaptation characterized by active exploration of the new environment, increasing familiarity, encouraging personal relationships, and diminishing tension. For most subjects, this adaptation is largely accomplished by the end of the first week. For five of the six groups, psychological stress was largely absent during the remainder of their stay. There were, of course, individual differences in this respect, but by and large these groups were free of heavy pressures and major uncertainties. However, one group (consisting of 12 students) was brought in under fundamentally different psychological conditions. These were marginal students in a local university, experiencing serious financial and academic difficulty. For them, the opportunity of living in the Clinical Center for two months while preparing for final examinations was a very favorable circumstance, since it relieved them of financial pressure for the time being. They were, however, under substantial stress as the examinations approached. Interestingly, this group differed sharply from all other groups in patterns of adrenal hormone excretion. Whereas all five other groups showed diminishing excretion of corticosteroids and catechol amines during the initial week in the Clinical Center, this group went up sharply as soon as the students returned to school (after a one-day orientation at NIH) and remained consistently high thereafter. We were also able to get some remarkable data during final examinations on additional endocrine variables, especially estrogen and androgen excretion.

In the past studies of the endocrinological effects of environmental stimulation have almost invariably focused upon the ability of a particular situation to bring about an increase in the circulating levels of certain hormones. Little or no attention has been paid to situations which may consistently lower such endocrine levels. In our Section on Psychosomatic Medicine, we have been attempting to develop experimentally controllable situations of mild stress, and have discovered several conditions which led to the lowering of plasma hydrocortisone concentrations.

Using young (18–25 years) normal volunteer adults of both sexes who spend several months living on the wards at the Clinical Center of the National Institutes of Health, attempts were made to determine if the showing in a group situation of certain selected commercial movies judged to be emotionally "arousing" could bring about consistent increases in plasma hydrocortisone concentrations. As control films, several Disney nature films were also shown. Bloods were drawn from the subjects prior to the showing of the films and immediately afterwards. In addition, self-assessment of mood was obtained from each subject by a modification of the Nowils-Green adjective check list before and after the showing of the films.

In order to control for the showing of either arousing or bland films, a second group of all young adult males had, in addition, bloods drawn during a comparable control period during which time the subjects followed their usual activities on the ward. In order to assess the effects of the possibly mild stressful situations of sustained attention to a long series of visual stimuli in order to respond differentially to them, a group of young adult male subjects were run on the Continuous-Performance Technique developed at NIMH. Bloods were drawn prior to and after each subject was run on the task. In order to replicate and expand upon the work of Persky, young normal adult subjects were placed in a hypnotic trance after which a particular emotion or emotionally arousing situation was suggested to them. Again bloods were drawn prior to the trance, after the induced trance, and after the presumably emotion-inducing hypnotic suggestion.

The difference in the mean pre-to-post-film changes in plasma hydrocortisone concentration for "bland" vs. "arousing" films corresponds to what might be expected from the nature of the stimulus.

For those subjects for whom control levels were also obtained, there is again a drop in level during the showing of bland films, with only a slight increase in levels during the showing to "arousing" films. The control period shows a drop that is appropriate in terms of diurnal cycle. ontrary to expectations, the subjects run on the Continuous-Performance-Technique also showed a drop in the levels of plasma hydrocortisone. As with Persky's findings, the induction of the hypnotic trance itself reduced the level of plasma hydrocortisone. Also, this effect seems to be stronger than that resulting from assumed emotionally arousing suggestions.

The three stimulus situations which have brought about the lowering of plasma hydrocortisone levels, while different in many respects, do have three major elements in common: (1) The experimental situations, per se, presents to the subjects bland, non-noxious stimulation. (2) The experimental stimulus is such that there results a narrowing of the subjects' attention away from other possibly stressful stimuli of either external or internal origin. (3) The subjects' anticipation of the experiment may serve as a stressor to bring about a rise in the hormone levels prior to the beginning of the experiment. This rise due to anticipation tends to predispose toward lowering the levels during the experiment itself. This may be due to an interaction of a physiological rebound phenomenon and of feelings of subjective relief at the blandness of the situation.

In any event, these findings call attention to psychological conditions under which adrenal hormone levels may be diminished—as well as the more familiar distress conditions under which these levels are elevated.

In the latter part of the year we began three new major stress studies in the neuroendocrine field. The primary stimulus for these studies is our ability to measure several additional hormones: (1) urinary excretion of aldosterone, androgens, and estrogens; (2) blood levels of butanol-extractable iodine as an index of thyroid function. We are interested in determining whether these hormones will be affected under conditions that have already been shown to be associated with elevated blood levels and urinary excretion of hydrocortisone, epinephrine. and norepinephrine.

The first of these major stress studies is being done through the cooperation of Dr. Gordon Zubrod and his staff in the National Cancer Institute. Dr. Stanford Friedman and associates are doing a study of coping behavior in parents of leukemic children, making serial hormone measurement in patients admitted to our unit for disturbed college students. Similarly, the third study, under the leadership of Dr. William Bunney, involves serial hormone measurement in patients admitted to the Clinical Center because of severe depressive reactions. In the latter two studies, the investigators are working closely with nursing staff to develop systematic observational techniques for rating of behavioral variables.

In most of our psychosomatic research, we are collaborating very closely with the Department of Neuroendocrinology in the Walter Reed Army Institute of Research under Dr. John Mason. Similarly, Dr. Joseph Handlon of the Laboratory of Psychology has been an active collaborator in most of these studies. Also, in several recent projects we have been collaborating with members of the Laboratory of Clinical Science: Dr. Philippe Cardon and Peter Mueller in measuring free fatty acids in relation to emotional distress; and Dr. Roger McDonald and Mrs. Virginia Weise in measuring VMA as an index of adrenomedullary function in depressed patients.

In striking contrast to the apparent importance of sleep and dreaming in human life is the dearth of established knowledge concerning these phenomena. Far from understanding the nature of their psycho-physiological functions, we are lacking even in adequate physiological description of them. It is evident that sleep is not a uniform state from hour to hour in the same night, from night to night in the same individual, or from one individual to another. There is reason to believe that the same is true of the physiological substrate and of the subjective experience of dreaming. Yet the parameters of such variability have yet to be conceptualized and made amenable to measurement. Only then will it be possible to make quantitative studies of sleep and dreaming in relation to the organismic economy. In view of the well known and apparently universal association of disturbed sleep and mental illness, it seems reasonable that attempts to define and quantify the parameters

of sleep might eventually lead to important clinical applications.

Dr. Frederick Snyder is investigating physiological and psychological concomitants of sleep and dreaming in an effort to further define and quantify their parameters. In its present phase the project takes the form of a series of small scale, hypothesis-seeking, exploratory studies. All-night records of electroencephalographic, electroculographic, and various other physiological measures, such as respiration, heart rate, plethysmogram, and skin temperature are obtained from sleeping subjects. Details of the procedure have varied among the sub-projects through which the study has progressed. For some purposes the recording has been done without disturbance of the subjects' sleep, and for other purposes subjects are systematically awakened by means of graded sound stimuli, at which time they attempt to describe their subjective experience prior to awakening, including whatever dream narratives they can recall. The subjects employed have been normal volunteer controls.

Three types of observations reported by other investigators have been repeated and essentially substantiated: (a) Dream recall is associated with a particular pattern of electroencephalographic and eye movements. (b) There is a regular and invariable recurrence of this physiological pattern three to five times in each night of sleep, taking up an average of 20% of the total sleep time. (c) Interruption of sleep at the onset of this pattern over a series of consecutive nights leads to a progressive increase in "dream attempts," and the nightly "dream time" is markedly elevated for several nights after the conclusion of the "dream deprivation" period. Several aspects of these phenomena which had not been mentioned in previous reports were noted: (a) A high degree of variability was found in the incidence and vividness of dream recall among subjects, in the same subject from night to night, and over the several dream periods of a given night. (b) There is a considerable range in the percentage of sleep time taken up by "dream sleep," to some extent apparently the expression of individual differences, but to a larger extent taking the form of night-to-night variation in the same subject. An attempt to account for some of the variability in percentage "dream time" through a hypothesized correlation with 17-hydroxycorticosterone levels was not supported. We have further sought physiological parameters of "dream sleep" which would relate to the vividness of dream recall. It was considered feasible to combine this objective with that of a reexamination of the question of sleep "depth." To these ends two additional facets have been explored: (a) Auditory thresholds for awakening and for signs of physiological arousal short of awakening have been obtained during various EEG stages of sleep, and in connection with the subjects' rating of the vividness of his dream memories. In keeping with the reports of previous studies, the auditory thresholds revealed a fairly predictable relationship between EEG patterns and the intensity of stimulus required for awakening outside of the "dream sleep" periods. It was noted, however, that this relationship was a relative one, with considerable variation in the absolute levels of stimulus required among subjects, or in the same subject under different conditions of "sleep need." Hence, it appears that the EEG pattern is not an absolute index of sleep "depth" as defined in terms of arousal thresholds. Within the periods of "dreaming sleep" the intensity of awakening-stimulus was extremely variable, with no constant relationship to thresholds required during other EEG stages, nor to the vividness of dream recall. (b) Physiological variables other than the EEG and eye movements have been monitored in the hope that they would provide indices which would relate to the vividness of dream recall, or to the depth of sleep as determined by arousal thresholds. Thus far these have included heart rate, respiration, plethysmogram, and skin temperature. This resulted in the finding that all of these measures tend to distinguish between "dreaming sleep" and "dreamless sleep," particularly in terms of the much higher degree of minute-to-minute variability of the measure during dreaming. This is especially striking with regard to the variability of respiratory rhythm. Early results are quite encouraging that this measure may relate to the vividness of dream recall. If further work bears this out, we will feel justified in postulating a parameter of "dream intensity." In relation to sleep depth, each of the autonomic measures is at least roughly related to the EEG

stages of sleep. Whether any of them might be a better absolute index of sleep "depth," as measured by arousal thresholds, remains to be studied.

The Field Study Group, under the leadership of Dr. Earle Silber, has been concerned with a naturalistic study of 20 students in the process of transition from high school to college. Our aim is to learn about some of the patterns of problem-solving in late adolescence. By the fall of last year we had selected 20 students from the senior class at the Bethesda-Chevy Chase High School and had completed a series of interviews with them while they were in high school. In addition, we had interviewed their parents, and all of the students in the project had been given a special projective test constructed by Dr. George Coelho and Dr. Silber. This test, patterned after the Thematic Apperception Test, was designed to elicit characteristic modes of dealing with certain potentially stressful situations in college. In the early fall, staff members who had interviewed the students in high school visited them at their respective colleges. In this interview we obtained data about the early weeks of college, the academic work, the experience with friends, roommates, dating, group activities, relationships with parents, impact of new values, satisfaction with college, expansion of new interests, and particular problem areas for the student. In addition, we toured the college campus with the student as a way of getting a view of the college through the student's eyes. We next met with the students when they visited home during the Christmas vacation. At this time the same areas mentioned in the original college interview were pursued, as well as focusing on current interactions between the student and his family while he was at home. During the Christmas vacation, the students were also again asked to take the projective test, which we have referred to as the Students' Thematic Apperception Test.

In the early part of this year a second interview was conducted with the parents by Mrs. Elizabeth Murphy. This interview was focused on the parents' perception of any change in the student and their response to these changes. In addition, we asked about any changes in goals that had been made by the student and the parents' response to this. We also obtained information about the parents' own education and explored with the parents their awareness of what contributed to certain assets or particular coping mechanisms in their children.

The students were seen again when they visited home for their spring school vacation. We continued following up all of the areas that were explored in the early interviews and we were also interested at that time in any change in their feeling about home and their parents following the Christmas vacation.

Students were seen after they had returned home from college in the early part of summer. We again pursued the topics covered in previous interviews with them; in addition, we heard about their plans for summer jobs, discussed the management of finances, and had the student view for us in retrospect some of the important tasks in the transition they experienced and how they were handled.

New pictures were designed for the Students' Thematic Apperception Test and students were again tested in the early part of June.

The summer months were used to conduct a series of joint interviews with the student and his parents. The purpose of this interview was to review with the student and his parents his transition to college from the point of view of the total family experience. These interviews were viewed also as an opportunity to make observations directly about patterns of family interaction.

Now that the data collection is completed, we are in the process of analyzing our material. We plan to maintain contact with each of the students in the project and to see them once a year until the completion of their college experience. We have already reviewed material related to the decision to go to college and factors involved in a choice of college by our students. We have been able to describe certain cultural forces which played a decisive role in directing them toward college. These students internalized an expectation that they would go to college and did not make a conscious decision about going to college. This was related to their family's socio-economic status and the role that education played in the social mobility of their family. In addition, the high school peer culture reinforces the trend toward college. Our students were active in using environmental re-

sources to learn about colleges. The process of selection involved the student's assessing his potential "fit" with particular colleges; that is, a simultaneous process of self-assessment along with an assessment of the college. The parents were active in the selection process, and they were explicit for the most part about their expectations and preferences, at the same time granting a good deal of freedom to the student in making a choice of college. When the student took a position contrary to the parents, the parents were able to accept this without it disrupting their relationship with the student.

We have also examined the coping mechanisms of our students during the time they were in high school, anticipating the change to college. We have been able to define certain personality attributes which would facilitate an active involvement in the tasks of the transition. Our students tend to value new experience, tend toward activity in facing new situations, and derive a good deal of pleasure in the sense of mastery of challenge. In addition, there were more specific mechanisms which could be viewed from the point of view of the student's developing and maintaining a self-image as adequate to the perceived requirements of the new situation. We have observed a number of mechanisms, such as: reference to analogous past experiences, reference to a present self-image, learning about the new situation in advance, role rehearsal, group identification, lowering the level of aspiration, and selectively perceiving encouraging elements in the new situation. In addition, our students were effective in maintaining distress within manageable limits. They viewed concerns that they had about college as being shared by others; some attempted to make their concerns seem desirable; they were active in dealing with issues that they felt they might have some concern about in the future; and finally, they used fantasy rehearsal as a way of dealing with some future contingencies in the college situation.

Certain characteristics of the parents of the students in the Field Study Group have been described. The parents fell roughly into three groups. One group was composed of two families we were not able to interview; in one of these the mother had been psychotic, and in the other there was severe marital conflict. A second group of 10 families were similar enough in their characteristics to be considered as a group. Four other families presented exceptions in certain areas. We were impressed with the range of patterning that was compatible with effective functioning in the student. Among the 10 families that seemed more homogenous as a group, we could describe such characteristics as: a stability and clarity of values, a persistence and complementarity of role in the family, the parents' acceptance of their children, the parents' self-acceptance, parents' encouragement and endorsement of the students' autonomy, confidence in the students' abilities, directness in communication and the parents' ability to perceive the students' assets.

At the present time we have completed a unit of analysis concerning the formation of friendships at college. We have been able to describe distinct phases in the pattern of friendships at college. Following an initial, almost indiscriminate, reaching out, there is a later selectivity and sifting based on common interests. The formation of friendships is an important vehicle in providing opportunities for further self-awareness, learning, exploration of new role possibilities. They provide an important sense of belonging, support at moments of crisis, and opportunities for intellectual stimulation. We are planning also to review material about the students' dating experiences and attitudes about sex and marriage. In addition, we will examine how the students dealt with the academic demands and new values at college.

Dr. Coelho has recently completed a report on the development of the Student Thematic Apperception Test. It includes the following material:

1. A description of the STAT instrument and rationale for its use as a projective device in the pre-diagnostic assessment of freshman coping behavior.

2. The operational definitions of three categories constructed for the analysis of STAT stories with respect to the variable, competence in problem-solving during the freshman transition in college. These rating categories are as follows: S, denoting the presence of a resolution in the story; A, denoting active efforts involved in effecting a resolution; F, denoting the presence of a favorable resolution in the story.

3. Presentation of quantitative results based on

comparisons of STAT protocols of three groups of freshmen: 10 students from the Field Group who were identified as exceptionally competent independently on the basis of interview data collected from the students' spring term of the senior year of high school and the fall term of their freshman year in college; 10 ward patients hospitalized in the Clinical Center for psychological difficulties experienced in their freshman year; 10 University of Maryland volunteers selected from an advanced freshman English section.

(a) Two gross indices of competence potential were derived from the preliminary results: an optimism index and an effectance index which differentiate between the competence levels, as defined, of the normal and patient groups of freshman subjects.

(b) An analysis of variance was carried out to test the levels of the group profiles and the shapes of the group profiles. The results show, on all three categories, significant differences between the competence levels of both the normal groups on one hand and the ward patients on the other. The differences between the Field Group and the University of Maryland freshmen are not statistically significant for A; they are statistically significant for F, but not practically significant.

(c) While the level differences are generally parallel between both of the normal groups on one hand and the ward group on the other, the biggest discriminators among the stories are Nos. 7, 9, 10, and 11 (namely, in the scenes—working to finish final exam under pressure of time (7), dealing with double date situation (9), calling home (10), day dreaming alone (11).

(d) A comparison of the STAT protocols of Field Group subjects tested 3 months before and 3 months after college entrance shows negligible differences in total positive ratings on any of the three categories S, A, F.

(e) A qualitative analysis is under way to investigate activity styles, resolution strategy, and hero imagery in the STAT protocols of the normal and patient groups of freshman subjects.

Dr. Coelho, Dr. Fredric Solomon, and Dr. Thomas Lewis have developed a group interview technique, using a small group of Maryland freshmen as informants, to explore the salience, intensity, and tempo of new environmental pressures experienced by freshmen. An observational and recording procedure was also developed for analyzing the content of the group interviews without the use of tape recordings. The investigators met with a small volunteer group for an advanced section of freshman English in one of the conference rooms of the Student Union of the University of Maryland. The arrangements were completed through the cooperation of Dean James Borrison. Our group interview data are based on the responses of a core group of five students, interviewed over seven sessions during their freshman year, the group size fluctuating from four to eight.

In addition to these data from Maryland University freshmen, comparative data were obtained, through the group interview method, on two other freshman groups.

1. Eight ward patients who were hospitalized in the Clinical Center, were interviewed over six sessions of 1½ hours each in an area apart from the ward unit, to emphasize the research focus of the group interview as distinct from group therapy.

2. Four freshmen from among the Bennington College normal volunteers were interviewed for five sessions of 1½-hour's duration each.

The following sets of data are now available: (1) An indexed table of contents showing topical areas of high salience, discussed by these three group of subjects; (2) a narrative account of the group discussions with section headings to illustrate the variety of topical areas; and (3) a summary of the highlights of the group interviews with special attention to comparative observations from the three groups of subjects.

An exploratory predictive assessment of freshman coping behavior at the University of Maryland is now being undertaken by Dr. Coelho, Dr. Solomon, Dr. Carl Wolff, using a sample of dormitory residents who were selected on the basis of Hi and Lo STAT ratings.

The specific research aims of this Maryland freshman study are:

1. To clarify in a predictive way the correspondence between fantasied coping behavior based on STAT performance and behavioral competence based on focused interview data collected during the second week of November 1960.

2. To explore the early coping behavior patterns of Maryland dormitory residents engaged in dealing with their new freshman situation.

A sample of 40 dormitory residents was selected from the total number of 350 freshmen to whom the STAT was administered in August 1960, during the precollege orientation program. The total group of 350 incoming freshmen were tested during the orientation program period, representing about one-fifth of the total number of students who attended the precollege orientation program on the Maryland campus in August. An interview guide focussing on specific questions in the following areas had been prepared:

1. Meeting of academic tasks, interest in course work, planning of tentative major and vocational directions.

2. Handling of separation from home, communication and visiting patterns with members of the family.

3. Initiation of friendships and involvement in group activities.

4. Experiences in the dormitory situation and with the roommate.

We have also approached the study of personality development in late adolescence by selecting a widespread and significant task of that developmental phase and studying individuals who manifest disturbance in their efforts to accomplish this task. The task we have selected is that posed by the requirements of the transition from high school to college. Under the leadership of Dr. Roger Shapiro, we have accepted as patients for our study young people who have had sufficient emotional difficulty during their freshman year at college to make it necessary that they drop out of school. We have conceived of the adjustment to college as containing many of the developmental tasks of late adolescence, which make demands upon the adaptive capacities of the individual in a number of areas. Among these tasks are: separation from parents and living away from the family for a prolonged period of time; living primarily with peers and having more significant relationships with peers; the demands of increasingly intense and mature patterns of sexual expression; the increasing intellectual demands of college work; an increasing requirement for decisions relating to vocational choice. We want to determine what current and past social experience interacts with the individual potentialities, to encourage the adaptive capacity of the individual in his mastery of the tasks of this phase, conceptualized by Erikson in their most generalized form as the achievement of a sense of ego identity. We want to understand what is lacking or inhibiting in the particular life experience of the individuals we study, both current and in the past, to interfere with the realization of their potentialities in late adolescence. Finally, we want to utilize our therapeutic program in a way which will help us to understand what aspects of current social experience can encourage realization of potentialities previously interfered with and allow for further personality development. We are concentrating our investigative efforts in two broad areas in our attempts to answer these questions. We are studying the recent situation in which the patient experienced acute difficulty, the developmental task with which he was confronted in college; and we are studying the family experience of the individual.

Our efforts to study the college experience of our patients has taken three major forms. Each of our patients is interviewed by one of the investigators in a series of research interviews which follow a schedule of areas of particular interest to us. Questions are asked in the following areas: What was involved in the decision to go to college; in the emotional preparation for college; in the initial adjustment to college; in friendship formation in college; in the reaction to the academic challenge; in other areas of difficulty in adjustment; in various reactions to these difficulties; in the crises and the departure from school. A second source of information about the college experience is contained in the picture obtained in psychotherapeutic work with the patient of what his college experience was. This is organized and written up by the therapist in a form covering the same areas of interest as the research interviews so that they can be compared. A third source of information about the college experience is a visit made by one of our investigators to the

college in which the patient had difficulty, to interview friends, teachers, and advisors of the patient in an effort to gain another vantage point for viewing the nature of the psychosocial environment with which the patient had to deal, adaptive efforts he made in the situation, and the nature of the relationships he formed. The patient's picture of these adaptive efforts and relationships is compared with the picture of those who knew him.

Our effort to study the family experience of the patient has in the past 6 months resulted in our utilizing the study of family sessions involving the patient, his parents, an adolescent sibling if available, the therapist of the patient, and the social worker. These sessions are held for one hour weekly and are observed by two investigators. They are integrated into our therapy program, which also includes two individual sessions a week for the parents with the social worker, and two sessions of group therapy per week for the patients.

We attempt to utilize interviews when family interaction can be observed, to shed light on the identity question in the following way: We pay particular attention to the interaction between parents and patient which gave some indication of the way in which the parents overtly and covertly identify the patient and the way in which he overtly and covertly identifies the parents. If we can see demonstrated relatively rigid and enduring delineations of the patient by his parents, which make for inflexibility in his sense of identity and anxiety over the changes in the concept of himself which the developmental phase requires, we will understand more of what contributes to pathological outcome in the period of identity crisis. We look also for evidence of inflexibility and stereotyped expectation on the part of the patient in the manner in which he delineates his parents, and undefensively keeps them in stereotyyped and rigid roles. With this identity question as the central focus of our research interest in the family sessions, we report on this aspect of the interaction in two ways. The therapist makes it a part of his summary of the interview; and observers follow each interview from this point of view rating on a prepared flow sheet sequences in the interview which reveal parental expectations of the patient and patient's expectations of the parents, delineations back and forth of what they can allow into their concept of the other. This includes their conscious statements about the other person; it also includes effective behavior toward the other person from which unspoken or unconscious attitudes can be inferred. We have two observers recording independent impressions in an effort to arrive at reasonably objective findings.

This investigation into problems in the personality development of the late adolescent has been in progress since January 1959. In these 2 years we have hospitalized 19 college students, predominantly freshmen, who were in sufficient emotional difficulty to make it advisable that they leave school. Interviews with these students about their college experience allow generalizations to be made in a variety of areas. We have summarized the data from interviews of 10 of these students under the following headings, and are now analyzing the remainder along these lines: (1) Attitudes toward new situations and change; (2) manner of arriving at the college decision; (3) dating and sexuality; (4) order, organization and control; (5) friendship patterns at college; and (6) grades, studies, intellectual achievement as a problem.

In connection with the study of freshman college students whose emotional difficulties prevented them from continuing with their college courses, it seemed advisable to develop a psychological instrument which might be of assistance in exploring the personality assets and liabilities of students with specific reference to their capacity for adjustment to their peers and to members of the hospital staff. We wished to set down in systematic and roughly quantitative form the observations of ward staff on personal relations of the student-patients. We are comparing these with the observations of another ward staff, using the same instrument, on normal volunteer college students. The development of this instrument was undertaken by Dr. Harold Greenberg and Dr. Sheldon Korchin.

Two sources were used in an initial effort to develop categories germane to an evaluation of the personality assets and liabilities of hospitalized college students: (1) Recorded interviews with the parents of these students in which the parents were asked for their assessment of their child's personality assets and liabilities; (2) opin-

ions of the nursing staff in this regard (favorable or unfavorable qualities which seemed of special import as staff members observed the student's relationship with them).

Using these data, nine general categories of "ego function" were developed which would serve as a basis for a more detailed evaluation of personality assets. Each of the nine categories was then subdivided into specific components. The nine categories were as follows: (1) Degree of initiative, activity, and thought process; (2) socialization; (3) work goals, productivity; (4) the capacity for effectiveness; (5) sense of identity—the integrity of the self; (6) dependency, independency; (7) flexibility; (8) impulsivity; and (9) character, assets, and liabilities.

In all, a total of 116 statements referable to the above categories was developed as a set of rating scales.

During the last several years, under the leadership of Dr. Lyman Wynne, the Section on Family Studies has been collecting a considerable body of data on, and has formulated preliminary hypotheses about, the place of family relationship patterns in schizophrenic illness compared to nonschizophrenic psychiatric illness. During 1960 the Section decided that the process of data collection and hypothesis formulation had proceeded sufficiently so far that it is now appropriate to shift the primary emphasis from clinical exploration of family patterns to a more comprehensive review and description of the available material and to a systematic evaluation of certain hypotheses in the light of this data. The shift to the latter emphasis is at present underway and it is expected that it will be possible to report substantive findings during the coming year. The present summary will not attempt to anticipate the statement of findings and conclusions that are emerging, but will be limited to an outline of the current trends in the work of the Family Studies program.

1. In order to create more time for writing and research evaluation of the material which has accumulated, the Section has reduced the clinical case load of the families being seen. However, to add at least slowly to the number of families studied and to keep the writing and research evaluation invigorated with currently "live" family observations, the section is continuing to see a small number of families clinically in family psychotherapy and family art therapy, with certain other families observed or treated jointly with Dr. Shapiro and members of the college student Personality Growth program.

2. The section is planning extensive, detailed, naturalistic descriptions of the family data in order to characterize as vividly as possible certain features of the family processes which have been observed. Family therapy sessions have now been held with 27 families one to three times weekly for periods of 6 weeks to almost 3 years. All sessions have been tape-recorded and most sessions, since September 1959, have been directly observed through a one-way mirror. Because conjoint exploratory therapy with families, especially with the families of schizophrenics, is a new and generally unknown approach in the psychiatric profession as a whole and because it taps details of schizophrenic family interaction not previously available to scrutiny, it seems highly desirable that our extensive experience be reported descriptively and naturalistically.

Although comprehensive descriptive reports remain as a current task, during the past year members of the section have presented several short descriptive papers at various professional meetings on the subjects of: sibling-patient comparison—the characteristics of the psychiatrically disturbed offspring compared with apparently normal siblings; clinical observations supporting the hypothesis that schizophrenic thought disorder may be a learned aspect of family life; observations on the nature of the experience of therapists while working intensively with the families of schizophrenics, as providing clues to intrafamilial experience; the use of family art therapy for understanding certain aspects, especially nonverbal features, of family interaction; and a consideration of certain over-all structural characteristics of family organization, especially intrafamilial alignments and splits, as a method of focusing on certain homeostatic family processes.

3. The Section is engaged in the construction of a comprehensive theory of psychological developments within the family setting. Such theory construction is necessary in order to distinguish the crucial from the trivial hypotheses

about family relationships. It is hoped that the formation undergoing development will facilitate interpretation of these underlying, genotypical features of family life which are especially pertinent to certain crucial aspects of schizophrenic impairment, especially the thought disorder and the incapacity to maintain major sets.

4. A central concern in the work of the Section is the development of two kinds of systematic comparison studies: first, comparison of families having schizophrenic versus nonschizophrenic psychiatrically ill offspring; second, the comparison of siblings, schizophrenic and nonschizophrenic, within the same family.

Data from 32 families are now available for these comparison studies. During the past three years families have been specifically selected with these comparison studies in mind. The criteria involved minimizing all presenting differences between the families except one: The presence of a schizophrenic versus a nonschizophrenic (but psychiatrically ill and hospitalized) young adult offspring. An effort has been made to match families in terms of social class and class-related values; each family has consisted of the two biological parents, the hospitalized offspring, and at least one offspring who is not overtly disturbed psychiatrically.

In order to correlate various aspects of family patterns with the degree of schizophrenic illness in the offspring, it has been necessary to rank-order the psychiatrically disturbed offspring along a dimension of degree of schizophrenic illness or tendency. During the past year a variety of ways of specifying the dimensions of "schizophrenicness" were empirically tried. A modified paired-comparisons method was used to make consistent rank-order ratings of all 37 patients seen in the program thus far. (Two families had multiple-birth patient-offspring, so the number of primary patients outnumber the families.) Dr. Margaret Thaler Singer, consultant to the project, similarly ranked 22 of the patients whose psychological tests she had seen. The clinical ranking and her ranking on the basis of the psychological tests she had seen. The clinical ranking and her ranking on the basis of the psychological tests proved to be almost identical. Thus, a satisfactory rank-ordering of the presenting patients is now available for use in the comparison studies of the families.

Dr. Singer has continued studies in which she matches "blind" the presenting patients with their families. Given the psychological test protocols of the family members except for the tests of the presenting patients, Dr. Singer has been able to make remarkably accurate predictions of the schizophrenic or nonschizophrenic characterists of the patient family member. Then, looking at the tests of the patient family members, she has attempted to match patient and family "blind," taking 4 to 7 families in a set.

She has now carried out this procedure with 22 families from the Family Studies program. In addition, she has matched 11 schizophrenic patients and families tested by the Yale Family Studies program headed by Dr. Theodore Lidz. Focusing particularly upon the form or style of thinking used within the families, Dr. Singer has been trying to specify criteria upon which she has been able to make her predictions and matchings. The next phase in the work will be to ask other judges to use her criteria to make the same differentiations and matchings "blind." The preliminary success which Dr. Singer has achieved is highly promising confirmatory evidence that the family characteristics of schizophrenics and of nonschizophrenics can in fact be differentiated and these families do have a subculture and a system of relations which involves the entire family, with the psychiatrically disturbed offspring constituting an integral part.

A second variety of systematic studies which is underway involves the use of excerpts from family therapy sessions. Several pilot studies have been conducted in which these excerpts are rated along dimensions which are relevant to the question of the connection between family relationship patterns and degree of schizophrenic illness in offspring. Excerpts from the family therapy with 14 families, seven having a schizophrenic and seven a nonschizophrenic psychiatrically ill offspring, have been selected for the comparison studies currently being conducted. Thus far excerpts have been selected in which the parents respond to a comment of a therapist and in which there is no indication of the diagnostic characteristics of the offspring. The preliminary results are encouraging with respect to the capacity of the excerpt technique for differentiating successfully the families of schizophrenics and nonschizophrenics.

A complex study being conducted by Mr. George Usdansky using this approach has yielded positive results in an extensive pilot study and is now being verified by rating of the family therapy excerpts made by two outside judges who are unfamiliar with both the hypotheses being tested and the diagnostic identities of the particular families whose excerpts are being used. In this particular study the families have been compared in terms of frequency of occurrence of certain kinds of interpersonal maneuvers which our previous hypotheses indicate may be particularly relevant. Using the rank-order ratings of degree of schizophrenic illness or tendency, it will be possible to compare these independent ratings of the maneuvers used by the parents with the degree of illness of the offspring.

5. As indicated in the annual report of last year, the use of conjoint family therapy as an exploratory research tool has led to a significant by-product: the development of the theory and technique of family therapy, an approach recently achieving wide recognition as a potentially valuable addition to the therapeutic repertory of psychiatrists. In the extensive use of this approach in the Family Study Section, many observations have been possible about the advantages and limitations, the indications and contraindications for exploratory family therapy. Ideas have been developed about technical problems peculiar to family therapy, about differences in technique of family therapy as compared to both individual and group therapy, and about the therapeutically effective aspects of family therapy. Although the work of the Section is primarily oriented toward untangling the relation of family patterns to schizophrenia, the experience in using exploratory family therapy with the families of schizophrenics has been perhaps more extensive than with any other group in this country and deserves report and discussion in future publications from the standpoint of therapeutic technique.

Child Research Branch

The general aim of the present Child Research Branch program, which was initiated at the beginning of the fiscal year 1960, is to develop a systematic longitudinal program of interlocking projects to explore the initial stages of family formation in volunteer subjects, subjects not receiving psychiatric treatment. Three research problems are central: (1) The developmental channels of two separate systems of behavior in the firstborn infant from birth to age 2½ years; (2) the development of the marital bond in various types of married couples from the stage of being newlyweds (they are studied at 3 to 4 months of marriage) to the stage of initial parenthood; and (3) the relationship of the initial interaction patterns, established between the new parents and their firstborn child in the first few weeks of life, to the infant's particular behavior pattern and to the marital type.

In order to implement this general aim, three projects have been under way over the past year. Dr. Richard Q. Bell has initiated his third study (Project No. MCR(C)11) of neonatal behavior characteristics, refining earlier measures of oral integration and of skin sensitivity in the 3- to 4-day old neonate. These infants are being studied in families who plan to continue to reside in Montgomery County (unlike the subjects for his previous two studies, who were infants of U.S. Navy transient families). The observations are carried out at the Suburban Hospital. The establishment of this study at the hospital in the spring of 1960 required extensive administrative efforts in order to secure the full cooperation of the staff of this hospital. To date 50 such infants have been processed; the plan is to follow up these infants as 2½-year-olds in the Nursery School observation situation between 1962 and 1963.

In the second project carried out this past year, Dr. Bell has developed a set of time-sampling observational methods to apply to the adaptation of 2½-year-olds to a nursery school experience of 4 weeks duration (see Project No. MCR(C)10). In preliminary data gathered on a group of 40 males it appears that there may be a significant correlation between these observed patterns of 2½-year-old behavior and the retrospectively parent-reported neonatal behavior patterns in this sample. It will remain for the first-mentioned study, however, to show that this correlation (if it is confirmed during the next year's work on females) is not derived from a distortion based upon faulty parental recall.

The third project of the Child Research Branch is the study of newlywed couples. Work

during the past year has involved the development of methods in a neglected area of developmental research. During this prepilot phase, 40 couples have been assessed in complementary ways. Based upon this experience, the exploratory foci for the study have been extended considerably beyond that envisaged a year ago (see footnote 2a, below). During 1961 a pilot study involving an intensive assessment of 40 new couples will be carried out, permitting the testing of a few hypotheses and the more rigorous exploration of the operational relations between various methods now in use and between the variables now being assessed. On the basis of this pilot study it should be possible to initiate a large scale study of approximately 150 couples, as the second cohort of the longitudinal study. (The 40 couples already seen this past year, as well as the 40 additional couples to be seen this coming year will also be followed into the neonatal phase, thus constituting the first prepilot and pilot "cohorts" of the study.)

The overall strategy of this longitudinal program is thus to move from our current position of carrying out three separate projects on newlywed couples, on neonatal infants and on 2½-year-olds to an intermediate position of carrying out during 1961–63 two overlapping projects, the one linking the phase of newlywed marriage to the neonatal phase and the other linking the neonatal behavior patterns to the 2½-year-old behavior patterns. Thereafter we expected to be in a sound position to carry out longitudinal studies linking all three developmental phases on the same subjects. More specifically, the program aims in the future to focus on a small number of theoretically and phenomenologically interrelated variables, studied on large cohorts of families. Intensive studies will be carried out only on a small number of systematically-selected families who will represent theoretically or clinically salient subsamples; these families will provide an opportunity to investigate the totality of family interaction in greater depth, and thus to feed back new information and to suggest needed modifications of hypotheses. Unlike many previous longitudinal studies, the program avoids continuous data-gathering on subjects through time, and concentrates on intensive investigation cross-sectionally at a given developmental phase. No single methodological approach is adopted but rather a combination of interviews, structured and unstructured observational and questionnaire methods is used. The focus of research questions is on both specific variables, for example, the later developmental expressions in the 2½-year-old of a neonatal pattern of vigorous effective oral behavior, and on general areas of descriptive data-gatherings, for example, the exploration of early marital events affecting the courtship and decision to marry. Since the progress to date in the projects on neonatal behavior (Project No. MCR(C)11) and on 2½-year-old behavior (Project No. MCR(C)10) has been adequately summarized in the Annual Project Reports, and since the exploratory nature of the study of newlyweds has made our current activities difficult to communicate in brief form, the remainder of this annual report of the Child Research Branch will be devoted to a more detailed description of the study of newlyweds who serve as the first cohort of the longitudinal study.

Study of Newlyweds

I. Aims

In January 1960, after approximately 5 months of planning activities by our interdisciplinary Couples Study Planning Group,[1] it was possible to prepare a tentative statement of aims and procedures for the study. The statement also contained a description of the place of the study within the total program of the Child Research Branch (Biosocial Growth Center). The purpose of the present report is to summarize developments in the project since last January. Our general goals for the study, general selection procedures and general study design remain essentially the same and will not be reviewed herein.[2] In the course of the past year, however, experiences in exploring 40 young couples' initial marital adjustment have helped us to reformulate hypotheses and concepts, and also to specify our methods.

In January 1960 we stated, "The study will

[1] This group is currently composed of Paul Blank, M.S.W.; John D. Campbell, Ph.D.; Naomi Costello, B.A.; Arden Flint, M.D.; Wells Goodrich, M.D.; Ann Lewis, B.A.; Harold Raush, Ph.D.; and Walter Sceery, M.S.W.

[2] For complete statements of the general goals and design for the project see

(a) "Developmental Patterns in the Young Family: A Research Problem," by D. Wells Goodrich, January, 1960.

(b) "A Research Program on Early Biosocial Growth," by D. Wells Goodrich, Richard Q. Bell and Harold Raush, July 1960.

assess several interrelated dimensions of family interaction which delineate developmental processes salient to the prepregnant, early marriage situation and to the neonatal period following the first child's birth. Each of these situations of "crisis," or change in family adaptation patterns, is viewed in dynamic and in structural terms,[3] the aim being to discover elements and processes within the initial marriage relationship which have predictive power for later patterns of family development. The variables chosen for study are relevant to the initial father-mother-infant relationship. The interest is equally in the influences of the infant upon his parents and in the influences of the parents upon the child. By testing several specific hypotheses in this problem area, we aim to make a contribution to knowledge of the transactional processes within the young family which in some measure determine the child's later ego strength."

As now formulated the project has four general foci:

1. The naturally-occurring events of the courtship and early marital relationship, and the initial stages of marital career development.
2. Developmental changes in the couple's perception of their preparental and parental role functions, from the phase of initial marriage to the paranatal period.
3. The development of the infant, in particular patterns related to Bell's[4] neonatal factors of skin-sensitivity and oral effectiveness (This portion of the work is at this time being carried out by Dr. Bell as an independent, but closely related project).
4. The initial triadic adjustment of infant-mother-father during the neonatal period. (This portion of the study will be initiated during the coming year with the pilot study of 40 couples.)

The project is concerned with several specific aspects of family interaction which serve to define the variables and to guide hypotheses about family relationships and developmental changes, including:

1. Feeding and eating as a setting for interpersonal interaction.
2. Affectional physical contact (touching, holding) as a setting for interpersonal interaction.
3. Freedom of expression of affect versus inhibition of affective expression in interpersonal interaction.
4. Modes of marital decision-making in new or problem ("crisis") situations.
5. Marital and parental role activities and perceptions, especially with regard to sexual adjustment, other forms of affectional contact, parental planning activities, feeding-eating activities, housekeeping activities, occupation, socialization activities with friends, and relationships with close family members (in-laws).

With reference to these general foci and specific aspects of family development, the project is studying both relationships (e.g. the couple as a unit, the mother-infant dyad as a unit, etc.) and attributes of individuals (e.g. of three-months' married husbands, of four-day-old infants, etc.) as illustrated in the diagram on the next page.

It will not come as a surprise to anyone familiar with the vicissitudes of scientific endeavor to learn that between January and December 1960 the project staff revised many of the initial plans. Between January and May, 9 couples were studied in an informal exploratory way. Since June, 31 couples have been assessed in a pre-pilot project by means of joint home interviews, individual tape recorded interviews, the color-matching test, the improvisations, and a set of questionnaires. As a result of this experience it has been possible to standardize the color-matching test, the questionnaires, and the improvisations procedures, all of which are essentially new and devised specifically for this project.

The development of theoretically meaningful, semi-structured interview techniques has, however, presented considerably more methodological, theoretical and clinical problems than had been anticipated. By September we had to face the fact that, while we had developed and tried out four separate interview schedules during the

[3] "Structure and Process in the Primary Crises of Personality Development," by D. Wells Goodrich, presented at the meeting of the International Preparatory Commission of the International Association for Child Psychiatry and Allied Professions, February 1960.

[4] R. Q. Bell: Relations Between Behavior Manifestations in the Human Neonate. Presented at the annual meeting of the Association for Research in Child Development, 1959.

summer, none met the requirements of the project. During this fall, the major effort has been to develop a clinically meaningful, standard and semi-structured interview form which will cover most of the areas of theoretical interest to us and also be reliable and suitable for various kinds of statistical analysis. A few couples have been seen since September for the purpose of testing portions of the interview procedure. Also this fall it has been possible to begin preliminary work on data analysis procedures.

During 1961, we anticipate studying 40 couples with the standard procedures which have been developed. This will represent a somewhat more extensive and expensive data-gathering effort on each couple than had been anticipated in the spring of 1960, but it will permit us to examine a large proportion of the phenomena which the planning group had decided would be worth examining. In order to be able to differentiate between changes in the marital relationship associated with simple maturation and changes associated with a specific developmental phase, at points in time (such as pregnancy or the neonatal phase) when the couples are reassessed, a control group of matched couples will be studied who, though married a comparable time, have not yet experienced the phase. The data from these couples will be "mined" intensively, exploring patterns of variables and relationships between theoretically connected data. The information on these 40 couples will serve as a basis for developing final data analysis procedures.

After these data have been collected, we expect to refine our assessment procedures, selecting only those most promising hypotheses and techniques, and beginning to assess each couple in a more "streamlined" fashion. By this means, after 1961, we expect to be able to increase our rate of assessment of new couples to 15 or 20 per month.

Two other research efforts are planned for 1961 (under the direction of Drs. Harold Raush and Arden Flint); the initiation of a small number of intensive personality studies on individual couples and the execution of a pilot study on the neonatal triad of infant-mother-father. Dr. Richard Bell's current project on the relationships of neonatal behavior characteristics to behavior patterns in the 2½-year-old will not be completed before 1963. We anticipate also that it will require 2 to 3 years more before a really meaningful integration of the Newlywed Study with the Infants Study can be achieved in operation.

II. Hypotheses

In our initial plan for the Couples Study, it was stated:

"It is our intention that the couples represent, broadly speaking, the socioeconomically middle group of suburban young families. In this sense, the social characteristics serve as control variables. It may be possible to include in the final proposal some examination of three questions of theoretical interest: (1) The relationships between grandparents' child-rearing behaviors around the issues of feeding and affectional contact and these areas of interaction in the new family; (2) the influence of intergenerational conflict, as it may be expressed in contrasting definitions of parental role, upon events in the neonatal period; and (3) the influence of such factors as sibling birth order, temporal spacing of children, number of siblings, major disruptions in early family relationships, etc., in the parental family of orientation upon the neonatal crisis."

The general hypotheses of interest in January 1960 were:

1. "Certain dimensions of the initial marital role relationship, expressed in the setting of affectional contact, will be predictive of aspects of the mother's contact with her newborn.

2. Certain dimensions of the initial marital role relationship, expressed in the setting of feeding activities, will be predictive of aspects of the mother's feeding of her newborn.

3. Extreme patterns of neonatal oral integration (vigor-and-effectiveness of eating) will influence the interactional events of the neonatal crisis in selected families.

4. Extreme patterns of neonatal skin responsiveness (sensitivity to light touch and temperature change) will influence the interactional events of the neonatal crisis in selected families.

5. The conflictedness of the mother's relationship with the maternal grandmother in settings involving affectional contact or feeding activities will be predictive of aspects of the mother-infant relationship in the neonatal period.

6. Certain social characteristics of the couple's families of orientation, if related to current social characteristics of the marriage, may predict transactional processes in the neonatal family crisis."

Illustrative specific hypotheses of interest to the Planning Group follow:

1. Parents with low mutual expression of affectional contact, if provided with a highly skin responsive infant, will foster greater bodily tension in neonatal infant-holding situations.

2. Parents with a marital interaction pattern of rigid maternal dominance and paternal passivity in feeding settings will foster greater tension in neonatal mother-child feeding settings than other marital interaction patterns.

3. Infants low on the vigor-and-affectiveness-of-eating factor, born to passive women (as defined in specific marital settings) will tend to show greater tension during mother-infant feeding activities than will other types of infant-mother pairs.

4. Couples with rigid marital role behavior patterns in the prepregnant phase will tend to show slower stabilization of neonatal mother-child interaction patterns, and more frequent infant disturbances, than couples with more flexible role behavior patterns.

5. Tension in mother and in infant, during neonatal situations of infant feeding and infant holding, will be observably higher in families where the mother-maternal grandmother relationship was conflicted in these areas than in families unconflicted in these respects.

On the basis of our experience during the past year the major questions guiding the current work of the Couples Study have been extended and further specified. We are now concerned with exploring systematically the patterns of initial adaptation between the newlywed husband and wife, as well as the patterns of individual development of each individual. These patterns, to be described rather briefly below, represent the major interests of the research planning group and have guided the development of our methodology. Formulation of these patterns at this time will serve to guide our initial efforts at data analysis.

In one sense the Couples Study is concerned with dimensions of the newlywed relationship which may be expected to relate to general effectiveness as future parents of a young infant. For example, the pattern of nurturance within the newlywed marital transactions themselves may be expected to bear a set of contingent connections to the nurturance-neglect dimension of the parent-infant relationship later on. The actual pathways of influence for such a general aspect of the initial marital adaptation, however, may not be feasible to specify at this time, or perhaps for a long time to come. These general aspects of the initial marital relationship have been selected for study, for the most part, because they are relevant to two pervasive interests of ours: an interest in the marital career as such, as it changes through time and without regard necessarily to child rearing, and an interest in the impact of these patterns upon several types of infants who may be born to the couple.

In addition to assessing the nurturance-pattern in the newlywed relationship, the project is exploring such general interpersonal patterns as collaborative or noncollaborative modes of decision-making, the shared or nonshared activities,[5] the relative salience of various newlywed activities [5] within their values about early family life, the currently-held prospective fantasies about initial parental experience, and the patterns of resolution of marital conflict situations involving either differing perceptions of reality or differing aims or intentions. Within the developmental stage of newlywed marriage, a major task is the mastery of initial sexual adjustment. This adjustment is explored in an individual interview with each spouse. Various forms (see "Structure and Process in the Primary Crises of Personality Development," by W. Goodrich, Feb., 1960) of mastery or failure to master this developmental issue will be described. The connections between these forms of developmental stage mastery and the general patterns outlined above may then be explored. Existing studies do not agree on the rate or form of resolution of initial sexual difficulties. Some reports suggest that sexual adjustment takes place in the course of the first several weeks of marriage, other studies suggest that sexual adjustment is not achieved in the average couple for many years.

[5] The activities which are most intensely studied, aside from the sexual area, are housekeeping, feeding, parental planning, general physical affection, socialization with friends, participation with close relatives, and occupational activities.

In addition, the couple's patterns of marital career development will be explored in a joint interview with husband and wife, focusing upon the major change events which took place at their first meeting, through the courtship, the wedding, and the honeymoon, and up to the initial establishment of a separate home life. We seek here a better understanding of major interpersonal influences within the relationship, or major external forces influencing the couple, which have shaped past decisions and marital career changes.

In a more specific sense, the Couples Study is concerned with dimensions of the newlywed relationship which grow out of the Infant Study, i.e., the oral effectiveness and skin sensitivity of the newborn. For example, variables within the Couples Study have been selected because they are assumed to reflect the latent capacity of the couple to respond (at a later date, after the arrival of the firstborn) effectively to a low-drive, low efficiency feeding pattern in an infant. This pattern would only be studied for those couples who receive such an infant, and obviously it would not be assessed routinely as an aspect of marital career development. The patterns which grow out of the Infant Study include also the capacity of the couple to respond to an orally demanding infant, their capacity to respond warmly to the skin-sensitive infant, and their capacity to accept a low-skin-sensitivity infant, without demand that it respond. These rather more specific interests have led us to include in the Couples Study an investigation of patterns of physical contact and of feeding activities in the newlywed marriage, as well as to focus heavily upon the individual history of each spouse with regard to physical affection and feeding activities vis à vis his or her parents while growing up.

Since the general dimension of expressivity or inhibition of affective expression appears to be emerging from the data of the first year's Nursery School observations as possibly related to the infant study variables, we are interested in this dimension as it may appear in the initial marital adjustment of each couple and as it may be reported to have existed for each spouse during his or her earlier parental relationships.

We also will be in a position to examine another set of relationships, namely, the relationships between the image each spouse has of developmental experience with his or her parents and the aforementioned patterns of newlywed adaptation or patterns of family adaptation to the arrival of the newborn. These areas of developmental experience, tapped in the individual interview of each spouse, include patterns of parental availability and nurturance, parental neglect and unavailability, parental behavior in relation to food preparation and family socialization at mealtimes, physical affection with parents and the expression or inhibition of impulses in each spouse's family while she or he was growing up.

III. Method

The Couples Study uses a combination of interviews, direct observations of marital interaction, and questionnaires.

A. Interviews

1. An initial two-hour home interview is carried out during which the couple's commitment to the study is outlined, identifying and social background data are obtained, information about the sequence of events and decision antecedent to the marriage is obtained, and exploration of the current marital relationship is initiated.

2. Two separate interviews at NIH are carried out with each spouse to explore (a) the history of developmental relationships with father and with mother (with regard to nurturance-availability, feeding, physical affection, and other areas of high satisfaction or conflict); (b) current sexual adjustment and current marital conflict or satisfaction; and (c) the prospective images of self and spouse as parents as well as of the fantasied first infant.

3. A final joint interview is then carried out in the home during which exploration of the current patterns of marital interaction is completed, any anxieties or questions stirred by our investigation are dealt with, and the conditions for possible future contact between the project and the couple are clarified.

B. Observations of Marital Interaction

1. The color-matching test: This 15-minute experiment presents the couple with 20 swatches of color in standard sequence, with the instruction to come to an agreement between themselves as to which color best matches the experimenter's colors. Unbeknownst to the couple, the situa-

tion is arranged so that in 10 out of the 20 attempts to match colors with the experimenter no agreement is in fact possible. In these 10 instances the couple is faced, in effect, with a situation in which each perceives reality in a different way and in which there is no immediate clue as to the source of the conflicting perceptions. Their responses are tape recorded and analyzed to reflect the degree of difficulty in finding a resolution for each conflict and the modes of decision-making employed in response to this type of ambiguity stress. We believe it may be valuable to note here which couples remain psychologically alined with each other against the confusing experimental situation and which couples are lured into a stubborn power struggle with each other. The maintainance or failure to maintain, psychological alinement under ambiguity stress may be predictive of subsequent coping behavior by the couple in new or strange situations.

2. The Improvisations: These are a series of four psychodramatic scenes, 4 to 6 minutes in length, in which the couple enacts a situation containing a conflict of goals (intentions). Here we record the verbal behavior and analyze for contrasting patterns of conflict resolution; we also observe certain nonverbal aspects of interpersonal behavior in the midst of conflict, particularly the employment of physical closeness or physical distancing maneuvers as part of the pattern of conflict resolution. The degree of empathy evidenced in communication and the degree of face-saving in decision-making are also of interest here.

C. Questionnaires

1. The Daily Dilemmas: This questionnaire assesses the relative salience of seven areas of marital interaction within each spouse's conscious value system. The form of the instrument permits a ranking of relative salience for parental planning activity, feeding activities, housekeeping, physical affection, occupation, contacts with close relatives and contact with friends, by the method of paired comparisons. In preliminary data on 22 couples it appears that newlyweds may consider the activity areas of parental planning, feeding and physical contact to be highly salient as sources of satisfaction and that the area of occupation is of low salience as a source of satisfaction. Studies in the literature suggest that disparity in values between middle class husbands and wives may accompany conflicted marriage.

2. The Daily Routines Inventory: This instrument is modeled after the approaches of Herbst, Hoffmann, and others, and provides an opportunity for each spouse to describe current activities and decision-making patterns for self and for spouse (in the seven areas noted above) in terms of relative frequency of participation. A second form of the Daily Routines gets at the "ideal" image of the partners' participation in these seven action areas at a future time when they have had a newborn infant to care for.

3. Marital Empathy Inventory: This questionnaire presents 14 situations of conflicting wishes between a hypothetical husband and wife pair. It provides an opportunity for the subject to choose between four resolutions for each conflict: a resolution in which only the husband's position is recognized, a resolution in which only the wife's position is recognized, and two compromise (empathic, "role taking") types of resolution, one in which the wife's and another in which the husband's needs take moderate precedence. These measures of empathy in the marriage can be examined cross sectionally in relation to observational data obtained during the color matching experiment and the improvisations and also longitudinally in relation to aspects of nonverbal communication in the initial parent-infant relationship.

4. The Locke-Wallace Marital Adjustment Inventory, an instrument which has been used in a number of published studies, is being used as a global index of "marital adjustment."

5. In order to supplement our interview data with certain concrete informational items more economically gathered in questionnaire form, we have devised an Interview Supplement Questionnaire. The interview itself focuses somewhat more on affect-laden aspects of the marriage, such as satisfactions or values with regard to current activities; this supplemental questionnaire includes such information items as the frequency of visiting with relatives, or the frequency of shared versus segregated housekeeping activities, etc.

In order to clarify the relationship of the questions asked by the Study to the methods de-

scribed above, we have prepared a series of charts to locate types of data (see appendices I–VII).

IV Data analysis

From the foregoing it is apparent that the Couples Study, as now designed in the pilot phase of the work, is oriented toward a rather wide range of problems. Over the course of the next year we anticipate reducing our focus considerably—particularly as regards the general dimensions of the marital relationship. The choice of patterns to explore further, we feel at this stage of our knowledge, would be best based empirically upon pattern analyses of data gathered by a wide net, on the initial group of 40 couples. In this analysis of data we plan wherever possible to use direct coding of interview content and behavioral indices rather than to rely solely on ratings or interviewer judgments. With regard to the statistical processing of the data, we intend to rely on a combination of factor analytic and other correlation techniques, and appropriate nonparametric tests. During the course of this research we will obtain from each couple some similar data at several points in time, we will be able to assess patterns of change by use of panel analysis procedures.

Future Directions

The program outlined in this annual report can be characterized overall as exploratory research. Because we are looking in new ways at phenomena which have, for the most part, not been examined in these ways, there seems little doubt that, at a descriptive level, observations of salient new patterns of early family development will be obtained. Since the approach includes systematic assessment techniques as well, we are confident that defined relationships between certain variables will emerge, which may clarify the nature of the early developmental tasks of the family or the nature of certain family styles of coping with these tasks. These research results of the program, gratifying as they will be, should they be produced, will not satisfy; for a primary scientific need is for new and powerful concepts. Theoretical concepts are needed which will integrate the complex and diverse events and processes within the family structure in new ways, events and processes which, under varieties of contingent circumstances, can become relevant to the mastery or the failure to master a developmental task (see footnotes 2 and 3).

The establishment of this developmental research unit has required two years so far, since its initial proposal; in some respects the operation is as yet in formative stages. One can legitimately inquire what opportunities for worthwhile extensions of the current program—using the current operations as a basic administrative structure—could be envisaged beyond 1963. Based upon the data of the 1961 pilot study, we now assume that several large samples of families can be usefully obtained, each sample being employed to test a different set of hypotheses, growing out of the pilot study. There also is the possibility that the data of the pilot study will indicate the need for a much heavier research investment in the study of pregnancy, as an intermediate phase between the newlywed and the neonatal phases, before very many valid conclusions can be drawn about developmental continuities from our data.

With the remodeling of several rooms in the building in the next year, it may be possible to vacate the apartment and make it available for the round-the-clock observation of infant behavior and of parent-infant interaction patterns in a way not previously possible. Sooner or later, too, it would seem worthwhile to relate these developmental studies directly to a study of emerging psychopathology or ego strength in the preschool child. Informal observations suggest the ready availability in currently observed samples of children of adequate numbers of cases of separation anxiety or of problems in impulse control (??pre-hyperaggressive children); it seems likely that some of our developmental variables have systematic relationship to the occurrence of such syndromes. Finally, it does not seem inconceivable that at some future date, a productive liaison may become possible with other laboratories at the National Institutes of Health, which are also interested in developmental problems, but from a more purely physiological point of view. This liaison would supplement our current collaborative arrangements with the Laboratory of Psychology and of Socio-environmental Studies.

Clinical Neuropharmacology Research Center

The past year has seen a further development of the collaborative program between CNRC and Saint Elizabeths Hospital, and given strong indication of the viability and potential of the symbiotic arrangement entered into by C.I., NIMH, and Saint Elizabeths Hospital. The Hospital has established eleven research positions for the development of research within the Hospital; these are being filled in consultation with the CNRC to complement the activities of the CNRC. Dr. F. N. Waldrop, Associate Director of Research for Saint Elizabeths Hospital (a Saint Elizabeth staff member) has done much to coordinate research activities, both within and outside the William A. White Service, and to further collaborative studies between Saint Elizabeths Hospital and CNRC staff. The Research Committee of the hospital (composed of senior staff members of the hospital, and of the CNRC) has continued to provide a useful planning board for the development of research throughout the hospital, and has served as a channel of communication between the NIMH and the hospital. A small Senior Staff Committee has recently been appointed to consider matters of planning and policy within the hospital, and make appropriate recommendations to the superintendent. Both the chief, CNRC (as director of research of the hospital) and the associate director of research of the hospital (Dr. F. N. Waldrop) serve on this committee. Among matters now being discussed by this body are the changing patterns of treatment throughout the hospital, and the evolution of new clinical care facilities (such as Day and Night Hospital, and possible O.P. Clinics) geared to the needs of the community of which the hospital is a part. A Program Consultation Committee has been created within the hospital to advise the various services in bringing about changes in their therapeutic programs. The Laboratory of Socioenvironmental Studies of CI, NIMH, as well as the CNRC, have been invited to participate in these various operations; and, thus, are likely to benefit from an unusual opportunity for studying a long standing and leading institution during a period of active change and evolution. Equally, the Pathological Laboratory Services of the hospital have been recently strengthened by the provision of new equipment, and the laboratory is now able to partake in research activities more actively than hitherto. The Personnel and Engineering Branch of the hospital have been helpful to the CNRC on many occasions. Throughout all these phases, the superintendent of the hospital, Dr. Winfred Overholser, and the assistant superintendent, Dr. Dale Cameron, have shown the CNRC and its individual members every consideration, courtesy, and support. The fact that a number of CNRC staff live on the hospital grounds is a fitting illustration of the many human links which have developed between the CNRC and the hospital, and which are closing the respective segments of the joint operating into a comprehensive and representative whole.

Dr. Irwin Feinberg, who joined the Center in July, 1960, from the Laboratory of Clinical Science is spending the present academic year in the laboratory of Dr. Jean Piaget, in Geneva, Switzerland. Similarly, the laboratories of the CNRC have accommodated three guest workers, during the past year: Dr. Leiv Gjessing of the Dikemark Municipal Hospital, Oslo, Norway, working with Dr. H. Weil-Malherbe; Dr. R. von Baumgarten, Professor of Physiology, University of Göttingen, Göttingen, Germany, working with Dr. G. C. Salmoiraghi; and Dr. R. Whalen, Research Fellow, from the Department of Psychology, University of California, Berkeley, Calif.

The CNRC has been visited by some 180 visitors during the past year. Twenty Guest Seminars have been held, some of them planned jointly with the hospital.

Psychiatry

As hitherto, the clinical studies of CNRC have centered on problems of the care and the treatment (including pharmacotherapy) of the chronic schizophrenic patient. This aspect was chosen for three separate reasons. In the first place, the chronic schizophrenic patient forms the numerically preponderant problem of the hospital, and it was hoped that any examination of this problem could be of service to the hospital. Secondly, the relative stability of a chronic population offers an opportunity for the calibration of research methods and instruments which are likely, at a later stage, to find their

application in the more mobile populations of an acute admission service. Lastly, the establishment of a sound clinical care program within the William A. White Service was judged a necessary preliminary to the pursuit of a more discrete program of clinical investigation of selected conditions and syndromes, including metabolic studies in man.

As mentioned in the last year's report, a comparative drug study involving two tranquillizers and a placebo was initiated by Dr. A. Hordern, visiting scientist, and Dr. J. Lofft, of the Saint Elizabeths Hospital staff. This study was planned to proceed simultaneously on six wards of the William A. White Service of Saint Elizabeths Hospital. As well as being a drug study, the investigation was intended to examine in some detail, and with special emphasis on sources of error, the effects of nondrug factors known to profoundly affect the response to medication in a typical mental hospital setting. Essentially, the study thus aimed at (1) determining the effect of physical environment on the responsiveness to drugs; (2) determining the effect of various types of nursing care and, particularly, nursing in small groups, on drug response; (3) the cultivation of native therapeutic and research skills often found, but ignored, in ward personnel; (4) the codification of a number of questions commonly entered into a drug trial in a mental hospital setting, (i.e., the Selection of Patients, Selection of Drugs, Selection of Experimental Design, the Technique of Drug Administration in a Blind Study, the Assessment of Side Effects, Management of Adequate Laboratory Control, and the Comparison of a number of Clinical Evaluative Techniques); (5) the monitoring of attitudes toward research; and (6) the institution of data reduction techniques suited to a drug study conducted in this manner. The dimension of the study was kept within the limits of applicability and feasibility in a mental hospital setting so as to encourage a re-testing of the conclusions in other Services of Saint Elizabeths Hospital and, conceivably, other state institutions. An analysis of the data, carried out by Dr. Wilson Taylor, though not yet complete, has pointed to several powerful sources of error. Some positive and empirical trends, however, are equally apparent. A coordinated program of clinical investigation proceeding simultaneously in a number of wards of a mental hospital building (rather than a single "research service") can profoundly, and with great economy, contribute to the raising of both therapeutic and research skills of personnel throughout such a building. The systematic review of methods and of data can become an instrument of education of personnel. Nursing in small groups, structured around the skills of attendants and of well preserved patients, can act as a pacemaker in profoundly changing the therapeutic climate of a chronic ward. Such a program, while contributing to the rehabilitation of patients, reduces the consumption of tranquillizers on a ward; it also changes attitudes of personnel. Rating scales, while having their uses, also have their serious limitations. Some documents furnish much higher inter-rater reliability than others, and some are more sensitive to fluctuations in the clinical picture over time than others.

The above study was replicated by Dr. M. Hamilton (Visiting Scientist) and Dr. F. N. Waldrop, in 63 male and 63 female patients selected for the degree of their chronicity and the severity of their symptoms, and transferred to the William A. White Service for the purpose. Prochlorperazine, Trifluoperazine, and inert placebo were compared, in a 3-way factorial design, with the effects of intensive nursing and social manipulative procedures; the design allowing for an evaluation of the effectiveness of drugs, and social therapy independently and in conjunction. Both drugs were found effective in the doses given, the effect being achieved in from 3 to 5 weeks. Social and group nursing techniques alone (i.e., in the absence of drugs) led to a significant reduction in symptoms on the male wards, but not on the female wards; the effects (in the male wards) being comparable to those achieved by drugs alone. On the female ward, the moderate improvement due to chlorproperazine could be enhanced by group nursing techniques. This study, like its predecessor, pointed to many pitfalls in any investigation in chronic schizophrenic patients which attempts to make due allowance for nondrug factors entering into a treatment program. It may be noted that cognate research designs are now being used in other parts of Saint Elizabeths Hospital, and that, at the initiative of the hospital staff, group nursing techniques as used in William A. White

Service are now being tested in other wards. The research nurse, Mrs. Helen Sainato, has been invited to present a paper on the work at the coming meeting of the American Psychiatric Association. With the cooperation of the Medical Arts Section of the Division of Research Services, NIH, the initial methodological study has been made the subject of a Teaching and Demonstration Exhibit. This was shown at the 116th Annual Meeting of the American Psychiatric Association in Atlantic City in May, 1960, and the 14th Clinical Meeting of the American Medical Association. Requests for the documents used in this study have been received from over 100 State institutions.

In the context of the above study, and also as a part of an independent program in Social Rehabilitation on his ward, Dr. Sheppard Kellam has now had one year's experience in the use of his Social Contact Matrix for measuring, in an objective manner, the amount and pattern of social contact of a patient in a ward setting. The instrument has been found useful and sensitive in quantifying changes brought about by psychoactive drugs, and group nursing techniques. Dr. Kellam is now attempting a correlation between Symptom Fluctuations in the individual patient and the pattern of social contact by the patient. He is also collaborating in the multihospital drug study organized by the Psychopharmacology Service Center, and will thus have a further opportunity to use the Matrix in a setting other than Saint Elizabeths Hospital.

A number of joint studies between the CNRC and other laboratories of CI, NIMH, are also proceeding. Dr. Irwin Feinberg, in conjunction with Dr. Roger McDonald of the Laboratory of Clinical Science, has examined the excretion of vanyllyl mandelic acid (VMA) in patients suffering from depression and treated with electro convulsive therapy. Data from only eight subjects are available to date. Some very high levels of VMA excretion before any treatment have been noted in these patients, suggesting, possibly, that such patients may be producing epinephrine in abnormally large quantities. Age as a variable, however, has not been allowed for in these subjects, and an extension of the series is needed before any conclusion can be drawn. In conjunction with Dr. Carlson, of the Laboratory of Psychology, Dr. Feinberg and Mr. R. Koresko have also examined the ability of schizophrenics to perceive velocity (i.e. predict the time of arrival of a moving line at a target point after its disappearance). This response has been studied in some 40 patients. It was found that many schizophrenics, while responding appropriately to varying speeds and target distances, still manifest a markedly prolonged delay. The evidence so far does not suggest that this delay may be necessarily related to a lengthening of the reaction time (a function notoriously disturbed in schizophrenia). In yet a third study, Dr. Feinberg has examined (in conjunction with Dr. Frederick Snyder, of the Adult Psychiatry Branch, and Mr. Richard Koresko), the quantity of dreaming in nonhallucinating and hallucinating schizophrenics. The technique of Kleitman and Dement (in which EEG and eye movements are used to monitor and signal dream activity) was used. The results so far suggest that the amount of dreaming does not differ significantly in the two groups. This study was carried out in patients hospitalized for brief studies only. It will be extended to more chronic patients during the coming year after Dr. Feinberg's return from Dr. Piaget's laboratory.

In sum, then, the clinical activities of the Clinical Neuropharmacology Research Center during the past year have been expended in creating within the William A. White Service a standard of care and observation which will serve as a necessary prerequisite for the more detailed studies which are now being planned. It is also hoped that the medicolegal problems connected with the prosecution of investigative work within the William A. White Service will have been resolved in the foreseeable future; Dr. Winfred Overholser is giving these endeavors his personal support.

Chemical Pharmacology

In pursuit of an old standing interest, Dr. H. Weil-Malherbe, together with Dr. E. R. B. Smith, has continued with studies aiming at developing reliable, sensitive, and specific methods for the routine assay of catecholamines in plasma. By combining an ion exchange procedure with chromatography on alumina, an additional purification of plasma extracts could be obtained without sacrifice in accuracy or sensitivity. In Dr.

Weil-Malherbe's hands the ethylenediamine method has yielded results in good agreement with those obtained by the trihydroxyindole method. The ethylenediamine method, however, resulted in a fourfold increase in sensitivity, thus potentially allowing the measurement of concentrations of 0.1 micrograms of catecholamines per liter. Similarly, Dr. H. Weil-Malherbe and Dr. E. R. B. Smith have developed methods for the differential estimation of metanephrine and normetanephrine, 3-4 dihydroxyphenylacetic and 3-4 dihydroxymandelic acid in urine. These methods will be put to use in material obtained from subjects in various stress situations. The NASA Space Task Group have invited collaboration in these studies. In a cognate area, Dr. H. Weil-Malherbe together with Dr. Axelrod and G. Hertting (of the Section of Pharmacology, Laboratory of Clinical Science) are studying the penetration of tritium labelled epinephrine and norepinephrine into various parts of the brain, and into the pituitary gland. Previous studies had shown that the amounts of radioactive epinephrine and norepinephrine penetrating into most parts of the brain are very small, and could probably be accounted for by the blood content of the tissue. In the hypothalamus, however, larger amounts were consistently found; though even in this area the uptake appeared slower than in most extracerebral tissues. It was shown that a number of drugs (including chlorpromazine, cocaine, and tyramine) which block the uptake of norepinephrine by extracerebral tissue (particularly heart) do not affect the accumulation of radioactive norepinephrine in the hypothalamus. The relative freedom of entry into the hypothalamus seems to be specific for norepinephrine and epinephrine. It was also found that the pituitary gland takes up circulating norepinephrine rapidly and with great activity. The results suggest that the so-called central effects of epinephrine may in part be mediated by the sensitivity of the hypothalamus to this agent.

Dr. Weil-Malherbe and Dr. H. Posner have also pursued further their studies on the effects of various drugs on the concentrations, and in the intracellular distribution, of catecholamines within the brain. Previous studies (by Dr. Weil-Malherbe and his colleagues) had shown that brain catecholamines may be separated into two intracellular franctions, one present in the cytoplasmic sap and the other sequestered in particulate matter. According to current concepts only the former is biologically active while the latter may serve as store. The effect of reserpine, phenylisopropylhydrazine (Catron, a monoamine oxidase inhibitor), pyrogallol, (an inhibitor of the enzyme catechol-O-methyl transferase), and 3:4-dihydroxyphenylalanine (DOPA) alone and in combination have been examined. In keeping with previous observations made by Dr. Weil-Malherbe, reserpine was found to cause a more rapid depletion of the soluble than the particulate fraction. This action was inhibited by phenylisopropylhydrazine (Catron), but not by pyrogallol. Phenylisopropylhydrazine alone induced a rise in norepinephrine concentration in the soluble fraction only, without significantly affecting the concentration of dopamine. Pyrogallol alone had no significant effect but was found to potentiate the action of phenylisopropylhydrazine, as well as of dopa, suggesting, for the first time, the involvement of the enzyme catechol-O-methyl transferase in the endogenous metabolism of brain catecholamines. These findings compel a re-evaluation of the suggested role of reserpine in terms of storage and release of catecholamines within the brain, and focus attention on the importance of intracellular economy and metabolic compartmentalization in the mode of action of the phrenotropic drugs.

In pursuit of previous studies, Dr. H. Posner has continued to examine the intermediate metabolism of chlorpromazine and related compounds in the human body. He has shown the existence of at least four different phenolic metabolites of chlorpromazine and promazine which are excreted in the urine as conjugates of glucuronic acid. The chlorpromazine sulfoxide would appear to be a minor metabolite of chlorpromazine. Owing to a lack of reference substances of known constitution, it has so far not been possible definitely to identify any of the metabolites. It is hoped, however, that as the substances become available the possible relationship between pharmacological activity of chlorpromazine and of its metabolic products may be further clarified. Such studies are being planned in conjunction with Dr. Cosmides of the Staff of the Psychopharmacology Service Center. In a cognate vein Dr. Posner, together with Dr. J. D.

Solomon (Director, Pathological Laboratories, of Saint Elizabeths Hospital) have examined the tests now available for the identification of phenothiazines in the urine and with special reference to the validity of the Forrest and Forrest Reaction. It was shown that whereas the test was reliable for chlorpromazine, it was apt to give false positive reaction for piperazine linked phenothiazines. Since the test is being used in the control of phenothiazine medication, a re-examination of it is in place.

Dr. Szara together with Miss F. Putney has continued to study the intermediate metabolism of psychotropic tryptamine derivatives with special reference to dimethytryptamine and N.N. diethyltryptamine. More recently he has added a third psychosomimetic indole derivative, -methyltryptamine to the compounds already examined. The side chain of the -methyltryptamine is identical with that of amphetamine, and it was found to undergo deamination similar to amphetamine. A scheme for the metabolism of -methyltryptamine has been proposed; this, once again, has stressed the importance of 6-hydroxylation in the metabolic handling of the drug. Dr. Szara has also improved his method for determining the 6-hydroxydiethyltryptamine in urine. This is now suitable for measuring small amounts of the substance in metabolic experiments in man.

These biochemical studies have been linked to behavioral observations, both in the experimental animal and in man. The animal studies (carried out in conjunction with Dr. Eliot Hearst) aimed at establishing the effects of psychotomimetic tryptamine derivatives, and their metabolites, on several types of behavior in various species and to correlate behavioral effects with metabolic variables in the same subject; an attempt is also being made to correlate behavioral effects with chemical structure and the chemical properties of various tryptamine derivatives. The early findings in rats (reported previously) have been confirmed and extended to both monkeys and pigeons. It was shown that the doses required to elicit a significant effect on performance (both in reward and avoidance situations) was lower for 6-hydroxydiethyltryptamine (the major metabolite of diethyltryptamine) than that for the parent substance (diethyltryptamine). Substances closely related in chemical structure to diethyltryptamine which are 6-hydroxylated (for example, -methyltryptamine) were found to have behavioral effects in rats and monkeys, whereas other structurally related substances (such as dihexyltryptamine) which are not hydroxylated exerted no behavioral effects. -methyltryptamine, which has a slower rate of hydroxylation than diethyltryptamine, also exerts a more prolonged behavioral effect than diethyltryptamine. It will be particularly interesting also to examine a number of inhibitors of 6-hydroxylation. Dihexyltryptamine inhibits the reaction competitively.

The effects in man of NN-diethyltryptamine are being examined in conjunction with several members of the Laboratories of Clinical Science and of Psychology, CI, in the multidisciplinary research ward at the Clinical Center. The project is intended to provide information on the relationship between the intensity of psychological effects of NN-diethyltryptamine and the metabolism (and especially the rate of 6-hydroxylation) of the drug. The drug is being given to chronic schizophrenic patients, and will be given to a number of acute schizophrenic patients and to normal volunteers. Since the study is in progress, no report can be made at the present time.

Behavioral Science

Following a study initiated last year, Dr. Eliot Hearst has continued to examine the generalization gradients for reward and punishment-controlled behavior in the Rhesus monkey. Baseline data in this area are lacking and are much needed for the appraisal of the effect of drugs on these functions in various motivational situations. It was assumed that the extent to which a subject may respond to a stimulus which resembles (but does not exactly correspond to) a stimulus used in an original conditioning situation may be a function of the type of motivation controlling the behavior at the time of the experiment. Dr. Hearst has used Intensity of Lighting (accompanying either reward or punishment behavior) as the conditioning stimulus, and measured generalization gradients between various intensities in conditions of reward and punishment. He has shown that generalization gradients for punishment controlled behavior were apt to be much flatter than for reward controlled behavior; there being much more stim-

ulus generalization under aversive conditions than under reward conditions. It was also shown that after discrimination training (i.e. the animals being specifically trained to respond to a bright light and not to respond to a dim light, and vice versa), both gradients were much steeper, but, nevertheless, avoidance gradients still were flatter than reward gradients. These studies are now being extended to auditory conditioning experiments and form a necessary preliminary to the study of effects of drugs and, quite possibly, of surgical lesions, or generalization. Since it has been suggested that the amygdala may have a specific function with regard to stimulus generalization, lesions in this area will be of particular interest.

Dr. Eliot Hearst has also examined the "resistance to extinction" function in pigeons, and is investigating the delayed alternating responding in pigeons, with special reference to the action of hyoscine, amphetamine, and barbiturates. Since this function shows some impairment in monkeys following lesions to the frontal lobe, and has also been shown to be affected by LSD–25, a study of this type of response in birds seemed worthy of examination.

In pursuit of studies carried on with the Department of Psychology at Berkeley, University of California, and also studies initiated at CNRC by Dr. R. P. Michael, Dr. Richard Whalen has embarked on a detailed examination of the environmental and physiological factors entering into sexual behavior in the cat. The pattern was chosen as a relatively stable pattern of behavior, and the physiological studies are designed to delimit the areas in the Central Nervous System respectively concerned with sexual arousal, ejaculation, and the effects of prolonged mating. It is hoped to develop a method for the local application of drugs to selected areas of the hypothalamus, and to record from relevant areas in the brain stem during mating. The studies should be of interest in view of the studies pursued in the Section of Limbic Integration in the Laboratory of Neurophysiology, NIMH.

Electrophysiological studies have centered on an examination of direct current (D.C.) changes in the cortex of the cat, an analysis of evoked potentials in the cortex and the geniculate body of the cat, and an examination of the patterns of discharge of respiratory and cardiovascular neurones in the cat, and in fish. Dr. Robert Gumnit, using a compact, sensitive, stable nonpolarizable electrode of his own design, has developed a method for recording Direct Current potential changes in the lightly anesthetized, and the conscious animal. The effect of auditory stimulation on these responses has been examined, and the localization of the changes in various areas of the cortex has also been studied. The method represents a definite technical advance in the recording of D.C. changes on the surface of the brain. The role of these slow shifts and their relation to the handling of sensory information by the brain is a matter of great interest, and, indeed controversy. The demonstration of the existence of these D.C. changes will provide a basis for further pharmacological enquiry. Of particular interest is the demonstration of D.C. potential changes (previously observed only in anesthetized animals) in the conscious, unanesthetized animal; and the response of these changes to auditory stimulation. These studies were carried out in conjunction with Dr. R. G. Grossman of the Walter Reed Army Institute for Research.

The subject of evoked potentials has also been pursued by Dr. Arthur S. Schwartz, who is studying the effects of small doses of LSD–25 on visual auditory evoked potentials, with special reference to differences in response in these two pathways. These studies represent an extension of the work done by Dr. E. V. Evarts, of the Laboratory of Clinical Science. Dr. Schwartz is also examining the role of the visual cortex in flicker discrimination and critical flicker frequence in the cat.

Dr. G. C. Salmoiraghi has pursued his independent investigation of the localization and patterns of discharge of the respiratory and cardiovascular neurones in the brain stem of the cat. In conjunction with Dr. Rudolf von Baumgarten, Professor of Physiology, University of Göttingen, Göttingen, Germany, who visited Dr. Salmoiraghi's laboratory for some six weeks during the summer, intra-cellular potentials from respiratory neurones in the brain stem of the cat were obtained. These represent the first intracellular recording from neurones in the lower brain stem. The data have amplified and extended the previously published data on the na-

ture of rhythmic discharge of neurones in the so-called respiratory areas. They have suggested that three factors may contribute to produce rhythmic discharge of respiratory neurones; namely, a self-reexciting mechanism within the inspiratory and expiratory networks of the neurones tending to maintain activity within these networks; some self-limiting mechanisms tending to limit the frequency and duration of the discharge; and an arrangement of reciprocal innervation which provides inhibition of one network when the other one is active. The findings have also suggested that the increase in the firing threshold, as a burst of action potentials progresses, may play a major part in regularly bringing the discharge to an end. A similar program has been followed in localizing the patterns of discharge of respiratory neurones in the goldfish. Respiratory potentials could be recorded from two relatively narrow strips, one on each side of the brain stem. These potentials could be divided into two groups depending on whether they occurred concurrently with the abduction or adduction of the gill covers. Neurones of either group, however, were found to behave rhythmically even after abolition of all respiratory movement by succinylcholine. The evidence suggested that the rhythmicity of discharge in the goldfish depends on mechanism or mechanisms not unlike those operating in the mammalian nervous system.

In a cognate vein, Dr. Salmoiraghi is now proceeding to localize the patterns of electrical discharge of cells related to the central control of blood pressure, and to analyze the mechanisms affecting repetitive discharge in these neurones. These physiological data will be used in subsequent analysis of the effects of drugs upon the central steering mechanisms governing respiration and blood pressure. If technically possible, drugs will be applied locally by multibarelled micropipette to cells in the relevant areas of the brain stem.

A welcome feature throughout the past year has been the marked interaction between the activities of the various sections. It is to be hoped that with the emergence of a stable organization, and the clear definition of some central themes to which the Center wishes to be committed, this interaction will increase, leading, in time, to a yield greater than the sum of individual endeavors.

Laboratory of Clinical Science

The Laboratory of Clinical Science has continued in its attempts to bridge the research areas between the basic biological disciplines and the problems of clinical psychiatry. The Laboratory represents a group of scientists and their associates, organized into sections representative of various biological and clinical disciplines, who pursue both independent and collaborative research relevant to mental illness. Their motivations and interests are strongly committed to the internuncial position which the Laboratory occupies in the Intramural Program, either by virtue of their own medical and psychiatric backgrounds or their experience and willingness to participate in a clinically oriented program. Because of the highly collaborative aspect of much of the research in the laboratory, the program is perhaps better outlined in terms of problem areas than by listing the contributions of the various sections. This report is, however, based upon original reports prepared by the various section chiefs:

Office of the Chief
Unit on Schizophrenia
Elwood H. LaBrosse
Unit on Psychosomatics
Philippe V. Cardon, Jr.
Section on Medicine
Roger K. McDonald
Section on Physiology
Edward V. Evarts
Section on Psychiatry
William Pollin
Section on Biochemistry
Marian W. Kies
Section on Cerebral Metabolism
Louis Sokoloff
Section on Pharmacology
Julius Axelrod

Schizophrenia

A current hypothesis relating to the pathogenesis of schizophrenia has postulated a defect in the metabolism of epinephrine in this disorder with the production of certain abnormal derivatives which may account for the major symptoms

of the disease. This hypothesis was developed at a time when the knowledge of normal metabolism of epinephrine was extremely vague and it was possible to account for only a few percent of the epinephrine released in the body, this being the epinephrine which was not altered by body metabolism but was excreted in the urine unchanged. In order to test this hypothesis in schizophrenia, it was first necessary to learn the normal pathways of metabolism. This, Dr. Julius Axelrod was able to do in a program of activities extending over the past several years so that in normal animals and man it is now possible to account for practically all epinephrine administered and also that presumably produced in the body in the form of two major metabolites, metanephrine and 3-methoxy-4-hydroxymandelic acid, and four minor metabolites including unchanged epinephrine. Based upon this work, Dr. Elwood LaBrosse and his associates in the past year have completed a definitive study of the metabolism of circulating epinephrine in a group of schizophrenic patients. The metabolites in this population were found to be qualitatively identical with that in the normal population with no evidence for the presence of abnormal metabolites. Only one compound, 3-methoxy-4-hydroxyphenylglycol, was excreted to an extent significantly different from that in normal volunteers (8.9 percent as opposed to 7.1 percent). This study argues against the presence of a major abnormality in the metabolism of circulating epinephrine in this disorder.

One of the interesting positive findings recently reported in a major segment of schizophrenia is that of Frohman and Gottlieb at the Lafayette Clinic, which suggests the presence in plasma of such patients of a factor which alters the carbohydrate metabolism of red cells. In the past year the laboratory provided an opportunity to Dr. Frohman to apply his test to schizophrenic patients and normal volunteers on our wards and to carry out the appropriate analyses in this laboratory. On the basis of blood samples submitted to him from 12 schizophrenic patients and eight normal volunteers and carefully coded to prevent their identity from being known, it was possible on the basis of his findings to predict correctly in a highly significant number the presence or absence of schizophrenia. On the basis of quite a different approach, Dr. Peter Mueller of the Unit on Psychosomatics, studying the response of free fatty acids to insulin, has found a reproducible alteration from the norm in a significant number of schizophrenic patients. These studies are being actively pursued with a systematic consideration of the many variables involved in an effort to determine to what extent either of these findings is basic to or characteristic of a subgroup of schizophrenic illnesses or is the result of adventitious factors.

In the family study area, a pilot study phase was completed by Dr. William Pollin and the Section on Psychiatry and a study of matched groups of families has begun. The emphasis continues to focus on an effort to use comparison of a schizophrenic patient and his nonschizophrenic sibling within the same family as an approach deemed likely to provide the maximal information concerning what types of emotional relationships and life history events may be related to the onset or course of schizophrenic illness. In the pilot study phase substantial differences were judged to be present in 8 of 10 families which seemed relevant to the appearance of schizophrenia in one child and its absence in his studied sibling. The matched study now beginning will compare differences in family relationships and life history events in one group of fifteen families where one of the children is schizophrenic and another is not, and in a matched group of 15 other families where one child is juvenile delinquent and the other is not, in an effort to answer the question: Which, if any, of the characteristics present in the families of schizophrenics and predominantly present in the life of the schizophrenic child, in comparison with his nonschizophrenic sibling, are actually unique to schizophrenia?

Biogenic Amines

One of the most interesting aspects of modern neurochemistry has been the finding that a number of amines with rather potent pharmacologic effects elsewhere in the body are differentially localized in various parts of the brain. These include norepinephrine, serotonin, tryptamine, histamine, among others. The distribution of these substances in certain functional areas of the brain and the alterations in their concentrations which occur in association with the action of potent psychotropic drugs such as reserpine or

iproniazid, have suggested that these agents play an important role in normal behavior or may even be implicated in schizophrenia. Although the synthesis, destruction and levels of these compounds in brain can and have been measured in animals and correlated with behavioral state, no such direct approach is possible in man for ascertaining the possible role of these substances in mental state or in the highly specialized behavioral patterns of normal and schizophrenic human beings. Studies in the blood or urine may serve to detect general abnormalities in the metabolism of these agents if they occur but are not sufficiently sensitive to elucidate their specialized metabolism in the brain. An ingenious technique based upon double-labeling has been devised by Dr. Irwin Kopin, which theoretically appears capable of yielding information on the brain metabolism of biogenic amines from studies of blood and urine, but numerous methodological problems remain to be solved before it can be applied to man.

Another approach has been carried out by Drs. William Pollin and Philippe Cardon, in which other members of the various sections have also participated. It employs the dietary or parenteral administration of certain known precursors of these and other amines in substantial amount, aided by the administration of drugs which inhibit monoamine oxidase and which would be expected to retard the destruction of brain amines and increase their concentration. Careful behavioral and psychiatric observation of patients under such conditions may serve to elicit mental or behavioral changes which may be characteristic of alterations in specific amine levels. In the course of the past year, there has been an opportunity to test a number of amino acids, including tryptophan, phenylalanine, histamine, glutamine, tyrosine, glycine and methionine, and two more immediate precursors of norepinephrine and serotonin. When approximately 10 times the normal dietary content of these amino acids is administered in successive studies, each for a period of one week, two of them were associated with significant behavioral change in a segment of the patients. Methionine was associated with increased activity and alertness, and an accentuation of some of the specific schizophrenic symptomatology which was followed by what appeared to be a moderate quiescence of certain symptoms in a number of the patients. Tryptophan, especially in conjunction with the monoamine oxidase inhibitors, produced a relaxation of inhibition and a freeing of association where these had been abnormally marked in several patients. These findings are being further evaluated in order to elucidate their significance to the nature of schizophrenia and the relationship of biogenic amines to behavior.

Fat Metabolism and the Nervous System

Drs. Peter Mueller and Philippe Cardon have been conducting a study of the relationship between plasma-free fatty acids and the nervous system. These substances represent the form in which, to a considerable extent, fat is mobilized from adipose tissue and transported in the blood stream. A number of interesting findings have come to light which appear to have special relevance to mental or neurological disease. It was found that vitamin C appears to be necessary for the mobilization of fatty acids which occurs in response to fasting. It was also found, in conjunction with Dr. Kies, that guinea pigs in which experimental allergic encephalomyelitis had been induced showed a marked increase in free fatty acids. When vitamin C was withheld and the animals became deficient in this vitamin, the appearance of the encephalomyelitis was entirely prevented. After restoration of the normal diet, the guinea pigs retained their resistance to the first and to a second inoculation with antigen.

Metabolism and Inactivation of Drugs and Hormones

Peripheral Metabolism and Inactivation of Catecholamines. This chemical group includes epinephrine and norepinephrine as substances of considerable biological and psychiatric interest. Dr. Julius Axelrod and his collaborators in the Laboratory have delineated the significant features of the metabolism of norepinephrine in the body and have demonstrated a number of areas in which the metabolism differs quantitatively from that of epinephrine. In addition and of considerable interest has been the work on mechanisms of binding and inactivation of norepinephrine at or near its site of action. Using tritium-labeled norepinephrine of high specific activity, they have demonstrated a rapid uptake

of this hormone following its injection into animals by certain tissues of the body which are richly innervated by the sympathetic nervous system. Denervation of such structures results in a loss in the ability to bind norepinephrine, and a number of drugs, including reserpine, chlorpromazine, amphetamine and cocaine, have the ability to prevent this binding or to cause the release of bound norepinephrine from these sites. The tranquilizers, reserpine and chlorpromazine, although having similar clinical effects have very few chemical ones in common, and this similar action on norepinephrine may represent an insight into the mechanism of their ataractic action.

Regional Neurochemistry

In the recent past neurochemistry has moved from a consideration of over-all aspects of cerebral metabolism to a recognition of the remarkable heterogeneity of the brain and the relationships between regional neurochemistry and the specialized functions of the nervous system. As an indication of the growth of interest in this area, it may be mentioned that the Organizing Committee of the International Neurochemical Symposia chose as the topic for the Fourth Symposium, held in the summer of 1960, "The Regional Chemistry, Physiology and Pharmacology of the Nervous System," the program of which the Chief of this Laboratory was asked to organize. A number of investigators in the Laboratory have contributed significantly to this field.

N- and O-Methyl Transferases in the Nervous System. Dr. Julius Axelrod has continued his investigation of these enzymatic processes which appear to be concerned with the interconversions of a number of substances present in the central nervous system and by means of which their activation or inactivation may be achieved. Imidazole-N-methyl transferase (an enzyme responsible for the methylation of histamine) and catechol-O-methyl transferase (responsible for the enzymatic inactivation of catecholamines) were found to be present in all areas of the central nervous system. The highest concentration of the histamine-methylating enzyme was found in the neurohypophysis while catechol methylation was highly localized in the adenohypophysis. The discovery of the histamine-methylating enzyme in brain, by Drs. Donald Brown and Julius Axelrod in 1959, in concentration higher than that in any other tissue is very suggestive of an important and as yet unknown role for histamine in the central nervous system.

Synthesis and Degradation of Melatonin. The pineal gland, a small structure directly in the center of the brain, has challenged the imagination of scientists even before the days of Descartes. Its function to date is unknown. Recently, Dr. Lerner at Yale Medical School isolated an agent, melatonin, which appears to be highly localized in the pineal gland, which structure alone in the brain has the ability to synthesize it. Drs. Julius Axelrod and Irwin Kopin of this Laboratory, with the collaboration of Dr. Herbert Weissbach of the National Heart Institute, have demonstrated the mechanism of the synthesis of melatonin by enzymes found in the pineal gland. These enzymes effect the acetylation and methoxylation of serotonin with the production of melatonin. The distribution of melatonin after it is formed is to all tissues including the brain, and its degradation is mainly by 6-hydroxylation with the excretion of the hydroxylated compound in the urine and bile. The function of melatonin in the brain is at the present time unknown, although in lower animals it is known to be the most powerful melanocyte-contracting substance known. The relationship between these cells which control pigmentation in lower animals and brain function is obscure.

Isolation of Antigenic Protein from Myelin. The Section on Biochemistry under Dr. Marian Kies has been engaged in a long-term program on the biochemical aspects of experimental allergic encephalomyelitis, a disorder produced in guinea pigs by the injection of suitably treated brain extracts. This disorder serves as a useful animal model of clinical demyelinative diseases, of which a specific example is multiple sclerosis. In previous years Dr. Kies has succeeded in isolating from brain extracts a purified protein which appears to be the antigenic agent. Since myelin is the part of the central nervous system which is specifically damaged by the host's reaction to the antigenic protein, it has been assumed but not demonstrated that the protein was located in myelin. Convincing demonstration of

this was achieved during the past year. Dr. Robert Laatsch, working with Dr. Kies, has isolated an active protein from purified myelin. Like the antigen prepared from whole brain, it is antigenic at levels from 1 to 5 μg and has similar physical characteristics. Dr. Kies has continued to pursue the provocative finding of the previous year that the water-soluble protein antigen is capable in itself of suppressing the behavioral and histological manifestations of the disorder. An understanding of the mechanism of this suppression may have some relevance to the ultimate prophylaxis or therapy of similar demyelinative processes in man.

Thyroxine

Dr. Louis Sokoloff and the Section on Cerebral Metabolism have concentrated their activities in the past year in a further definition of the significant finding of an action of thyroxine on protein synthesis, directed toward an elucidation of the mechanism of this stimulation. It has been conclusively established that mitochondria are essential for the thyroxine effect, but the actual effect is the stimulation of amino acid incorporation into protein. In addition, there is also need for an oxidative phosphorylation system. Although energy derived from glycolytic processes appears adequate to support amino acid incorporation, the thyroxine stimulation is observed only when the energy is provided by oxidative phosphorylation. The work gives a basis for postulating the formation of an intermediate which then stimulates the amino acid incorporation. The intermediate has not thus far been identified, but it is planned to emphasize this aspect of the project during the coming year. Efforts have been directed in the past year toward demonstrating the relationship between the effect on protein synthesis which was observed and the physiological action of thyroxine. Studies with thyroidectomized rats have confirmed the expectation based on such a relationship of a decreased rate of amino acid incorporation. Studies with various analogs of thyroxine have also indicated a parallelism between physiological activity and effectiveness on the amino acid incorporation system.

Neurophysiology

Dr. Edward Evarts and the Section on Physiology have continued their definitive studies of the activity of single neurons in unanesthetized cats during sleep and wakefulness. There appears to be a greater differentiation of neuronal activity during waking than during sleep. Neurons in the reticular formation show a reduction of evoked responses during sleep together with an increase in spontaneous activity. Neurons here and in the visual cortex show an increase in the ratio of evoked to spontaneous activity during waking. This may explain the increase in sensitivity to environmental stimuli which appears to occur during the waking state.

Studies of evoked potentials in man, made possible by the development of an improved evoked response detector by Mr. Robert Cox, have been carried on in normal subjects during sleep and wakefulness. These have indicated that sleep leads to an augmentation of the initial phases of cortical response to retinal stimulation and to disappearance or reduction of the latter phases of the response. Preliminary studies in schizophrenic patients have failed to reveal any abnormalities of evoked potentials.

New Techniques

One of the important accompaniments of human stress is the release from the adrenal gland and the sympathetic nervous system of the important hormones, epinephrine and norepinephrine. Although measurement of the amount of such release and its correlation with other measures of stress in health and disease is a much sought after bit of information, the available techniques for deriving such information have been quite unsatisfactory, depending upon analysis of the urine for epinephrine or norepinephrine which are excreted unchanged in the amounts of only a few percent of that which is released. Since more than 95 percent of these hormones are converted to other substances after their release, any attempt to estimate the amount secreted from an analysis of this extremely small residue is associated with error which may be several hundred percent in magnitude. On the basis of new knowledge acquired by Dr. Axelrod in this Laboratory and other investigators elsewhere, the metabolic products of these catecholamines have now been identified. Mrs. Virginia Weise, working with Dr. Roger McDonald, has in the past year developed a technique for the determination of 3-methoxy-4-hydroxymandelic

acid (VMA) in the urine of man. Since this compound in the urine accounts for some 40 percent of injected and presumably endogenously produced epinephrine, it is a much closer indicator of catecholamine secretion in various states. These workers have begun to apply their method in a number of clinical states and have found, for example, that there is a high excretion rate in patients with involutional depression and that certain drugs or procedures, like reserpine or electroshock, cause marked increases in VMA excretion.

Monoamine oxidase inhibitors constitute a class of drugs which have been utilized in the treatment of depression. In evaluating the enzymatic effectiveness of various members of the group under clinical conditions and in order to monitor dose levels, it is of some importance to have available a measure of the degree of enzymatic inhibition under clinical conditions. Measurement of the excretion of tryptamine, suggested by investigators at NHI, has served as a useful procedure. This excretion, however, is not only a function of monoamine oxidase inhibition but also that of dietary tryptophan intake. Drs. Irwin Kopin and Elwood LaBrosse, utilizing the knowledge that indole acetic acid excretion is also proportional to dietary tryptophan, have suggested and used the ratio of tryptamine to indole acetic acid excretion in patients under iproniazid treatment. This ratio appears to be a better index of monoamine oxidase inhibition than the tryptamine excretion alone.

For many years it has been possible to record electrical activity evoked from the cerebral cortex of animals by auditory or optical stimuli through electrodes placed directly on the exposed cerebral cortex. In intact man, such signals, having to penetrate the skull and scalp, are lost in the background noise of the electroencephalogram. Although a few other groups have found it possible to extract the evoked waveform from the background noise in human recording by a system of algebraic addition of repeated responses, the techniques available have been extremely complex and prohibitively expensive, or quite cumbersome. Mr. Robert Cox, working with Dr. Evarts, has developed an evoked response detector which appears to be able to carry out this averaging process effectively and neatly at a relatively small cost, making possible the more widespread utilization of such studies in electroencephalographic laboratories.

Laboratory of Psychology

The primary areas of research carried on in the combined laboratory have remained essentially the same this year, though the experimental work has shown the natural development. The Section on Animal Behavior has continued its attempt to define the behavior served by association cortex in man, chimpanzee, and monkey, and to define the neural mechanisms underlying this behavior. In addition, considerable effort has been expended in attempting to specify the neural mechanisms underlying motivation and attention in monkeys and man. The section on Learning and Perception has, on the one hand, continued its studies of the effects of the physical structure of the environment on instinctive and social behavior in rats and on the other its studies of the nature of the perceptual process as reflected in size-constancy and in velocity. The Section on Aging has expanded its previous studies of the behavioral and physiological changes associated with age, concentrating especially upon the age-related impairment of higher cognitive processes. The Section on Child Development has advanced in its attack on problems of behavioral development, particularly during the first year of life, using statistical analytic techniques on longitudinal data, normative and experimental studies. The Section on Personality has become heavily involved in a series of experimental studies on processes affecting cognitive restructuring which were only in the planning stage last year. The Section has, however, continued its studies on the noncontentual components of speech as related to the communication process, and on psychosomatic studies related to stress. In the Office of the Chief the major concern has been with two areas: (1) psychology of schizophrenia—the problems of preparatory set, cognitive and perceptual processes, and the relative influence of environmental psychological and hereditary factors; and (2) the psychotherapeutic process—the microscopic aspects of the communication of affect in therapy, and the limits of psychotherapy for realizing potentialities.

The relevant aspects of the reports of the several section chiefs follow.

Child Development

The concern of the Section on Child Development is to increase understanding of the processes of behavioral development, primarily during the first years of life. The emphasis is on the normal processes as necessary bases both for promoting healthy growth and for evaluating and diagnosing the pathological. Its work is based on the hypothesis that genetic determiners of behavior become manifest during the processes of maturation, and that the maturation of the organism occurs only as it interacts with relevant aspects of an environment which is in varying degrees favorable to the infant's growth and development.

Certain studies are designed to observe and describe, under standard conditions, the central tendencies and normal variations in mental, motor, emotional, and motivational development of infants in the first 2 years of life. In this program Dr. Bayley is revising, restandardizing, and extending her infant scales of mental and motor development. In doing this she has worked with the Collaborative Project of NINDB, as a consultant and by training their psychologists in testing procedures, in return for which they have furnished tests on 1,200 infants aged 1–15 months. On the basis of these data, with the addition of about 300 tests given locally, preliminary revision of forms of the mental and motor scales have now been set up and are being used on a trial basis. The Infant Behavior Profile for evaluating normal development aspects of attitudinal and emotional behavior is still in the state of preliminary analysis. Further testing and statistical procedures for use in scoring the tests are currently under way as well as item analyses that may permit early diagnosis of central nervous system damage and other forms of retardation. The impetus for undertaking this program was the great need for an adequately standardized set of tests of infant development. The current battery was designed to include a broad band of relevant behaviors, to be standardized on a geographically and ethnically wide selection of infants, to bring the normative data for standardization up to date with current tests, and to clarify and improve the directions for observing, recording, and interpreting infant behaviors in response to standard materials and conditions, in the light of present psychological theory and knowledge. The many requests already received for the tests indicate the need for such instruments by physicians and psychologists who are making diagnoses and carrying out research programs with infants.

Another aspect of the standardization of the infant scales is the preparation of a motion picture which will demonstrate, for training purposes, testing procedures and characteristic responses of infants at successive ages. Pictures of the testing situations have been made, and are now in the process of being edited.

The program of development of the mental scales leads naturally into plans for more detailed studies of the mental processes involved. Thus, within the broader framework of standardizing the tests for evaluating general behavior development of infants, the section hopes to institute a program to explore in more detail the early processes of perception, concept formation, and the general area of cognitive functioning in the first two years of life.

Dr. Rheingold's program of research is another approach to the study of normal developmental processes of infants. Her purpose is to investigate the process by which the human infant's response to social and nonsocial objects is modified by the responses these objects give to his exploratory activities. Classifying this behavior as a homeostatic drive, (the forerunner of behaviors labeled as sociability, curiosity, manipulatory drive, and play) she hypothesizes that at first the infant's responses to people and to things are similar and that differentiation of response occurs by a process of learning based on the different nature of the responses given to the infant's own overtures. At present she is studying the dimensions of novelty and complexity of stimuli, both spatial and temporal. The dimension of familiarity will also be explored. Maternal care is, of course, and important part of this program, as it is a major source of environmental stimulation for the infant, and operates both as evoking and reinforcing stimuli. Another part of this general program is a comparative study of social and exploratory behavior in other young mammals, together with their maternal care.

In this program Dr. Rheingold has employed three methods, depending upon the stage of the problem and the nature of the questions being asked. The first is the naturalistic observation of sequences of interaction between mother and child in their usual environments (in her study of measurement of maternal care). In the second method these naturally occurring sequences of behavior are reproduced under controlled conditions, but still in the infant's usual environment (for example, the study of social conditioning of vocalizations in the human infant). The third method, which is now being perfected, seeks to control the presentation of the stimuli which result from the infant's own behavior, and to record the infant's response. This method of recording sequences of behavior with precision requires laboratory space and instrumentation which at present are inadequate. This experimental method is a very important step in making the detailed analyses of the processes observed in the naturalistic situations.

So far in these studies, observation has revealed that a considerable amount of time and energy is spent by the young infant in seeking a response from his environment, more from certain objects than from others. Measures of the responses to people and to things of 3-month-old infants receiving different amounts of environmental stimulation (home and institution) revealed few differences. What differences did appear showed that infants receiving less day by day stimulation responded more quickly and more fully. Some nonsocial objects were effective in producing the smiles and vocalizations usually associated with responses to social objects, suggesting that the origins of the smiling response may be based on the stimulus properties of the object itself and not on the history of need-reduction (e.g., maternal care). Pilot studies with 3- and 4-month-old infants indicate that panel-pressing may function as a free operant and that illumination of these panels when made contingent upon the infant's press may function as a reinforcer. In Dr. Rheingold's continuation of comparative studies of maternal care in mammals, the data on detailed observations of dogs is now being analyzed, but has not yet reached a state in which results can be reported.

A related area of research in which Dr. Schaefer has been active, in collaboration with Dr. Bayley, has been the exploration of maternal behavior, child behavior, and their interrelations in available data from her Berkeley Growth Study. Several articles have already been published from these studies, and two monographs are now in preparation. The first of these monographs, Maternal Behavior, Child Behavior, and their Interrelationships from Infancy through Adolescence, is almost ready for publication. Because of the currently strong interest in and the various theories about the effects of maternal care on the child's adjustment and personality development, these long-term records on a group of children who started life as healthy newborns, should yield important relevant information.

Data on the social and emotional development of 54 subjects had been systematically collected at frequent intervals from their birth in 1928 until they were 18 years of age by Nancy Bayley at the Institute of Human Development, University of California, Berkeley, Calif. Observations of maternal behavior during infancy, interviews with the mothers at preadolescence, and the data on child behavior were organized and the interrelationships of the variables were computed at NIMH. A few of the many findings on consistency of both maternal behavior and child behavior through time, and on the relation of maternal behavior to child behavior are summarized below.

Maternal behavior from infancy to preadolescence is relatively consistent for a dimension of acceptance versus rejection but is relatively inconsistent for a dimension of autonomy versus control. Child behavior from infancy to adolescence is rather consistent for a dimension of extraversion versus introversion but is relatively inconsistent for a dimension of adjustment versus maladjustment. Maternal behavior is more highly related to child adjustment than is socioeconomic status, although maternal behavior is related to socioeconomic status, financial stress, and poor physical health. Maternal acceptance versus rejection is more highly related to child adjustment than is maternal granting autonomy versus control, suggesting that the controversy about permissiveness may be emphasizing a less important aspect of parental behavior. Maternal behavior during infancy is more highly related to sons' positive task-oriented behavior at preado-

lescence than it is to the sons' behavior at 3 years, suggesting there may be a cumulative effect of maternal behavior upon sons' behavior. Maternal behavior during infancy is more highly related to sons' behavior at 9 to 12 years than is the sons' own behavior during infancy, suggesting that early behavior may be more predictive of the child's future adjustment than is the child's own early behavior. For this sample boys' behavior is more highly related to earlier mothers' behavior than is girls' behavior although girls' behavior does relate to current maternal behavior. These results suggest that girls may respond more to their current interpersonal situation while boys may reflect their past relationships more. Additional data on the relationship of maternal behavior and the social and emotional behavior of the child to intellectual development are currently being analyzed.

Dr. Schaefer's interest in maternal behavior, methods of observing and recording it, its organization into a conceptual model, and its relation to child behavior, has led him into a series of related studies. He has checked his work on the Berkeley Growth Study material by successfully applying the methods to the tables of intercorrelations of maternal and child behavior variables derived from the studies of other investigators. He has worked on further development of methods for the study of maternal personality and behavior variables, in cooperation with the NINDB Collaborative Project. For this project he has developed two objective inventories, a Pregnancy Research Inventory and a Postnatal Research Inventory, for assessing the perinatal adjustment of mothers. Both inventories investigate psychosomatic symptoms of the mother and her psychological response to her current situation. These inventories were given to pregnant women and women soon after the birth of their child, and for a small sample the same women were given both inventories. The inventories were found to measure reliably several patterns of response. For the Pregnancy Inventory these are: 1. Dependency, Fears for Baby, and Fears for Self; 2. Depression, Irritability, and Lack of Desire for Pregnancy; and 3. Numerous psychosomatic symptoms before and during pregnancy, at menstruation, and at times of emotional stress. For the Postnatal Inventory, they are: 1. Fear or Concern for the Baby, Need for Reassurance, and Intrapunitiveness; 2. Negative Perception of Aspects of Child Rearing, Irritability, Depression, and Extrapunitiveness; and 3. Psychosomatic symptoms. There was evidence of consistency of characteristic patterns between the Pregnancy and Postnatal Inventories. The inventories have now been turned over to the NINDB Collaborative Project for actual use.

Dr. Schaefer has used yet another approach to measuring parent behavior by developing an inventory to administer to school children, on their perceptions of their parents' behavior, both mothers and fathers. He was able to develop a reliable instrument which revealed two major dimensions—love versus hostility, and autonomy versus control. Thus these replicate the conceptual structure of the model of maternal behavior previously obtained from observations of mothers' behavior. The children tended to describe both parents as behaving toward them in a very similar way, i.e., both hostile, or both loving, etc. In an exploratory trial of the inventory significant differences were found between groups of delinquent and normal boys, but these findings need to be checked further. Also, checks on children's perceptions with independent observations and interviews of the same parents will be needed to answer questions about the accuracy of the children's perceptions, and the possibilities of significant biases in the perceptions of certain children. Such tests are planned.

Dr. Gewirtz, who is on leave, is carrying out a program in Jerusalem, which is correlated in many ways with the studies here described. His work will permit cross-cultural comparisons on underlying constancies in infant behavior—both the developmental aspects, and the effects of social deprivation versus enrichment on such functions as conditioning and social responsiveness and adjustments.

Personality

Members of the Section on Personality have conducted research programs in three major areas: (1) Psychosomatic studies; (2) noncontent components of speech; and (3) processes affecting cognitive restructuring. The major effort of the section has been in the third area.

Psychomatic Studies. Dr. Handlon has continued to work with the Adult Psychiatry Branch

in their studies of emotional behavior and adrenal function. In addition to his active participation in the planning and execution of a variety of studies, he has contributed significantly to methodology by devising two techniques. The first is the Daily Mood Check List, which is self-administered. The Daily Mood Check List has been found to correlate with hormonal output as well as ward behavior. The Mood Check List has been recognized as such a reliable index of change in the individual that it has been used as an indicator of when to make hormonal determinations. Intensive studies of mood fluctuations in individuals are currently under way. These involve the utilization of an individual's Daily Mood Check List over long periods of time. The Mood Check List has also been useful in establishing an individual's affective response base line. By an analysis of the individual's Mood Check List responses during the first and second weeks of hospitalization, it has been found possible to predict the relative hormonal levels of such an individual in subsequent weeks of the study period.

The second measure is the Defensive Maneuver Instrument. This Q-Sort has provided an opportunity to determine whether an individual's characteristic defensive maneuvers are related to the over-all level of hormonal output. This seems to be a reasonable assumption in view of the fact that it has been found that a subject appears to have his own idiosyncratic pattern of hormonal response. Such response involves not only his basal level of output but also his propensity to respond. The data analysis have not yet been completed to evaluate the effectiveness of the Defensive Maneuver Instrument.

Dr. Handlon has continued to work with Dr. Margaret Thaler-Singer to determine whether there are patterns of response to Rorschach tests which correlate with patterns of hormonal output.

Noncontent Components of Speech. The basic assumption underlying Dr. Boomer's interest in nonlexical speech is that speech mechanisms represent complex phenomena which are sensitive to and reflect transitory changes occurring in ego functioning. It is further assumed that the individual can exercise less conscious control over the nonlexical aspects of speech than over the content per se. It is hoped, therefore, by the further development of measures and techniques that a valid index may be derived of some basic psychological processes. At the present time psychology does not have instruments to monitor the moment-to-moment transitory changes in an individual's psychological functioning. The widely used physiological indices which are presumed to relate to anxiety, arousal, etc., are open to serious criticism.

The major aspect to Dr. Boomer's research has been focused on the identification and measurement of noncontent variables such as speech disturbances, rates of speech, respiration rates, etc. During the past year he has undertaken to interrelate these variables as well as correlate some speech disturbance measures with variables believed to be relevant to the psychotherapeutic process, i.e., changes in anxiety level, period of treatment, etc. In the analysis of certain categories of speech disturbances occurring in patients' communications during a prolonged period of therapy (one to two years) it was found that patients who are judged by their therapist to be making good progress revealed a striking diminution of certain categories of speech disturbances. This along with other suggestive findings have encouraged us to undertake the development of a computer program which will permit the more rapid analysis of larger amounts of data. The data currently being analyzed include not only tape recordings of psychotherapy interviews but tapes provided by the University of Michigan of the responses of subjects under the varying post-hypnotic suggestions of "expression," "suppression," and "repression," of anxiety-laden subject matter.

Cognitive Restructuring. During the past year the work of Drs. Berlyne, Caron, and Parloff in the area of "creative problem solving" has been based on the essential assumption that central to creativity is the cognitive restructuring involved in recognizing new meaningful relationships among seemingly remote concepts and ideas.

The investigations have centered mainly on evaluating the influence of motivational factors. Two types of motivational influences have been studied: (1) Those affecting the individual's commitment and persistence in attempting to solve a problem; and (2) comparison of the relative ef-

fectiveness of intrinsic and extrinsic motivation. A second approach to creative problem solving has concerned the study of enhancing and inhibiting influences of different social interactions on members of dyads as they attempted collaboratively to solve open-ended problems.

Motivational Studies—Commitment and Persistence. Dr. Daniel Berlyne conducted a series of experiments designed to investigate some of the basic processes underlying the arousal of "curiosity." It is assumed that curiosity is a prerequisite condition to creative thinking. Dr. Berlyne demonstrated that the amount of curiosity and interest evidenced by subjects was significantly related to the degree of "uncertainty" induced by confronting the subjects with sets of two and three response alternatives of approximately equal strength. He also conducted a set of experiments to determine the influence of such variables as "conflict," "novelty," "surprisingness, "complexity," and power to induce "uncertainty," on the orientation reaction. In this instance the orientation reaction was measured by the Galvanic Skin Response. All hypotheses were supported except the one concerning "complexity."

Two papers were prepared based on this work. One entitled "Collative Variables and Arousal" was read at the APA convention in September, 1960; the other entitled "Conflict and the Orientation Reaction" has been submitted for publication.

Dr. Parloff and Dr. J. Fishman, of Adult Psychiatry, have investigated the variable of "subjective probability of success" as it effects factors related to creative thinking. The aim of the study was to determine whether increasing an individual's self-confidence would enhance his "creative thinking" as measured by actual test performance on Guilford's "Creativity Factors." The technique for increasing self-confidence was hypnotic suggestion. The preliminary findings are very encouraging; however, there appears to be a condition-order effect. The individual's best test performance on any of the three matched test forms occurs under the hypnosis self-confidence suggestion condition as compared with hypnosis —no suggestion, and the nonhypnotic test conditions, provided, however, that the hypnotic suggestion is the first testing condition to which the individual is exposed. Efforts are now being made to determine whether this finding is due to the subject's witting or unwitting inhibition of performance on subsequent test conditions.

Motivational Studies—Intrinsic and Extrinsic. Dr. Albert Caron conducted a study of some 1,200 10th grade students in Montgomery County to compare the effectiveness of intrinsic motivation—desire to learn because of the student's inherent interest in the topic, and extrinsic motivation—desire to learn in order to conform to some external standard such as grades, recognition, etc. The task was to learn and comprehend some complex scientific principles and to apply them in a new test situation. The motivational states were induced by experimental manipulation of the learning conditions. In addition, such independent variables as IQ, test anxiety, need for achievement, desire for success, rish taking attitudes, and under- or over-achievement patterns were also measured. The analysis of the data has not yet been completed; however, contrary to prediction it appears that subjects in the extrinsic motivational set outperformed those under the intrinsic motivational set for both comprehension of principles and rote learning. In comparing the test performance of males and females there is some evidence that the usual finding that girls' academic performance is superior to that of boys may refer particularly to superiority in rote memory performance and not to functional understanding of abstract principles.

Collaborative Problem Solving. In recent years the need for collaborative research between individuals of different disciplines has been widely recognized; however, so many problems arise in such collaborative settings that much disenchantment has been expressed with the entire notion of collaborative or interdisciplinary efforts. A study undertaken by Drs. Parloff and Handlon investigated the hypothesis that creative problem solving in teams would be significantly affected by the degree of mutual respect existing among team members and the ability to suspend premature criticism of ideas. A replication of their earlier dyad-team problem solving study was completed this year. It appears that sig-

nificantly more "good" ideas are presented under conditions of suspended critical judgment and that this suspension is more likely when the team members are mutually respectful of each other. At present we are trying to derive a model to explain the psychological processes underlying these findings.

The essential goal of research in this area remains that of learning more about the conditions and processes which underlie the utilization of one's capacities. The aim of this section is to deal with a wide range of variables and conditions which may influence cognitive restructuring and to assess how much of the variance each of the factors explain. To this end it is planned to further investigate some of the mediation models suggested by Hebb, Osgood, Malzman, and others. In addition the section will continue to utilize some of the fundamental psychoanalytic concepts regarding processes underlying creative thinking. Some of the specific problems to be pursued grow out of the questions raised by the research thus far undertaken. Further, with the addition of Dr. Sanford Unger to the staff, it is anticipated that he will assist in the development of research along the lines of mediated association, manipulation of sets, and the role of affective variables on cognitive restructuring.

Office of the Chief

The Office of the Chief has, as in recent years, continued to concentrate on the psychology of schizophrenia and the nature of the therapeutic process.

In addition to participating in the current experimental program on schizophrenia to be reported below, Dr. Shakow has carried out further analysis of an accumulated body of past experimental data. Based on both groups of studies on schizophrenia, he presented a paper at the 16th International Congress of Psychology at Bonn, and is in December presenting the Collier Lecture at the University of Rochester. Aside from other values of last summer's visit to Israel, Germany, France, and England, the trip afforded Dr. Shakow the opportunity to confer with investigators who are also working on the experimental psychology of schizophrenia. He has also devoted time during the year to the preparation of a monograph on "The Impact of Freud on Psychology" which he is writing in association with Dr. David Rapaport of the Austen Riggs Center, and which is to be completed during the coming year.

Dr. Rosenthal's research has centered on various aspects of schizophrenia. Studies of the role of heredity and environment in the etiology of schizophrenia have uncovered the following points: (1) There are at least two kinds of disorder which traditionally come under this diagnostic label. In one, hereditary factors seem to be contributing almost all the variance; in the other, hereditary factors seem to account for little of the variance; (2) if one samples from among resident hospital populations, heredity will be said to account for more of the variance of the disorder than if one samples from consecutive hospital admissions; (3) the incidence of schizophrenia is no higher in twins than nontwins, a fact which rules out such a psychogenic theory of schizophrenia as "confusion of ego-identity," according to which higher incidence in twins would be predicted; (4) concordance rates with respect to schizophrenia are higher for female than male pairs and for same-sexed than opposite-sexed pairs of relatives when the familial relationship is primary (parent-child or sibling) but not when the familial relationship is more distant (grandparents, uncle-aunt and nephew-niece, cousin). These data strongly suggest that psychological factors are contributing at least some of the variance to the incidence of schizophrenia.

In addition to participating in the continuing studies of mental set in schizophrenia, Dr. Rosenthal has begun a new series of studies, using a battery of tests, related to cognitive and perceptual processes of schizophrenics, and in one study, of the parents of schizophrenics. The preliminary findings from these studies show: (1) In testing a small sample of parents of schizophrenics, at least one parent from each pair evidenced schizophreniclike thought disorders, even though none of the parents had ever been diagnosed schizophrenic or hospitalized for mental illness; (2) chronically ill schizophrenics tend not to show perceptual closure to the extent that normal subjects do. Acutely ill, recent admissions diagnosed as paranoid schizophrenic tend to show a greater amount of perceptual closure than normal subjects; (3) although schizophren-

ics are more variable than control subjects on a wide variety of tasks, when they are specifically asked to vary their performance to product contrasting sized drawings (big-little), they do not vary their behavior as much as control subjects do.

With Dr. Rosenthal and Dr. Shakow, Dr. Zahn has continued investigating preparatory set and the arousal mechanism in schizophrenia using the methods of reaction time and psychophysiological recording.

In one reaction time study, schizophrenic and normal subjects were presented preparatory intervals (1″–15″) of ascending and descending lengths. The findings showed that although the reaction time of the schizophrenics was approximately the same for long preparatory intervals under each condition, it improved on shorter intervals only under the ascending but not the descending condition. The normal subjects improved on shorter intervals under both conditions. In another reaction time study, the preparatory intervals were presented in an irregular ("random") manner. Under these conditions the shorter preparatory intervals produced longer reaction times than the longer intervals, a result which appears to be dependent on the previously presented preparatory interval. The effect was less in the normal subjects. A control reaction time study using varying intertrial intervals was directed at determining whether more slow pacing of events rather than maintenance of preparatory set was involved. It showed that a long intertrial interval by itself produced no appreciable detrimental effect on the reaction time of either schizophrenic or normal subjects. These results suggest that schizophrenic patients persist in a poor preparatory set which appears to be based on a much narrower range of information than that of normal subjects. The relationship of poor set to disturbance in the arousal mechanism will soon be investigated by recording psychophysiological measures simultaneously with reaction time.

In collaboration with Dr. Hamilton, Dr. Zahn tested a small sample of schizophrenic patients at Saint Elizabeths Hospital for the effect of two phenothiazine drugs and a placebo on reaction time. The drugs appeared to improve the performance of most of the patients. However, the results were not statistically significant.

In a continuing study of psychophysiological responsivity and adaptation to visual and auditory stimuli, the GSR, heart rate, and respiration of 20 chronic schizophrenic patients were recorded and are currently being analyzed. The data from conrtol subjects are still being collected.

Dr. Dittmann continues his work on the analysis of psychoanalytic interviews from tape recordings and motion pictures. One of the main projects has been to index these interviews in a form most meaningful for future use. At the present time some 60 interviews have been indexed—the first 30, together with another 30 spread out over a long period—in which 84 topics, with a minimum of overlap, and nine time referents have been delineated.

Dr. Dittmann has completed studies of foot movement associated with speech disturbances, body movement with different moods, and judgment of mood from restricted cues, for which segments of interviews were evaluated by judges. The results from the foot movement-speech disturbance study were contradictory: in the first sample a significant positive relationship was found; in the second a negative trend showed up; and in the third there appeared no association at all. The findings from the body movement-mood study were that frequency of head movements differentiated among the moods at the 0.01 level of significance, while hand movements differentiated at the 0.001 level. The measurement of distance and duration of hand movements showed that distance differentiated at the 0.001 level, but duration was unrelated. Rate of hand movement was similar to that of distance. The pattern of movements in relation to mood was also analyzed, showing, for example, that the "calm" mood was accompanied by the fewest head movements and the most frequent short hand movements, while the "angry" mood was accompanied by the most frequent head movements and very few but widespanning hand movements. The agreement of three judges, who were presented material from silent film and filtered tapes for the judgment of mood from restricted cues was not high, although there was no systematic bias from judge to judge. Enough questions were left open that replication with more carefully selected samples seems called for.

Dr. Dittman has also been continuing a study

of judgments of affect from motion pictures which has thus far given equivocal results, primarily because of methodological problems. This study will continue, however.

During the first half of the year, Dr. Bergman worked on the clinical testing of some hypotheses derived from the "General Theory of Psychotherapy" that he had formulated last year. Counter-conditioning was the key concept of this theory. The observations made in cooperation with a group of colleagues (Drs. Jenkins, C. Elkes, and Waskow) seemed to support the view that this direction of therapy was productive of rapid symptomatic improvements, but did not fulfill the theory's expectation of helping patients to optimal personality development. The question then arose whether the group should continue to explore the hypotheses, which were now considered limited in value in order to establish clearly these limitations. It appeared to be a job of technological and argumentative relevance, but not of theoretical and intrinsic interest. Dr. Bergman and the group, therefore, decided to turn to new hypotheses which appeared to have greater promise. He consequently dropped his plans of controlled experiments which were meant to isolate and study the process counter-conditioning.

The new hypotheses, supported by clinical testing, are related to the current trend of experiential psychotherapy. Granting that this approach to psychotherapy has, in principle, given a workable, though not exclusive, solution to the problems of therapy, the plan is to investigate an area of extension of its practice which seems to hold intrinsic theoretical interest. The hypothesis is that the "normal" state usually achieved by successfully adjusted persons or successfully treated patients is not identical with an "ideal" state of mental health, and that this "ideal" state can be approximated by prolonged psychotherapy. Dr. Bergman and his group plan to work with subjects who are not severely ill at the beginning of the therapy. Some of the questions to which the group is seeking answers are: Can an "ideal" state free of anxiety and self-consciousness be achieved or approximated? What are its empirical concomitants in physiological, behavioral, and intellectual areas? Can everybody achieve such a state or does it depend on certain constitutional or experiential givens?

Dr. Kendig is completing her study on the self-concept and the body image as related to disease susceptibility and organ choice. All the interviews on which the study is based were scored from the tapes by the principal investigator as they came in and 104 of these by an independent trained worker as well. On this group the inter-scorer reliability was 0.91. The sample, totalling 150, includes a group of 50 arthritic cases and a group of 25 leukemia cases with a like number of controls for each, individually matched on 6 variables—race, sex, age, education, occupation, and religion. The arthritics were divided into two groups of 25 each, partly in order that statistical analysis could be begun at once on half the total, partly than an analysis of the second half could serve as a replication study. A preliminary survey of the gross pathology scores on Arthritics I showed that they did not differ significantly from those of the control group. The data were then subjected to item analysis but only 4 items showed differences significant at the 0.05 level. This, it was recognized, could well be by chance as indeed proved to be the case when completion of the interviews on the second group made possible comparison on these items. Item analysis on the second group of arthritics and the leukemia group is now being set up for machine processing. A design for "cluster" analyses is also being developed on the hypothesis that while the comparison of patients and controls on individual items may not be statistically significant, when related items are considered together, significant differences may emerge. For this approach 6 new scales have been developed and the scores they yield are to be analyzed with the assistance of the Computation and Data Processing Branch.

Animal Behavior

The research of the Section on Animal Behavior has continued to be concerned with (a) defining the behavior served by association cortex in monkey, chimpanzee, and man (b) determining the neural mechanisms underlying this behavior and (c) specifying the neural mechanisms underlying motivation and attention in monkeys and in man. We have added to our usual techniques those of electrical stimulation and recording with very encouraging early results.

In addition to confirming our earlier findings

that the chimpanzee, unlike the monkey, recovers from an initial deficit on delayed-response problems, it has been shown that this recovery may not be complete, a residual deficit persisting for as long as two years. It has also been shown that the difficulty of the delayed-response problem may be increased such that frontal-lobe lesions result in a substantial deficit from which the chimpanzee does not recover, at least within a two year period. The differences in the effects of frontal lobe lesions in the chimpanzee and monkey thus appear to be quantitative rather than qualitative.

The results of three experiments involving both chimpanzees and monkeys were consistent in showing significant impairment following unilateral prefrontal lesions. Thus, whether the measure was of learning or retention, on delayed alternation or delayed response, in monkeys or in chimpanzees, there was no overlap in the scores obtained by frontal animals and those obtained by their operated or unoperated controls. The deficit produced by unilateral frontal damage was incomplete and with sufficient training the animal's performance frequently approached control levels. These results encourage continued search for an impairment, analogous to delayed-response impairment, in man in whom the frontal injury to be studied is most usually unilateral.

An earlier study had implicated posterior midline structures near the splenium of the corpus callosum in delayed alternation performance. It was not clear, however, which of several possible structures were specifically involved in this behavior. Recent research suggests that it is most likely the posterior columns of the fornix.

An intensive analysis of the problem-solving behavior of monkeys with frontal lesions has led to the hypothesis that their impairment is due to abnormally strong perseverative tendencies. The hypothesis provides a consistent account of the frontal animal's difficulty on learning set, discrimination reversal, and one-trial discrimination learning. Even more important, however, the hypothesis offers an alternative account of the frontal animal's deficit on the delayed-response test, a deficit which has been traditionally ascribed to a "loss of recent memory." This latter conception has faced many difficulties, among which has been the failure of a recent study (in which this section collaborated) to find any evidence of memory loss after frontal-lobe injury in man. Conversely, the "perseveration" hypothesis appears to be consistent with a large body of evidence pertaining to the effects of frontal injury in man.

While the "perseveration" hypothesis is thus seen to be potentially useful in ordering a large literature on the effects of frontal-lobe damage in both man and animals, it is recognized that the conception is not yet sufficiently precise. A number of on-going experiments give promise of leading to a sharper definition. For example, that perseveration must be distinguished from loss of inhibition is suggested by the results of experiments in which animals with lateral frontal lesions are compared with animals in which orbital frontal cortex has been removed. On tasks which demand inhibition of food-getting responses, the orbital animals generally perform more poorly; on tasks which demand frequent switching between responses, the lateral frontal animals perform more poorly. Further investigations along these lines may be expected to yield still sharper distinctions between perseverative tendencies and superficially similar defects.

A detailed picture is beginning to emerge of the nature of the interaction between the primary visual and inferotemporal areas. Having already demonstrated that the two areas are interconnected by ipsilateral and contralateral pathways (the latter crossing in the posterior third of the corpus callosum), the work in the past year has attempted to determine whether these connections are direct or indirect, and, if the latter, what intermediate cortical areas are involved. Both neuroanatomical and behavior-ablation experiments agree in implicating prestriate and parietal areas as intermediate relay stations along the striate-inferotemporal pathways.

It has been demonstrated in other recent experiments that the visual impairment which is produced by severing these pathways is not simply a by-product of visual field defects. The impairment thus appears to be qualitatively similar to that produced by bilateral inferotemporal lesions. However, the nature of this associative defect in vision still eludes precise definition. Further studies aimed at distinguishing between

two possible interpretations—one following a perceptual model, the other a conditioning model—are currently underway using stimulus generalization techniques.

While the research outlined above has provided a sound rationale for ascribing visual functions to the inferotemporal cortex, other studies, from this laboratory and elsewhere, have reopened the issue as to whether the functions served by inferotemporal cortex are exclusively visual, or whether they embrace olfactory functions as well. A study aimed at precise localization of the temporal-lobe region serving associative functions in olfaction is now in progress. Similarly, recent studies have suggested that frontal association cortex may be implicated in auditory functions, thus reopening the issue as to whether or not frontal cortex serves specific sensory functions. A study aimed at re-examining this question is now in progress.

Electrical stimulation and recording techniques are being used to construct a volumetric map of areas in the forebrain of the monkey which affect food and water intake. To date, using six animals, approximately 3,000 points have been explored. Food and water ingestion and food (and possibly water) ejection can be elicited and localized separately, each from many points in the forebrain. The most effective of these points are selected for chronic studies in which each point is evaluated from the standpoint of motivating self-stimulation and escape behavior, autonomic effects, and such interrelated effects as facilitation, inhibition, and transmutation. Remote stimulators and telemetric devices are being developed to enable assessment of these effects in animals in a free-ranging situation.

Points have also been localized from which aggression or penile erection may be evoked by electrical stimulation. Modest success has been achieved in conditioning these behavior patterns by using classical conditioning procedures. However, many artifacts confound the results making conclusive statements unwarranted at this time. Nevertheless, the early successes are extremely encouraging and suggest that these evoked responses can be conditioned. In connection with these stimulation studies a technique for localizing electrodes in the brain was developed. This method is based on the fact that the impedance between an exploring intracerebral electrode and some other fixed point changes in a consistent fashion as the electrode is advanced through the brain. If one knows from a stereotaxic atlas what structures are likely to be encountered in an electrode track, close monitoring of impedance changes as the electrode is moved can signal precisely the passage of the electrode tip into a given target. Also, a very compact pulse-subtraction amplifier has been developed making it rather easy to monitor a variety of signals from an animal while he is in the stimulating circuit.

The studies of attentive behavior in man have been concerned with comparison of behavioral and physiological concomitants of impaired performance and with description of the nature of the behavioral impairment. The information which is now available indicates certain similarities and differences among the effects of the various agents that have been studied which appear to have significance for the specification of the central mechanisms necessary for consciousness and wakefulness. Thus, although sleep deprivation, acute chlorpromazine administration and petit mal epilepsy all produce marked impairment in the performance of tests of sustained attention, only sleep deprivation and petit mal epilepsy can be characterized to any significant extent as showing simultaneous changes in the EEG. The fact that the behavioral changes may occur independently of the EEG changes (i.e., with chlorpromazine) would seem to imply separate neural regulation of these phenomena and, possibly, independent neural systems. Although the effects of sleep deprivation and petit mal epilepsy are similar in the correspondence between behavioral and EEG changes, they too differ, but with respect to the autonomic accompaniments of the period of impaired responsiveness; opposite peripheral circulatory changes accompany the two states. This suggests further specificity and complexity within the underlying neural regulatory systems. The notion of independent systems subserving behavior and EEG changes receives additional support from the analysis of the time relations between changes in behavior and EEG changes in petit mal epilepsy. Evidence has been gathered which indicates that the behavioral impairment accompanying the spike and wave burst characteristic of this disorder precedes the burst in time and begins to subside before the burst has ended. Other

information suggests that the perceptual and motor aspects of the attention deficit may have differing time courses. These out-of-phase relationships may be interpreted as indicating separate, although closely related systems being affected differentially in time by some central epileptogenic process. In the coming year these studies will be directed in part towards further analysis of the relationships among behavioral, EEG and autonomic information. Other research will attempt to specify and contrast the nature of the deficit in petit mal patients and in normal subjects under the influence of centrally-acting drugs using appropriate normal and neurological patients as controls. In addition, a study is currently under way in which direct brain stimulation of trained monkey subjects will be carried out, simultaneous with performance on an attention test, in an attempt to test directly hypotheses concerning the differing neural systems underlying the regulation of behavior, EEG and autonomic functions.

Participating in various aspects of these studies have been Dr. H. E. Rosvold, Chief of the Section, and Drs. M. Mishkin, A. F. Mirsky, M. K. Szwarcbart, and B. W. Robinson. Also participating have been Dr. Charles Butter, NIMH Post-Doctoral Fellow, Dr. Stefan Brutkowski, a Visiting Scientist from Warsaw, Dr. H. Kuypers of University of Maryland, as a Consultant in Anatomy, and Mr. Thomas Brown, NIMH Pre-Doctoral Fellow.

Perception and Learning

The Section on Perception and Learning has two quite separate areas of research—that of Dr. Calhoun at Rockville and that of Dr. Carlson on perception.

Rockville Farm Project. This year saw the completion of Dr. Calhoun's first pilot studies begun in 1958 and the initiation of a further series oriented toward evaluating factors which have emerged as major variables in the pilot studies. Dr. Calhoun, with the assistance of Dr. Barbehenn, post-doctoral fellow, has studied the effects of several different physical structures of the environment on socially relevant aspects of the behavior of rats.

In an environment where movement patterns had been biased to favor one subarea over others, abnormal aggregations of animals developed. As much as 80 percent of feeding activity, for example, occurred in that subarea, even though all subareas would meet the requirements of basic needs equally well. Paralleling the development of this abnormal aggregation, normal patterns of maternal, nest-building, and sexual behavior became disrupted, and adult females experienced a high rate of mortality due to reproductive abnormalities. Liver and serum analysis suggested that these and other symptoms might be due to Vitamin A dysfunction. By varying the degree of prior opportunity to adjust to strange stimuli and the number of new stimuli in a test field, it appeared that fewer prior opportunities for adjustment led to greater avoidance of the test field and, for those rats entering the test field, hyperactivity persisted longer with greater numbers of strange stimuli.

As a consequence of these and other results a factorial study has been initiated involving large and small social groups, different levels of Vitamin A in the diets, and stressful and nonstressful conditions. Large-scale groups appear to offer more opportunity for adjusting to new situations; an increase in Vitamin A, however, may work in the opposite direction, decreasing the ability of rats to adjust to change.

This year has also seen the completion of a long-term study begun by Dr. Calhoun in 1946 on the evolution of social groups among mammals. He has elaborated a general formulation on this topic, tracing interactions in space which successively lead from random distribution with poor communication, to gradually condensing groups with mean size of twelve individuals and enhanced communication, to herd-type animals living in large groups and capable of reducing or ignoring communication among associates. This formulation has been made possible through data contributed by a number of field workers, who have executed studies under Dr. Calhoun's direction.

Perception. The dichotomy between sensation and perception has been a source of fundamental difficulty since the beginnings of experimental research in perception. It is manifested in many ways in present-day perceptual theory, especially with respect to the problem known as object-constancy. Perceived object-size remains functionally invariant with varying object-distance

in spite of the fact that retinal image-size varies inversely with distance. The general resolution of this apparent discrepancy has been to assume that the primary sensory datum is the angular size of the retinal image, which is integrated perceptually with other elementary cues to object-distance, perceived object-size emerging as a complex, judgmental result. One of the most compelling supports for this view is the observation that physical extents, immediately and directly, "look" smaller as they are farther removed from the observer. It is readily verified by most observers, for example, that railroad tracks appear to converge in the distance.

Dr. Carlson has formulated an alternative hypothesis, which postulates that veridical size-perception is the basic perceptual fact and seeing-in-perspective is a culturally conditioned, attitudinal bias. Two experiments were completed during the year which offer strong support for this argument. In the first, experimental judgments of overestimation of size-at-a-distance were shown to be a function of nonperceptual factors. In the second, it was demonstrated that administration of the centrally acting drug LSD produced underestimation of size-at-a-distance only when the drug condition represented the subject's first experience with the experimental task. This result can only be interpreted as an interaction between drug effect and response-bias, not as a direct effect of the drug on the perceptual process itself.

Another fundamental problem in perception is concerned with perceptual adaptation to motion. Theoretical accounts of this phenomenon have generally treated it as analogous to color or temperature adaptation. By means of an aftereffect experiment, Dr. Carlson has obtained results suggesting that this analogy can only be partially correct. The zero-point of perceived velocity was shifted appropriately as a function of adaptation; but there was no effect of adaptation to a given velocity on a subsequently experienced velocity of opposite direction, a kind of aftereffect which occurs rather strikingly in color and temperature perception.

A special test for measuring perceived velocity has been developed in connection with the foregoing velocity adaptation experiments. The essential feature of this test is that response time is utilized as a correlate of perceived velocity. Dr. Feinberg, of the Clinical Neuropharmacology Research Center, and Dr. Carlson, in an initial exploration of the response of schizophrenic patients to this test, discovered that schizophrenics appear to manifest extraordinarily long response times while at the same time reacting appropriately to variations in the stimulus parameters of the task. A larger scale study has been set up at Saint Elizabeth's Hospital to evaluate this preliminary finding, and collection of the data is nearing completion at the present time.

Aging

The program of the Section on Aging is directed at securing, through experimental investigations of the behavioral capacities and physiological status of animals and humans, an understanding of aging as a pervasive natural phenomenon. Through the research and theoretical efforts of the staff members of the section the subject matter has undergone what, in retrospect, seems to have been a rapid transition from a descriptive naturalistic phase into a more tightly organized state of knowledge. Though it has several facets, the research program of the section has two principal foci: (1) The accumulation of systematic information concerning behavioral and physiological changes associated with age; (2) the systematic investigation of age-related impairments of the higher cognitive processes in man, and the study of possible methods of preventing or overcoming such deficits. Work has continued on the analysis of age changes in learning ability and the speed of psychological processes. The cellular components and extracellular relations in the nervous system have also continued to be studied in the hope of identifying some of the biological concomitants of the behavioral changes in aging.

Eleven of the fifteen chapters planned for a monograph reporting the results of the extended, interlaboratory study of aging have been completed, and publication has been scheduled for the spring of 1961. In collaboration with several laboratories at NIH, the Section on Aging also has in progress a set of followup studies on the survivors of the group of aged subjects from whom the original data for this study were obtained during 1955–57. Selected reports on work presented in the seminar at Berkeley and at the meetings of the International Congress of Geron-

tology in San Francisco will be collected into two volumes that are being prepared for publication early in 1961.

Dr. Birren reported at the Berkeley seminar on preliminary results from his and Dr. K. Riegel's response time study of age-differences in primarily mental activities as compared with predominantly motor performances. In this experiment, a set of 22 problems, programed by the Psychomet and differing widely with respect to their demands on associative facility, were administered to two contrasting age groups. The results indicate that the relative speed-impairment associated with aging increased as the complexity of the mental functions required by the problem increased. The apparent age-decrement in speed of performance on tasks requiring such mental operations as color-symbol matching, superordinate, coordinate and part-word association far exceeded that which could be understood in terms of the purely motor deficits found in analogous tasks that did not require these cognitive functions. During the current year, data will be obtained from additional subjects and the full set of results will be analyzed to determine the relative variances contributed by the associative processes that are found to deteriorate with age.

Dr. Birren, using a two choice point watermaze, has continued his study of age-differences in the psychomotor performances of rats. The initial learning portion of this experiment has been completed and the results show no dramatic effect of age on learning proficiency. The animals will now be retrained on the same maze after a 6-month interval without practice. Having satisfied a relearning criterion, they will be given a new problem. Learning under these interference conditions will then be measured. When this study is completed, the Section on Aging will have available for comparison four sets of data, obtained under a variety of conditions by different experimenters, all relevant to the study of the effects of age on learning efficiency and interference. Coordinated analyses of these results are expected to provide rather precise identification of conditions conducive to the occurrence of age-differences in the learning ability of rats.

Dr. Botwinick's comparison of the reaction time of young and old human subjects in relation to set and sense modality has continued. 64 young controls and 15 elderly subjects have already been tested, and 49 elderly subjects in good physical condition are to be added to the group. The results obtained thus far indicate that changes of the reaction time response and the ability to maintain set are associated with the aging process.

Four papers are in preparation by Dr. Jerome on related studies of learning and transfer of training in senescing rats. They provide evidence to the effect that (1) aging animals are not more stereotyped in their free choice behavior than are young adults, (2) they do not manifest a greater tendency to higher order (sequential) stereotypes, (3) senescing animals, under some conditions at least, learn as rapidly as young adults and (4) aging animals transfer their learning about as efficiently as younger ones. A collaborative study with Drs. Goodfriend and Kaufman is in progress on the effects on learning of several phenylalanines, possible causative agents of phenylpyruvic oligophrenia. Additional collaborative work is being carried forward with Dr. Streicher in screening a set of drugs for differential age effects on behavior.

The results from Dr. Jerome's study of the effects of aging on the higher cognitive processes in man reflect a marked impairment of the heuristic controls relevant to the solution of the set of problems programed by the Logical Analysis Device. Though there were marked individual differences among the old subjects, even the best of them was not as proficient as the worst of the young subjects. The following specific deficits were prominent in the performance of the aged: (1) Failure to secure a clear indication of subgoals before engaging in random search; (2) failure to take advantage of mnemonic aids urged upon them by the procedure; (3) inability to develop solution strategies designed to reduce the cognitive strain of the problem solving effort; (4) inability to apply solution strategies that were repeatedly explained; and (5) failure to draw inferences appropriate to information at hand. These results have emphasized the urgency of investigating the feasibility of retraining in the basic heuristic processes that appear to have so seriously deteriorated in the process of aging.

Employing an original technique in which a

single word or single phrase is played repeatedly to the subject from a loop of recording tape, Dr. Warren has made a comparison of the perceptual processes of old and young adults. This repetitive stimulation gives rise to illusory perceptual changes frequently involving considerable phonetic distortion. Young and old subjects differed markedly in their reactions to this type of presentation, the illusory transitions occurring about four times more rapidly in the young than in the old. In the older subjects, moreover, the stimulus word's frequency of occurrence in English appeared to influence the illusion more than it did in the younger subjects, the latter appearing to give greater emphasis to phonemic aspects of the stimulus. It appears therefore that the manner in which stimuli are organized perceptually changes with age—increasing reliance on the probability of occurrence and increasing perceptual stability being associated with age.

Dr. Streicher's investigation of the thiocyanate space of various rat organs and tissues was terminated this year. For the brain the extracellular space was found to be a function of dosage. The extrapolation of the curve relating extracellular volume to plasma level indicated a space approximately 2 to 5 percent in young and old animals alike, whereas for other tissues, heart, skeletal muscles, testes and kidney of senescent animals showed significant increases in extracellular volume. There was, however, some evidence that thiocyanate was eliminated from the brain of older animals more slowly than it was from the younger. Finally, animals in which cerebral edema was induced experimentally showed no change in the distribution of thiocyanate, although the sodium content of the brain was elevated 50 percent and that of the spinal cord approximately 100 percent. From these studies it was concluded that if such hypothesized age-related changes in the brain as loss of cells, dehydration, gliosis, etc., occur in the rat, they are not reflected by alterations in the extracellular space which serves as a sensitive indicator for similar changes in other organs. Dr. Streicher, in collaboration with Dr. Rall of the Laboratory of General Medicine, NCI, and Dr. Patlak of the Section on Theoretical Statistics and Mathematics, NIMH, has also undertaken an investigation designed to identify and measure the rates of those processes which are responsible for the movement of charged, lipid-insoluble material into and out of the cerebral spinal fluid. Preliminary results indicate that the rates of entry of some materials are a function of their concentrations in the blood plasma. More detailed conclusions will be available when the mathematical analyses are completed.

The several lines of investigation being explored by the Section on Aging are providing accumulating evidence to the effect that the cognitive processes of lower animals are only slightly, if at all, impaired by age. This raises doubts about a commonly accepted assumption that the deterioration of the mental capacities in man with age is an intrinsic component of an ontogenetically determined process characteristic of all biological levels. Similarly, though our physiological studies have revealed age-related deteriorations in several classes of tissues, the differences observed would explain deficiencies in reproductive, vegetative and motor activities more directly than they would impairments of tissue more directly connected with mental capacities. Diligent search in our own laboratory and elsewhere has failed to uncover any convincing evidence of cortical or subcortical degeneration of neuronal materials. This may be interpreted as not consonant with a position that the intellective faculties of man are structurally predestined to degradation with age. From the study of human subjects, the evidence of impairment of the perceptive processes combines with consistent indications of an age-related decrement in the speed of reaction to suggest a curtailment of interactive capacities. This in turn explains, to some extent at least, the observed deficit in associative facility, since a decrease in interaction would naturally entail associative disuse. Some appreciable decrement in the ability to deal with problem situations is, of course, to be anticipated from a curtailment of interaction and a reduction of association facility. But the marked collapse of the elementary heuristic system appears to suggest a more pervasive failure for which one would have to invoke a theory of generalized decay of organization at the neuronal level. At the level of heuristic regulation, the relevant organization, as distinguished from gross architectonics, could reasonably consist of a delicate tuning of synaptic resistances and dendritic elaborations too subtle to be detected by bio-

chemical analyses. According to Hebbian theory, such regulation is originally generated by prolonged training and is maintained only at the expense of assiduous exercise, so that reduction of interaction would potentially result in a decay of organization sufficient to explain the phenomena of cognitive impairment commonly observed in experiments with the aged. The Section on Aging will probably continue to direct much of its future work toward the search for specific age-related deficits, both behavioral and physiological, and toward the determination of the extent to which the heuristic deterioration commonly associated with aging can be modified. It is expected that from such studies of modification, additional important principles of the aging process will be uncovered.

Laboratory of Socioenvironmental Studies

Dr. John A. Clausen, chief of the laboratory since its inception in 1951, has left the National Institute of Mental Health to become director of the Institute of Human Development at the University of California at Berkeley. Dr. Clausen created *de novo* what was at first a small program of applied sociological research and what later broadened and deepened into a far-ranging basic research program in sociology, social psychology, and anthropology. After 8 years of building staff and program, he leaves behind a solid, functioning enterprise.

During this past year, the laboratory has been most fortunate in recruiting Drs. William Caudill and Stanley Diamond. Dr. Caudill, now chief of our Section on Social Studies in Therapeutic Settings, is widely known for his pioneering studies of the social organization of the mental hospital, as well as for his research on Japanese culture. Dr. Diamond brings to the laboratory his wealth of anthropological field experience in Israel and Nigeria.

The departure of a laboratory chief is necessarily a time for stocktaking. We have now reached the stage in an organization's history where it is no longer necessary to be preoccupied with creating a new staff. We have a first-rate core staff in all three of the social sciences most relevant for an NIMH research program—sociology, social psychology, and anthropology. The state of the job market for the past 2 or 3 years has been such that we have not been able to attract as many people of the requisite quality in sociology as in anthropology and social psychology; we shall have to put special emphasis on seeking out sociologists. But, by and large, the growing stage is largely past, and we are now in a position to build on the people we have and the momentum they have developed.

In general, it may be said that the laboratory as a whole is concerned with one major problem —the effects of social structure upon personality and behavior—with the sections, and the individuals in those sections, approaching the problem from a variety of vantage points. To some degree, the Section on Social Studies in Therapeutic Settings is marked by its interest in the structure and functioning of the mental hospital and by a cross-cultural orientation, the Section on Social Developmental and Family Studies by its interest in family relationships, and the Section on Community and Population Studies by its interest in how the organization of the larger society affects personality and behavior. But these are distinctions of emphasis.

Almost half of the current research in the Laboratory is focused on the family and personality development. Current studies range from broadscale attempts to delineate variations in parent-child relationships associated with the family's social position, to more intensive studies of how particular aspects of parent-child interaction affect children's personality development. Considerable effort is being devoted to the twin problems of developing better concepts for interpreting family relationships and developing better methods for securing data. Methodological research on the use of observational data, on the validity of inference based on retrospective reports, and on the consistency of reports given by various family members have been integrated into several of the substantive studies.

The concentration of attention on the family reflects its strategic importance for the personality development of the child. But the program includes, too, studies of personality in later years: research on the self-concept in adolescence, on the emotional reactions of men in their middle working-years to job stress, and on social aspects of gerontology.

During the past few years the laboratory has developed an intensive program of research on

the functioning of the mental hospital, a program now accounting for about one-third of our resources. Present studies include a comparison of Japanese to American mental hospitals, a large-scale survey of nurses' attitudes and behavior toward patients at Saint Elizabeth Hospital in Washington, D.C., participant-observational studies of the functioning of the mental and experimental studies with schizophrenic patients.

This represents a research program of considerable scope and diversity. It is a rich program, for it is made up of capable people pursuing the objects of their individual curiosities under the conditions of freedom essential to scientific accomplishment. But are we making the maximum use of the diversity of talents, interests, and points of view in the laboratory? We probably have not done enough to establish the free marketplace for intellectual interchange that makes it possible for each investigator to benefit maximally from the presence of other people tackling similar problems in different ways. It should be possible to create working conditions so that as a group we accomplish far more than would be possible if each of us were working in isolation from the others. The challenge is especially exciting to social scientists, for the problems with which our science deals.

The section reports which follow present our program in greater detail.

Social Studies in Therapeutic Settings

During the past year the members of the section have been continuing their studies of therapeutic settings in the United States and in other cultures. Although many of our studies are carried out in therapeutic settings as these are narrowly defined in terms of the hospital, the clinic, and the doctor's office, we have also interpreted our mission more broadly so as to include consideration of the therapeutic potential of such important social structures as the family, the school, and the peer group. A therapeutic setting such as a hospital exists, in one sense, only as a nexus of relations among staff and patients which, for members of both groups, is tied to other clusterings of significant relations in their past, their present, and their future. Although a hospital in one culture may appear superficially similar to a hospital in another culture, the manner in which these institutions, and the lives of those who work and live in them, are caught in the wider network of family and community organization may be remarkably different. This question is one of the reasons we have chosen to include a comparative cross-cultural dimension in our work.

During the year there have been two additions to the professional staff. In July, Dr. William Caudill, an anthropologist with research experience in American and Japanese psychiatric hospitals, joined the section as chief, coming from Harvard University where he had held the posts of lecturer in the Department of Social Relations, research associate in the Department of Psychiatry, and chief of the Department of Social Science at McLean Hospital. In October, Dr. Stanley Diamond, an anthropologist with field experience in an Israeli Kibbutz, an Arab village, and a primitive community in Nigeria, joined the professional staff of the section, coming from Brandeis University where he had held the posts of assistant professor in the Graduate Department of Anthropology, and research anthropologist in the Department of Psychiatry at Massachusetts Memorial Hospital.

Through June of the past year the section continued to be host to Mr. Yngvar Löchen, a sociologist and visiting scientist from the Dikemark Mental Hospital in Oslo, Norway. Mr. Löchen continued his comparison of certain aspects of organizational structure in Norwegian and American psychiatric hospitals. He was particularly interested in the relation between the formal and informal structures of the hospital as this relation affected the balance between considering the patient as a "case" and as a "person." The section profited greatly from its contacts with Mr. Löchen, and we hope in the future to continue to provide opportunities for mutual exchange with visiting scientists from other countries.

Dr. Leonard I. Pearlin, a sociologist, in collaboration with Dr. Morris Rosenberg of the Section on Community and Population Studies, continued his studies at Saint Elizabeths Hospital in the search to identify therapeutically crucial attitudes among nursing personnel, to develop methods for their measurement, and to investigate the meaning of these attitudes in their interrelation with the differing social structure to

be found in a series of wards in the hospital. The results of this study are described more fully below, and Dr. Pearlin plans in the future to make use of the methods and results of this study in a 10-hospital cooperative study initiated by the Psychopharmocology Service Center. During the year, Dr. Pearlin also continued his work with several members of the Personality Development Section of the Adult Psychiatry Branch on a study of how competent adolescents cope with the problems generated by transition from the senior year of high school to the freshman year of college.

Dr. Carmi Schooler, a social psychologist, continued a series of studies with schizophrenic patients. Dr. Schooler's basic interest is in the experimental study of the relationship between various psychological and social background factors and the regressed chronic schizophrenic's reluctance to affiliate with others. One experimental study in this area of interest was carried out during the past year at Springfield State Hospital, and the results are presently being analyzed. Another somewhat similar study is being planned which will investigate some possible factors in the chronic schizophrenic's attitudes toward social interaction by asking subjects to select the valued behavior patterns they prefer in individuals with whom they are expected to socialize. Dr. Schooler's interest along these lines has also led him to study the relationship between birth order and schizophrenia.

As is indicated by the range of academic disciplines represented in the section, our studies have been carried forward using a variety of methods: anthropological field work, historical analysis, formal and informal interview techniques, surveys carried out through the use of schedules and questionnaires, and experimental designs. Some of the details of this work are indicated in the following descriptions of several of the projects being carried out in the section.

Dr. Pearlin, in his study at Saint Elizabeths Hospital, devised and used two instruments. One was a structured questionnaire, completed by 1,138 members of the nursing service. This questionnaire was designed to measure therapeutically relevant attitudes and also asked for material regarding the social and personal characteristics of each staff member. The second instrument, intended to gather information about characteristics of patient populations, programs, policies, and staffing patterns, was addressed to each of the 156 wards of the Hospital. With these data, it has been possible to examine a broad range of factors influencing attitudes relevant to patient care. One type of factor found to influence these attitudes concerns the social values of individual nursing personnel, many of these values being acquired as members of the larger community. Characteristics of wards to which personnel are assigned are also important determinants of attitudes. Most instrumental in attitude formation, however, are not staff or ward characteristics alone, but both of these together in certain combinations. One of the attitudes examined in this way is "status distance," defined as a withdrawal from patients by setting one's self above patients. Status distance is most likely to occur among staff assigned to wards containing patients of low social standing, but this is particularly true for personnel with the following characteristics: a relatively high position in the nursing order, an obeisant regard for authority, and blocked aspirations for occupational mobility. The importance of the interaction between individual and ward characteristics in shaping attitudes is also demonstrated in the case of "personal distance." Personal distance, defined as emotional indifference to patients, is most frequently exhibited by staff working with patients whose ages are markedly different from their own. Nursing personnel working with patients of their own sex, however, express more personal distance than staff working with patients of the opposite sex. Thus, the study has isolated and explored a series of nurse-patient combinations that serve to reduce or expand psychological distance.

The first of Dr. Carmi Schooler's experimental studies with schizophrenics referred to earlier was based on a modification of a method for studying affiliative behavior developed by Dr. Stanley Schachter. In this modification each subject was brought to the experimental setting and told that she was going to be seen by a psychologist; the subject was then asked to wait because the psychologist was not yet ready for her. The subject was led into a waiting room containing two alcoves—an empty one, and one in which an assistant of the experimenter, dressed in the manner of a patient, was sitting. The

subject was told she could wait in either alcove, and her choice of alcove as well as her reactions to the assistant served as measures of her desire for social affiliation. After 4 minutes of waiting, the subject was taken to a psychologist and given a battery of tests. Although the data from this experiment have not yet been fully analyzed, a preliminary analysis indicated that the chronic schizophrenics conformed to a pattern found by Schachter among normal subjects in which subjects born in earlier birth ranks were more likely to meet stress in a social manner than subjects born in later birth ranks.

The above finding led Dr. Schooler to study the relationship between birth order and schizophrenia. The method used for investigation consisted of an analysis of data from a 25 percent sample of the schizophrenic female patients in Springfield State Hospital, and a reanalysis of data collected in other published and unpublished studies. As a result of this investigation, Dr. Schooler has found that: (1) In a sample of hospitalized female schizophrenics, significantly more patients came from the last half of their sibling group than from the first half. (2) In a sample of discharged patients there were disproportionately large numbers of last half catatonics and first half paranoids. From the data at hand, however, it cannot be concluded whether this finding can be true only for discharged patients since a similar, though not significant, trend exists among the hospitalized population. (3) In general, when the data from the various studies were combined, significantly more of the subjects were last born than first born. This difference, however, appears to be entirely due to the subjects from families of four or more. (4) It is impossible with the data at hand to separate the effect of birth order and maternal age. Because of this a followup study is now being planned to evaluate the relative effect of birth order and maternal age on schizophrenia.

Dr. Diamond, as indicated earlier, has done extensive anthropological field work, and he now plans to bring together Israeli, Arab, and Nigerian materials in a study of comparative socialization. He reasons that the Israeli Kibbutz represents a type of modern collectivism, and the Arab village represents a hierarchical and patriarchal society in transition to more or less "Western" modes of behavior, and that the Anaguta of Nigeria represent a type of primitive, communally organized society. These three levels of organization—contemporary, transitional, and primitive—imply an over-all historical schema under which studies of socialization relative to the specific history and structure of each society can be subsumed. The objective of this inquiry, then, is to help broaden and develop the comparative-historical approach to those culturally determined aspects of the individual's life cycle. This inquiry may also provide us some keys in tracing the etiology and characteristic course of certain emotional disorders.

Dr. Caudill's current research is concerned with cultural and psychodynamic factors in the occurrence and treatment of psychiatric illnesses in Japan. Using mainly observation and interviewing, studies were made during 1958–59 of the organization and operation of three contrasting small Japanese psychiatric hospitals located in metropolitan Tokyo. In terms of treatment, the first hospital was organic in its emphasis, the second specialized in Morita psychotherapy, and the third utilized psychoanalytically oriented psychotherapy. In addition to these intensive observational studies, information was obtained on the history and present organization of psychiatry in Japan. A schedule calling for psychiatric and sociological information was constructed and completed for the approximately 600 patients who were admitted during 1958 to the three hospitals which were studied, and for an additional 200 patients who were admitted in 1958 to a large psychiatric hospital run by the city of Tokyo. A questionnaire asking for information on personal background and nursing career was administered to approximately 200 student nurses at two schools of nursing in Tokyo. A picture interview, using a series of eight pictures as visual questions, was devised and used with 80 subjects (divided among doctors, nurses, and patients) in the attempt to obtain data on what impulses were allowable or needed restraint in various life situations.

Specific findings are not yet available as analyses of the several parts of the data from Japan are still in progress, but it is hoped that this study will help determine the extent and nature of cultural influences on types of psychiatric illnesses and their treatment. On a descriptive level this involves identification of patterns of

symptoms and preferred modes of care that are characteristic for a particular culture. On a more conceptual level this study should provide information on the effect of different cultural settings on the development of character structure and ego defenses. In line with this, the data should be of use in furthering understanding of the interplay between the human organism and its "average expectable environment" as this concept has been developed by Hartmann and carried further by Erikson. The reasoning in this regard is that both the patterning of the defensive structure of the personality and the nature of the "average expectable environment" are subject to cultural influences throughout the varying stages (or transitional crises) of the life cycle.

Social Developmental and Family Studies

The Section's primary research emphasis is on child development. The developmental areas in which the research falls can be summarized as three: (1) Parental-child relationships; (2) developmental problems of a social psychological nature; and (3) problems of methodology in child development research. A secondary focus in the section is on problems of human aging. The disciplines of social psychology and sociology are represented in the section, bringing a wide range of theory and methodology to bear upon developmental problems. Interview and questionnaire techniques, field and laboratory experiments and controlled observations in natural settings of peer groups and family are being used.

In the search for critical variables in the socialization of the child, research in general stresses the mother-child unit, and in particular the mother's attitudes and practices of child-rearing. However, a mother rears her children within various family and cultural contexts which may directly or indirectly affect the mother-child relationship. It is with these contexts and their possible influences that several of the projects are concerned.

Investigations of the mother role in terms of the family's social class, racial membership, and the mother's employment status are being carried out in two related studies. Mr. Gillette has been interested in how differing combinations of class, race, and employment statuses operate to influence: (1) the nature of husband and wife participation in family functioning and (2) the mother's concept of herself and her satisfactions in her various roles. Questionnaire data were obtained from 700 mothers from different social class levels in Negro and white groups in the Greater Washington area. The degree and types of father and mother participation in various family tasks and the degree of consensus between husband and wife on family norms are shown to differ significantly by class and race. Middle-class fathers are more likely than working-class fathers to share in household tasks. Fewer discrepancies between husbands and wives in attitudes toward family issues are reported by middle-class than by working-class mothers. These class differences are accentuated by controlling for maternal employment status, with greater participation by the husband and greater husband-wife consensus in families in which the mother is employed. Mothers' concepts of themselves and their satisfactions from mother, wife and career roles also differ by class and race. Employment is less likely to be seen as incompatible with the mother role by Negro women than by white women, regardless of class.

Dr. Yarrow has used a subsample from the preceding study to investigate associations between mother's employment and child rearing practices. The combination of mother role and work or career role introduces a number of changes into the family. It may introduce a factor of maternal separation or multiple-mothering in the upbringing of the child. It might also be hypothesized that specific rearing philosophies and practices would be associated with the maintenance of a work role by the mother. Of particular interest are mothers' child rearing practices with regard to independency training, permissiveness of aggression, achievement motivation, and sex role typing. At present, only preliminary analyses have been completed. They indicate very slight differences between employed and nonemployed mothers in child rearing philosophies and practices. when class and race are controlled.

The unique position of each child in his family group, in terms of his sex role and sibling order, is being studied as another context within which the mother-child relationship develops. Dr. Campbell and Dr. Yarrow are be-

ginning studies in this area. The basic research tool of these studies is the interview.

Drawing upon various cross-cultural studies of primitive societies, Dr. Burton has analyzed a number of structural characteristics of families, with an interest in the formulation of a theory of identification which would integrate findings from varied cultural sources. He has shown in this analysis that the absence of the father or the exclusive mother-son relationship in infancy and early childhood is associated with certain developments and customs during adolescence. These associations in primitive cultures appear similar to associations found between the family structures of certain lower class subgroups within our society and the incidence of delinquent behaviors in adolescence in these groups.

As indicated earlier, a number of studies in the section share a common focus upon social psychological development of children. A study of children's cognitive processes in social experiences—their appraisal of other persons, their sensitivities to and interpretations of interpersonal relations—has been completed by Dr. Campbell and Dr. Yarrow. Two hundred sixty-seven children between 8 and 13 years of age were interviewed and observed in resident camp settings. Sensitivity in social perceptions—measured by the child's ability to give complex descriptions of others and to integrate diverse characteristics of the other person into an organized impression, often including inferences regarding the motives underlying observed behavior—shows an increase with the age of the children. Cognitive complexity is also associated with a child's adjustment to his group. High status among peers is associated with higher scores on the several measures of cognitive complexity. Children showing a high frequency of interaction in the peer group, as contrasted with a withdrawn pattern, tend to have the higher ratings in quality of social perceptions. Boys and girls do not differ in these respects, but they tend to emphasize different features in their reports on the characteristics of others; boys report more on aggressive features of interaction, girls on nurturant aspects.

Dr. Burton has underway a series of experimental studies on the processes of internalization of rules, standards and values, with special emphasis on resistance to temptation and guilt. What are the conditions which dispose young children to conform to established rules or to yield to the temptation to break rules? Further, what are the psychological after-effects of resistance or yielding? What are some of the child-rearing antecedents to variations in children's internalization of moral standards? Measures of conformity to established standards are obtained in experimentally controlled test situations. Psychological effects of the temptation effect are measured through the use of incomplete-story techniques. Observations of mother-child interactions in controlled situations furnish data on child-rearing variables.

A number of projects in the section have methodological interests as their focus. As a methodological by-product of the study of children's social perceptions, a study of intermeasure correspondence in the appraisal of social relations has been undertaken. Three different sources of data—children's ratings of one another, adults' ratings, and observational records of interaction—provided data from which to compare various measures on the children. A wide variation in degree of intermeasure correspondence appears: For some variables, such as popularity status, a considerable degree of intermeasure agreement is obtained. For others, such as appraisal of anxiety, considerable intermeasure discrepancy exists.

A major methodological study is the investigation by Yarrow and Campbell of the nature of mothers' retrospective reporting on events and conditions pertaining to the earlier development of their children. The technique of recall has considerable prominence among the techniques of research used in investigating the significance of childhood experiences for later development. Most often the parent is asked to reconstruct his precepts and practices in caring for his child or to describe the characteristics of his child at some designated earlier date. The aims of the study 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 time interval, intervening events, and present psychological situation. The data collection on 250 families is very nearly completed and some analyses have begun.

In the area of human aging, Dr. Yarrow has

collaborated in the NIMH interdisciplinary study of healthy aged men and, with the interlaboratory group, is now completing a book on the biological and behavioral aspects of human aging. This investigation brings the perspectives and measurements of physiology, psychiatry, psychology, and sociology to the examination of factors in human aging. The social psychological interests lies in (a) analyses of the psychological environments of the aged, (b) the attitudes and behavior of the aged in their present life circumstances, and (c) relationships between these factors and the data from physiology, cognitive functioning and psychiatric assessments. Significant relationships have been found between environmental losses suffered by the elderly, particularly losses in love-objects, and many aspects of their daily functioning and adaptive capacities. The individual's social adequacy, measured by the complexity of his daily behavior and his maintenance of goals and social relationships, shows significant relationships to laboratory measures of cognitive functioning, reaction time, cerebral metabolism, the subject's own evaluations of age declines, and depressive symptoms.

A followup study is just now beginning of the men who were studied intensively 4 years ago. The objectives of the followup are to examine stabilities and changes that have occurred with time and to determine the predictive value of certain physical and psychological indicators present four years ago for current status and functioning.

Community and Population Studies

The three studies being conducted in this section, quite disparate in focus, share a common concern with the impact of the organization of the larger society upon personality and behavior. In the first study, Dr. Stephen Boggs is concerned with the ways in which one's work career affects one's values and one's emotions. The second deals with adolescents, rather than adults: it is an exploratory study, conducted by Dr. Morris Rosenberg, of the social determinants and consequences of adolescents' self-conceptions. In the third, Dr. Melvin Kohn, Dr. John Clausen, and Miss Eleanor Carroll have been examining the effect of social class upon parents' values and practices.

The first of these studies is based upon a survey that Dr. Boggs conducted among the research technicians and assistants in the various laboratories of the NIH. He concentrated on men in the age group where the realities of limited further advancement become inescapable. Thus far in his analysis he has dealt principally with what happens to men's values in the course of their careers. One pregnant finding is that as men hit the promotion ceiling, they become preoccupied with advancement, rather than intrinsic job interest or even security. Men farther along in their careers, frustrated in their desires for promotion, are even more likely to be advancement-oriented than are the younger men who have not yet come face-to-face with limited opportunity.

Dr. Rosenberg's study of adolescents self-conceptions is based on a questionnaire he has administered to a representative sample of 5,077 high school students in the State of New York. The questionnaire is broad-gage: it attempts to secure information about these adolescents' self-evaluations and their ideals, about those personal and familial experiences that may be relevant to their self-conceptions, and about those aspects of their behavior that would presumably be most directly influenced by their self-conceptions. The data are in hand and the analysis underway, but it is too early to report results. One unusual aspect of this research might be noted, however. While there has been much informed speculation about the social determinants of self-conception, the research that has been done on this problem has taken little account of the multifaceted nature of self-conceptions or of the complexity of inter-related variables with which any study in this area should deal. Dr. Rosenberg, by using a questionnaire rather than more intensive interviews, has chosen to sacrifice the opportunity for securing a great deal of information about each of his respondents in favor of having at his disposal sufficient information about a large number of individuals to be able to do the complex multivariate analysis the problem demands.

The work done this past year on the study of social class and family relationships represents the culmination of a long-term study; it may thus be useful to review not just this year's work, but the project as an entity. (The study is based on interviews with mothers in a representative sample of 339 Washington, D.C., families, plus

independent interviews with fathers and children in one-fourth of those families.)

Rejecting the oversimplications that working-class parents differ from middle-class parents principally in a greater propensity to use physical punishment and a readiness to permit their children a greater range of freedom, the investigators approached the problem by attempting to understand the ideologies of parents in both social classes and then tracing out the behavioral consequences of these ideologies. The study has shown decidedly different emphases in middle- and working-class parents' values for their children. The dominant motif of middle-class parents' values is that the child develop his own standards of conduct: desirable behavior consists essentially in the child's acting according to the dictates of his own principles. By contrast, the dominant motif of working-class parents' values is that the child's actions be reputable: desirable behavior consists essentially in his not transgressing parental proscriptions.

Both middle- and working-class parents use physical punishment—when milder methods of discipline do not work. Further, parents of one class use physical punishment about as frequently as do parents of the other. But they resort to the hand and rod under quite different circumstances. Faced with the problem of whether or not to punish a child's misbehavior, middle-class parents look to the child's motives and feelings. Working-class parents look, instead, to the act itself; their attention is focused on the direct consequences of misbehavior. The middle-class child is more apt to be punished for loss of self-control, the working-class child for disobedience.

Having seen the implications of parents' values for their disciplinary practices, the authors considered what the effects on the overall structure of family relationships might be. They assumed that inherent in parents' standards for their children must be conceptions of their own responsibilities as parents. Specifically, it seemed that middle-class values imply a parental obligation to be sensitive to the child's thoughts and feelings; working-class values imply a parental obligation to make clear to the child what rules are to be obeyed. One consequence should be for the ratio of support to constraint in parents' handling of their children to be higher in middle- than in working-class families. Another consequence should be for mothers and fathers to divide the responsibilities for the support and constraint of the children differently in the two social classes. In particular, given their own and their wives' ideologies, middle-class fathers should be expected to play a much larger part in providing support to the children than do working-class fathers. It would seem more appropriate to working-class ideology that the father's role center on the imposition of constraints.

Both expectations are borne out by the data. This has important theoretical implications above and beyond an interest in the impact of social class on family relationships. Most theories of personality development have been based on the model of a family in which mother's and father's intra-familial roles are necessarily differentiated, with mother specializing in providing emotional support and father in imposing constraint. However useful a first approximation this may be, both middle- and working-class variations on this general theme are sufficiently great to compel a more precise formulation.

The data are at least partly consistent with the mother-supportive, father-constraining formulation, for even in middle-class families almost no one believes that the child turns to father *more* readily than to mother. Yet, in a sizeable proportion of middle-class families it is mother who takes primary responsibility for imposing constraints on sons, and father is at least as supportive as mother. And although middle-class fathers seldom seem to be as supportive of daughters as are their wives, it cannot be said that fathers typically specialize in constraint even with daughters. In most middle-class families, mother's and father's roles do not seem to be sharply differentiated. To the degree that they are, a new division of responsibilities is developing, with each parent taking special responsibility for support of children of his own sex.

Mother's and father's roles are more sharply differentiated in working-class families, with the children almost always feeling that mother is the more supportive parent. Yet, despite the high valuation put on the constraining function, fathers do not necessarily specialize in setting limits for the children, even for the sons. In some working-class families mother specializes

in support, father in constraint; in others—perhaps in most—the division of responsibilities is that mother raises the children, father provides the wherewithal. This pattern of role-allocation probably is and has been far more prevalent in American society than the formal theories of personality development have recognized.

Office of the Chief

Dr. Gordon Allen (the sole geneticist in a social science laboratory) has devoted himself to two principal projects. The first is an assessment of factors in mental deficiency, based on a reporting system set up in 1937 by Dr. Allen's collaborator, Dr. Franz Kallmann, which provides index information on a large number of mentally subnormal twins in New York State. Dr. Allen studied many of these twins, 178 pairs in detail, classifying them as to zygosity, and obtained relative concordance rates for two major diagnostic classes. In the more severe types of mental defect, with evident congenital or neonatal pathology, he found a clear contrast between monozygotic and dizygotic twins. This he interprets as evidence that genetic factors play an important role, interacting with nongenetic factors that may also be elucidated by further analysis of the data.

Dr. Allen's other study is more methodological in intent; he is trying to develop better techniques for the determination of zygosity in triplets and quadruplets as well as in twins. For a set of twins there are only two possible hypotheses about zygotic origin. For triplets there are five and for quadruplets, fifteen. Some of the triplet and quadruplet hypotheses cannot be differentiated on the basis of simple genetic traits like blood groups, and quantitative differences must be brought into the calculations. This can apparently be done by combination of individual probabilities for separate pair differences within the set. A likely formula has been found for combining the probabilities, but it still lacks theoretical and empirical validation.

Dr. Melvin Ember has formulated a study of authority in the mental hospital. In this study, he will use anthropological field methods similar to those he developed in research on the legitimacy of authority in American Samoa. He plans to study three large nursing units selected as manifesting differences in the degree to which nursing staff accept as legitimate the formal authority structure. Intensive observation and interviewing will be conducted in one of these units, for the purpose of developing quantifiable measures of the amount of authority accorded by each member of the unit to the others. The indices developed on that nursing unit will then be used on the others. The rationale here is to use the phenomenological approach of the anthropologist who goes into a new field with a formulation of objectives and overall research design, but develops the niceties of design and the specific indices out of the material the culture presents to him—in this instance, the "culture" being the mental hospital.

Addiction Research Center—NIMH

Introduction

The mission of the NIMH Addiction Research Center is the conduct of research on the causes, prevention, and treatment of drug addiction. Although this may seem a narrow scientific field to many persons, it is actually a broad area. Many years of experience has shown that addiction is a very complex phenomenon with pharmacological, physiological, biochemical, socioenvironmental and psychological components. The study of addiction is much more than the study of a few types of drugs. Addiction has roots in the neuroses and behavior disorders, and is therefore related to the entire field of human behavior. The work of the Addiction Research Center is pertinent not only to drug addiction specifically, but to the broad area of nervous and mental disease in general.

Thus the section on opiate addiction carries on studies of the addictiveness of new analgesics, partly, as a service function to prevent introduction of potentially addictive materials into clinical use without sufficient and proper warning. Despite the time required for this routine task, the section was able to develop new methods for evaluating quantitatively the subjective responses following administration of opiates. The methods are simple, can be carried out by ordinary ward personnel, correlate well with the objective physical manifestations created by the drugs, and they constitute in fact a prototype technique generally applicable in clinical psychopharma-

cology. Simultaneously, a more sophisticated approach to evaluation of the subjective effects of drugs has been carried on by the psychological staff. Inventories have been developed which differentiate between different types of drugs and yield quantitative dose responses. Factor analyses of the data show that the information gathered in the inventory is not only useful in the evaluation of different drugs, but may also yield data of fundamental importance to the psychological modes of action of the various drugs.

The most interesting drug studied by the section on opiates during the year was a compound developed in the Hoffman-La Roche Laboratories at Basle, Switzerland—1-(p-Chlor-phenethyl)-2-methyl-6, 7-dimethoxy-1, 2, 3, 4-tetrahydro-isoquinoline hydrochloride (NIH-7672A, or ARC I-K-1). Adequately controlled clinical studies have shown that this drug is at least as effective as codeine in relieving pain when administered either intramuscularly or orally. Unlike other analgesics, I-K-1 does not induce clearcut morphine-like subjective effects of any intensity in former morphine addicts following administration of the highest doses that can safely be given. It is only a very weak suppressor of abstinence from morphine, being only 1/7th as potent as codeine in this respect. It does not induce a significant degree of physical dependence following administration of the highest doses compatible with safety for 30 days or more. This compound may represent the greatest dissociation between analgesia and physical addictive properties than has yet been achieved.

Experiments on the effects of drugs on the electroencephalographic and peripheral vasopressor responses to stimulation of the midbrain reticular formation, carried out by the neurophysiological section, have led to hypotheses that both "muscarinic" (blocked by atropine) and adrenergic synapses are involved in these responses. Alternative pathways may exist which take over the function of the primary pathways when either the muscarinic or adrenergic synapses are blocked. These concepts may be of fundamental importance in neurophysiology, and alternative pathways may be involved in tolerance to drugs.

The psychological unit continued work designed to elucidate psychological mechanisms involved in pain and its relief, a project obviously of great significance to clinical medicine in general. Work was initiated on probability learning in psychopaths, in the hope that light may be shed on the reasons for this puzzling pattern of behavior which is important in the areas of alcoholism and delinquency as well as in the field of opiate addiction. The psychological unit also continued work on conditioning factors of possible importance in relapse to opiate drugs.

A modest beginning was made on problems of alcohol. It consisted of elucidation and quantitation of the subjective effects of alcohol in human subjects and comparison of these with the effects of other centrally active drugs in the same patients. Also pertinent to the problem of alcohol was the project carried out in the neurophysiological section on the mechanisms of convulsions and delirium following withdrawal of barbiturates and related drugs. This study suggests that the cerebral cortex is not necessary for the development of withdrawal convulsions.

Addictive Properties of New Analgesics

These studies are designed primarily for the purpose of providing 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 evaluation of 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 improvements were made in methodology for measuring the overall abuse-liability of new drugs. Thus "subjective" and "objective" rating scales of behavioral change (with particular reference to "euphoria") were tested for several new drugs under double-blind conditions. As noted in the last annual report, these quantitative data revealed a high degree of concordance between the ratings of our experimental subjects ("subjective") and the ratings of the observers ("objective") in the case of the potent analgesics such as morphine and heroin, but considerable discordance when weaker analgesics were tested. The addictive "ratings" of opiate addicts for a series of morphinelike drugs, respecting the parameters (1) identifica-

tion as "dope" (opite), (2) "strength" (potency), and (3) the extent to which they "would like to take each drug daily" covaried with the intensity of the abstinence syndrome which developed when each of these drugs was discontinued abruptly. The degree of "acceptance" of drugs by addicts was significantly influenced by the route of administration; for example, positive "acceptance" of morphine orally was zero, 40 percent when given subcutaneously, and 54 percent by the intravenous route. Heroin had an "acceptance" rating of 32 percent subcutaneously as compared to 76 percent intravenously.

Since addicts in the United States prefer to take their drugs intravenously, an intensive comparison was made of intravenous morphine and heroin, using single doses and four levels of each drug in order to obtain the relative potency of each. In addition, both morphine and heroin were administered intravenously to postaddicts in an 18-day direct addiction test. All studies utilized a "cross-over," "double-blind" design. Using a variety of objective and subjective measures, it was found that 1 mg of heroin was equivalent to 1.80 to 2.66 mg of morphine sulfate. Postaddicts could identify morphine and heroin as such with a high degree of accuracy when these agents were administered intravenously, either acutely or chronically. Though they showed no preference for a single injection of one or the other of these drugs, they expressed a preference for heroin in the short-term addiction study (76 percent positive "acceptance" for heroin, and 54 percent for morphine). (In the text, Addiction Research Center numbers (ARC) or generic names will be used to designate compounds.)

1. *1-(p-Chlor-phenethyl)-2-methyl-6,7-dimethoxy-1, 2, 3, 4-tetrahydroisoquinoline HCl (NIH-7672A, ARC I-K-1)*

This is a new isoquinoline derivative reported to have analgesic potency in man comparable to that of codeine, but tests in postaddicts show its addictiveness is less than that of codeine, and even less than that of *d*-propoxyphene. This means that it shows a favorable dissociation between analgesic and addictiveness. Clinically it would probably be satisfactory only for oral use because it is very insoluble and a potent tissue irritant when injected, a feature which greatly reduces its abuse liability.

When given orally to former addicts, I-K-1 did not induce euphoria nor suppress abstinence in morphine dependent patients, nor did it induce any detectable degree of physical dependence when administered on an addictive schedule for 60 days. Although it partially suppressed abstinence when given intramuscularly, it was only about 1/7th as potent as codeine in this respect, and such large doses of an acid solution were required that protracted inflammation at the site of injection ensued. In single intravenous doses of 60 and 120 mg it induced subjective effects somewhat similar to those of comparable doses of codeine, but it was not feasible to administer intravenous doses repeatedly because of chemical thrombosis of the veins; hence any danger of addictiveness by the intravenous route is minimal. Work on I-K-1 has been completed and will be reported to the Committee on Drug Addiction and Narcotics of the National Research Council, and prepared for publication.

2. *2-(Beta-hydroxyphenethylamino)-pyridine . Hcl (Phenyramidol, ARC I-L-1)*, and
3. *N-Isopropyl-2-methyl-2-propyl-1,3-propanediol dicarbamate (Carisoprodol, ARC I-M-1)*

Phenyramidol and carisoprodol will be described together. They were investigated because both were alleged to be internuncial neuronal blockers and both compounds were being marketed as orally effective muscle relaxants with analgesic properties.

Phenyramidol showed no evidence of addictive properties.

Carisoprodol, in large single doses, induced barbiturate-like effects; it partially but significantly suppressed abstinence in doses of 15 to 20 times the dose of morphine; and, although tolerance to sedative effects developed in the direct addiction tests, it was not identified as an opiate by former morphine addicts and there was no abstinence syndrome when the drug was discontinued abruptly. Carisoprodol has less addictiveness than codeine and *d*-propoxyphene.

Work on phenyramidol and carisoprodol is complete and will be reported to the Committee on Drug Addiction and Narcotics, NRC.

4. *Alpha-dl-3-acetoxy-4,4-diphenyl-6-methylaminoheptane hydrochloride (NIH-7667, ARC I-C-25)*

This is a derivative of methadone. Subcuta-

neously it is more potent than morphine in inducing morphinelike euphoria and in suppressing abstinence from morphine. A report to this effect will be made to the Committee on Drug Addiction and Narcotics, NRC.

5. *6-Acetyl-3 ethoxydidhydromorphine (NIH–7623, ARC I–A–38)*

This compound is related structurally to both morphine and heroin. Subcutaneous doses of 100 to 200 mg induced consistent morphinelike effects and it was about one-half as effective as morphine in suppressing abstinence. It is considered to have abuse liability greater than that of codeine but possibly less than that of morphine. A report to this effect will be made to the Committee on Drug Addiction and Narcotics, NRC.

6. *N-(1-Methyl-2-piperidinoethyl)-propioanilide . Hcl (NIH–7602, ARC I–I–1, Phenampromid)*, and
7. *N-(2[Methyl)-phenethylamino]-propyl)-propioanilide (NIH–7603, ARC I–J–1, Diampromid)*

Phenampromid or diampromid administered orally induced morphinelike subjective effects and behavior in nontolerant former morphine addicts, and both suppressed symptoms of abstinence from morphine.

A report on phenampromid and diampromid was made to the Committee on Drug Addiction and Narcotics, NRC, in January 1960. The Committee advised the Commissioner of Narcotics that both compounds should be placed under control of the narcotics law of the United States. The President, after appropriate action by the Commissioner, promulgated an order to this effect in October 1960.

Acute and Chronic Intoxication with Nonanalgesics, Barbiturates or Alcohol

Cross Tolerance Between LSD and Psilocybin. Two experiments were completed during the year. In the first, 10 subjects received LSD in doses increasing to 1.5 mcg/kg over a 6-day period, and on another occasion the same patients received psilocybin in doses increasing to 150 mcg/kg over the same period of time. The patients were then tested with the drug they had been receiving, and "challenged" with the drug they had not been receiving. In the second experiment, 9 patients received LSD in doses increasing to 1.5 mcg/kg over a period of 12 days, and on another occasion, psilocybin in doses increasing to 210 mcg/kg over a period of 12 days, following which they were tested with the drug they had been receiving and "challenged" with the converse drug. The second experiment was carried out to determine if administration of psilocybin over a longer period of time would result in a higher degree of cross tolerance to LSD. The results in both experiments were similar. Definite tolerance to psilocybin developed in both experiments, as manifested by the reduction in both "objective" and "subjective" parameters of response. The patients tolerant to psilocybin also showed a reduced response to LSD. Conversely patients tolerant to LSD also showed a reduced response to psilocybin. Direct tolerance to LSD was greater in degree than direct tolerance to psilocybin. Increasing the dose and length of time over which psilocybin was administered did not increase the degree of tolerance and cross tolerance. This demonstration of cross tolerance between the two drugs reinforces the hypothesis, which was based on the similarity of the clinical patterns of effects induced by both, that they induce psychic aberrations by acting through common mechanisms or through mechanisms serving a final common pathway. The study suggests that psychotomimetic drugs can be classified into definite groups by studying tolerance and cross tolerance.

Cross Tolerance Between LSD and Mescaline. As the initial step in this project, a comparison has been made of the objective and subjective effects of LSD and mescaline in 10 patients. Patterns of effects induced by both drugs were very similar. Objective findings after both included elevation of temperature, elevation of blood pressure, diminution in threshold for the kneejerk, and pupillary dilatation. Subjective reports after both drugs included anxiety, feeling of unreality, perceptual distortion (particularly visual), and hallucinations. LSD is approximately 3,000 times as potent as mescaline. Cross-tolerance experiments between both drugs will be completed early in 1961.

Psilocin. This compound is simply dephos-

phorylated psilocybin. Its effects, in comparison with those of mescaline and LSD, were studied in 10 men. In the doses and manner used, it proved to be the most reliable psychotomimetic drug we have ever studied. All 10 patients who received 150 mcg/kg of psilocin intramuscularly developed marked symptoms of depersonalization, vivid visual hallucinations, and many of them lost insight. Onset of action of psilocin is extremely rapid and the effects are largely dissipated in less than 4 hours. Because of its short length of action, psilocin may be preferable to LSD or mescaline as a psychotherapeutic tool or as a possible psychodiagnostic agent.

Alcohol, Barbiturates, and Related Drugs

During the year work progressed on the psychopathology of alcoholism as compared with that of narcotic addiction, on acute effects of alcohol and barbiturates on verbal report, and on testing for possible correlations between such drug effects and personality characteristics. Previously reported were findings on the first application of factor analysis to personality profiles (MMPI) of three institutionalized groups, 200 alcoholics, 200 narcotic addicts, and 200 criminals. This analysis indicated considerable similarity of these populations, especially with regard to psychopathic traits. During the present year an inverted, rectangular factor analysis was applied, using all profiles to check and refine previous results. From these analyses emerged five factors similar to those found earlier but more clearly defined. The first and common factor, on which practically all profiles regardless of group were heavily loaded, was interpreted as social deviance. The characteristic was so strong in the three populations that no factor independent of such psychopathic traits was found. In Factors 2, 3, 4 and 5 the dominating influence of social deviance was combined with various neurotic and schizoid tendencies, none of which however were sufficiently differentiating to even approach diagnostic significance. This study, for which early publication is planned, strongly suggests that psychopathy or social deviance is of specific etiological significance to neither alcoholism nor narcotic addiction, nor is it of such significance for the addictions in general. However it does appear that such measures as were used in the study are general predictors of asocial or antisocial actions, including addictions of various sorts. Predictors of the specific directions of such behavior apparently must be sought elsewhere, perhaps they may still be sought partially within the framework of social deviance, with emphasis on the level of availability of deviant modes of action and on the lack or impairment of behavioral controls which result in such deviancy.

Measurement of subjective effects of three doses of alcohol and one of pentobarbital in acute studies were continued, using the Addiction Research Center Inventory (ARCI). Previous pilot work had established a dose of pentobarbital, and initial and maintaining doses of alcohol which produced appropriate intoxication for a period of about three hours. During the year, gathering of data on the main study was completed. The data were analyzed in the same manner as those discussed under Psychological Studies of Addiction (see VII below). Two scales of discriminating items were developed for both alcohol and pentobarbital (3.00 cc/kg of 30 percent alcohol with maintaining doses, and 200 mg of pentobarbital). Publication of the studies as separates and in manual form is anticipated. Work is also going forward, by means of correlational and other techniques, to ascertain common and specific effects of these drugs within the limits of the ARCI. Administration of the Guilford-Zimmerman Temperament Survey to post-addicts, under conditions of no-drug and the above dose of alcohol, failed to support Eysenck's theory concerning the actions of alcohol on individuals showing different degrees of introversion-extroversion. Significant correlations were found on four scales of this survey. They indicate that the emotionally labile, depressed individual is most strongly affected by alcohol. These data are to be reanalyzed before being prepared for publication. Other personality-drug effect correlations will be implemented as ARCI data are collated with those from the MMPI.

Biochemistry of Addiction

Excretion of Heroin and Codeine. Since the toxicology laboratory of the University of Wisconsin and this laboratory encounter some of the same problems concerning the routine analyses

of urines of suspected addicts for the detection of opium alkaloids and related drugs, it was thought that a joint study would be mutually beneficial. The purpose of the study was to determine just how long and how much morphine could be detected in the urine of subjects possessing high and low tolerance to heroin after abrupt withdrawal of the drug, and how much and how long morphine, codeine, and other codeine metabolites could be detected in the urine of subjects possessing high and low tolerance to codeine. Extraction and chromatography methods devised by Dr. G. J. Mannering were used for the study.

The unexpected finding that nicotine has the same R_f as morphine in the chromatography solvent system used for the heroin experiment necessitated a change in the proposed procedure for the quantitive assay of the chromatograms for morphine. Much time was spent in developing a method that excluded nicotine. A method of eluting the chromatograms and reacting with silicomolybdic acid which gave suitable recoveries, without interference from nicotine, was eventually devised. Our half of the duplicate samples from the heroin study have been quantitated; however, the results are not reported herein since Dr. Mannering has not completed his samples. The codeine phase of the experiment has been completed through the initial extraction procedure, and will be finished when Dr. Mannering decides on the best procedure to follow for the final quantitation of the residues.

Opiate and Synthetic Drugs Excreted in Urines by the Clinical Service. The introduction of routine chromatography in the tests for urinary morphine has resulted in tests which are free of interference from newly introduced drugs. Urinary demerol has been separated by chromatography into demerol and nor-demerol.

Chronic Intoxication of Barbiturates and Related Drugs

Elevation of Electrical Seizure Thresholds. Daily induction of convulsions by slowly increasing the voltage of the stimulating electrical currents until a fit ensues results in a progressive increase in the voltage necessary for production of a convulsion ("ECT" tolerance). Such tolerance occurred in adrenalectomized animals maintained on fixed amounts of adrenal corticosteroids and therefore is not likely due to increased adrenal cortical activity arising from the stress of repeated convulsions. If one pair of stimulating electrodes are implanted anteriorly and another pair posteriorly, daily stimulation of the anterior pair results in increase of the threshold through the posterior pair, even though stimulation is applied to the posterior pair at intervals widely separated in time. This suggests a general cerebral change rather than a focal change directly beneath the stimulating electrodes.

The project is to be continued. If elevation of the ECT threshold can be induced in rats by daily stimulation through corneal electrodes, the rate of progress can be accelerated. It will become possible to determine whether daily electrostimulation results in an increased production of a hypothetical chemical by the brain which is a "natural" anticonvulsant.

Effects of Bilateral Decortication on Barbiturate Withdrawal Convulsions. Three totally decorticated dogs and one decorticated cat did not have *grand mal* seizures following abrupt withdrawal of barbiturates after long intoxication, whereas all unoperated controls did have convulsions. Thus it seems that the cerebral cortex is necessary for *grand mal* seizures during withdrawal of barbiturates. Pentylenetetrazole (metrazole) induced typical *grand mal* convulsions in a totally decorticated dog. Determinations of the amounts of pentylenetetrazole required for the induction of convulsions in decorticated dogs and their controls should be necessary before a clear interpretation can be made.

These investigations are to be continued, using higher dose levels of barbiturates and longer periods of chronic intoxication. In addition, electrical recordings from subcortical structures will be made in order to determine whether barbiturate withdrawal seizures originate in subcortical structures. In the latter case, the effect of decortication might be due to simple elevation in the threshold for *grand mal* convulsions.

Effect of Dilantin on Barbiturate Withdrawal Convulsions. Maximum tolerated doses of dilantin orally, intraperitoneally and intra-

venously did not prevent convulsions due to abstinence from barbiturates in chronically intoxicated dogs. Further pharmacological analysis of the mechanisms of barbiturate withdrawal convulsions will be carried out, using such drugs as scopolamine, physostigmine, and sodium hydrazide.

Effect of Unilateral Cerebral Cortical Lesions on the Occurrence of Convulsions During Withdrawal of Barbiturates from Small Dogs. Barbiturate withdrawal convulsions did not occur in any of 4 animals in which focal cerebral cortical lesions were induced by injection of either aluminum hydroxide cream or by freezing with ethyl chloride. Convulsions did occur in the controls. The project is to be continued utilizing higher doses of barbiturates, other species of animals such as rats and monkeys, the effect of topically applied acetylcholine during withdrawal of barbiturates, and the thresholds for direct cortical responses to electrical stimulation during abstinence from barbiturates.

Psychological Studies of Addiction

Gathering and analysis of data on the Addiction Research Inventory for differentiating subjective effects of drugs continued to be the main clinical study of drug actions in psychology. This work has been progressing for several years through construction of a 550-item inventory from an original pool of 3,400 questions (by means of exploratory work on this pool with various drugs) to development of various drug scales. The final form of the inventory was printed and administered to approximately 175 postaddict subjects under the following conditions: no-drug, placebo, morphine, alcohol (4 dose levels), pentobarbital, chlorpromazine, LSD–25 (2 dose levels), amphetamine, and pyrahexyl compound (2 dose levels). All individuals were also given two different personality inventories and a test of literacy and intelligence. During 1960, gathering of data was completed. The data from subjects who produced valid tests, as indicated by a rational scale of consistency, were transcribed to punchcards and processed in various ways by the Computing Center of the University of Kentucky.

By means of an item analysis all other conditions were compared with placebo on a first group of 50 subjects. This method uses the frequencies of "true" and "false" responses, and a technique for determining statistical significance to isolate all items of the inventory which discriminate separately between placebo and every other condition at or beyond the 0.05 percent level. These procedures resulted in initial scales of differentiating items or questions. Similar data on a second group of 50 subjects were processed in the same manner. Items were then chosen for the final scales which maintained discrimination in both groups at less than the 0.05-percent level of significance. These final scales were then tested on a third group of 30 subjects for purposes of validity generalization. Scales derived in this way were termed "primary," since they were composed of items showing the strongest drug effects. In addition, "secondary" scales were developed concurrently with the above. These scales consisted of items selected in the placebo and the other conditions in the same manner, but that differentiated placebo and the other conditions in the total at the 0.05-level of significance. Although only superficial analysis of scale content has been made, the primary scales appear to be heavily loaded with sensory and affective or mood changes, and alteration in the stimulation-depression continuum. The secondary scales are composed of items which appear not to be as physiologically based. However in the case of LSD–25, the only drug to which factor analysis has been applied, it was found that apparently all "secondary" effects are dependent upon the occurrence of the "primary" effects. Such interrelationships are to be investigated in the other drug conditions. Separate publication is anticipated for each of the developed drug scales. These initial publications and the preparation of a test manual, however, cannot include detailed analyses of the actions of each drug and their interrelationships. Such analyses of common, multiple, and specific drug actions have been started, but they are so complex and refractory to statistical techniques that they must be dealt with as individual studies. Factor analysis is by no means ideal for most of the work, and development of original methods may be a necessity. The work of discovering possible correlations between drug-effects and personality characteristics is also one of the main objectives

of these investigations. Preliminary work is encouraging, but sustained effort must await completion of several other phases in this area. As time and personnel permit, the ARC Inventory will be standardized on normal populations with and without drug medication. It will be used for investigating alcoholism and schizophrenia, and should be especially useful in measuring psychopathy or social deviance.

Other studies on human subjects continued during the year included the gathering of Minnesota Multiphasic Personality Inventory profiles on physician addicts, and investigations of probability learning in former addicts. Both have progressed rather slowly but for different reasons. Previously, addict physicians were tested 30 days or more after their admission to the hospital. For some unknown reason, the number of available subjects was not maintained at the rate found in the earlier part of the study. Testing at 10 days after admission during the past year was instituted to facilitate gathering of an adequate sample, and to compare such profiles with those obtained after 30 days. The study is being continued for the next year, but prospects are not encouraging because of the current low admission rate.

The probability learning study, which is now in progress, is one of a number of different procedures which will be directed toward comparing variables which control decision making and thus behavior in the "psychopath" and normal subject. Is the behavior of the psychopath controlled to a greater extent by immediate rather than remote contingencies, by negative rather than positive reinforcements, and more nearly by immediate personal gratification and relief from frustrations than is the behavior of the "normal" individual? As appropriate procedures are developed for answering these questions, drug produced alterations in the controlling variables will be introduced. During 1960 data were gathered on 60 subjects, using a probability learning procedure. This study was postponed to allow collection of data on the ARC Inventory, and the addition of several more groups is necessary to draw more than tentative conclusions. The procedure involves an estimate or prediction of future events in a simple situation where one of three lights will be lit. The task of the subject is to predict which light will be lit on successive trials. His only source of information is his experience in the situation. He does not know that the separate lights are presented 70, 20, and 10 percent of the trials in randomized order. Data accumulated to date indicate that addicts overestimate the most frequent alternative and underestimate the least frequent. Thus far this finding is the opposite to that found in normals. Also such "choice" behavior in the addict is altered in the direction of "normalcy" by administration of appropriate doses of morphine. Various other types of reinforcement will be used under control and drug conditions.

In rats, the practical and theoretical aspects of anxiety reduction by potent analgesic drugs were pursued further. This series of studies was originally begun on human subjects to test the hypothesis that reduction of pain-conditioned anxiety is a necessary action of potent analgesics, and to develop a method for detecting such drugs. A very promising procedure was devised and published. The theoretical basis for the study was strongly supported. Continued work with this procedure in man was not feasible since it was very penalizing to the subjects and to the experimenter as well, because it was necessary that he test the apparatus on himself. An animal method was then set up which complemented the human work and involved the same general principles. Rats were trained to press a lever in a Skinner-box to obtain food. A 4-minute tone terminated by a brief, strong electric shock was applied to the feet of the rat during each daily session. Shortly, the tone acquired an inhibiting function, producing cessation of lever-pressing for its duration. This inhibition was assumed to represent the effects of pain-conditioned anxiety or a conditioned emotional response (CER). It was then hypothesized that potent analgesics would reduce this form of inhibition, as had occurred in man, and that other classes of drugs would not. Work on this technique, which extended over several years and which is being continued in modified form, was completed on 10 drugs in multiple doses. It was found that opioids produced significant dose-effect curves of CER reduction; optimal doses practically eliminated effects of the tone. Significant dose-effect curves were found for no other drug except pentobarbital and then only at 20 minutes after injection. Effects of drugs other

than these two classes are to some extent dependent upon the intensity of the tone and other parameters. Publication of several papers on this CER work is being postponed until ancillary studies clarify further the actions of pertinent drugs on auditory discrimination, sufficient work on other supplementary procedures having already been completed. In this work the most encouraging procedure is the imposition of a tone discrimination upon an escape response. Apparently this type of conditioning has never been accomplished previously. Shock intensity is extremely critical. The rat runs from an electrified start box, down a short alley to escape in one of two end boxes. Tone is in one end box, signalling the "hot," or nonescape side. Apparently no discrimination involving choice can be established when the shock is at such a level as to be a prepotent stimulus. In the limited number of animals that have thus far been successfully conditioned in this way, when using optimal doses found in the CER work, morphine and pentobarbital abolish avoidance behavior, raise the "escape" shock level, but have no effect on discriminative escape, while amphetamine has an effect on only the latter, disrupting it. These studies of the CER and ancillary investigations may be of considerable practical and theoretical value in several different areas other than pain and analgesia.

Interoceptive conditioning has been studied little in the United States and such endeavors have been sporadic. It would appear that at least some behavioral effects of drugs can be reasonably subsumed under such learning or conditioning principles. Work on the actions of drugs at the ARC assumes that one mechanism by which drugs produce effects on behavior is through alteration of internal stimuli. Such changes may produce effects in behavior by facilitating or inhibiting responses directly, or drug-produced changes in the organism may come to act as occasions for responses in their own right, i.e., become stimuli to which responses are conditioned (The studies to be described later, on a pharmacodynamic theory of relapse to narcotic drugs, are partially based upon such reasoning). Previous annual reports summarized studies on rats in which support for such a position was obtained and in which amphetamine was found to act as a "depressant" rather than a stimulant. Initial work in rats on the effects of autonomic blocking agents was also described. Studies on the latter are being continued. The hypothesis being tested is that while autonomic effector activity is not essential for the acquisition of a traumatic avoidance response, such activity serves to increase resistance of the response to extinction. To test this hypothesis, acquisition and extinction of a traumatic avoidance response under nonmedicated conditions are compared with these procedures under the ganglionic blocking agent Ecolid. Because there is hazard in assuming that drugs always act according to published descriptions, it was necessary to demonstrate initially that Ecolid is an autonomic blocking drug. After several tedious and discouraging pilot studies, partially shown in the previous report, a procedure was found which demonstrated that Ecolid does significantly reduce blood pressure in the otherwise unmedicated rat. To our knowledge this is the first demonstration of such effects. A method was then devised for producing traumatic avoidance conditioning. As yet the experimental design has not been completed, about half of the groups remain to be tested. However, the data show (contrary to expectation) that Ecolid slightly impairs conditioning to asymptotic performance, but does indeed hasten markedly the extinction or "dropping-out" of the traumatic response. As shown in other studies, shock intensity during conditioning is an important parameter in determining the strength of the conditioned response as measured by resistance to extinction. In this study the least effect of the drug was found on extinction after conditioning on the highest of three shock levels. This work is being continued. Investigations of this nature may have significance for predicting and therapeutically controlling emotion and some forms of mental illness.

Central Nervous System Depressants

During the last year work on the mode of action of drugs on brainstem integrating and regulatory mechanisms has been limited to investigations of acute tolerance and physical dependence on opiates in the dog and cat. Various aspects of these problems have been studied in the intact dog, the spinal dog, and the *cerveaú isolé* cat.

In addition to evaluating the effect of mor-

phine and nalorphine on behavioral states in intact and spinal dogs (e.g., sleep, sedation, restlessness, etc.), observations have been made on pulse rate, respiratory rate, body temperature, pupillary diameter, skin twitch, and withdrawal reflexes (intact dog), patellar reflex (S.D.—spinal dog), ipsilateral flexor reflex (S.D.), contralateral crossed extensor reflex (S.D.), and the extensor thrust reflex (S.D.). The effects of single subcutaneous doses of morphine sulfate (10 mg/kg) and nalorphine HC_1 (20 mg/kg) were evaluated for a 5-hour period following injection to provide a base line for the determination of tolerance and to evaluate morphine-nalorphine interactions in both intact and spinal dogs.

Morphine sulfate was infused at a rate of 3 mg/kg/hr in intact dogs for 7½ to 8 hours. Maximum sedation was observed 2 to 3 hours after onset of infusion; thereafter dogs that had shown severe depression (no spontaneous activity and unresponsive to both nociceptive and nonpainful stimuli) became progressively more responsive, and by the end of the infusion some dogs would stand, turn and walk spontaneously. A concomitant temporal pattern of alteration was seen for depression of the skin twitch, withdrawal reflex and miosis; these effects were maximal after 2 hours of infusion and thereafter diminished in magnitude. A 10 mk/kg subcutaneous test dose of morphine (17 hours (circa)) after the end of infusion produced a smaller degree of miosis and significantly less depression of the skin twitch and withdrawal reflex than this test dose did prior to infusion, despite the fact that dogs had not completely recovered from the infused morphine, as evidenced by a subnormal pulse rate and body temperature. These findings indicated that tolerance developed during the course of infusion and was clearly demonstrable 17 hours after the end of infusion.

Administration of nalorphine (20 mg/kg) following a 7½- to 8-hour infusion of morphine (3 mg/kg/hr) in the intact dog produces a syndrome which consists of marked restlessness, violent tremors, lacrimation, salivation, rhinorrhea, defecation, urination, acceleration of respiratory rate, marked tachycardia, mydriasis and a rapid increase in body temperature.

Nalorphine (20 mg/kg) produced, in the intact dog, only minimal changes, the most striking of which was miosis. Although nalorphine produced no discernible depression of the skin twitch or withdrawal reflexes in the intact dog, it produced a decided depression of the ipsilateral flexor and the crossed extensor reflexes in the spinal dog. These reflexes were even more profoundly depressed by morphine. No signs of recovery of these reflexes were observed in the spinal dog during an 8-hour infusion of morphine. In this respect, tolerance to the effect on reflex responses elicited by nociceptive stimulation in the spinal dog developed at a slower rate than did tolerance to morphine-induced alterations in reflexes evoked by nociceptive stimuli in the intact dog.

In the spinal dog, administration of nalorphine (20 mg/kg) following an 8-hour infusion of morphine (3 mg/kg/hr) evoked not only the above signs of abstinence, but in addition caused extensor hypertonus of the hindlegs, inhibited the extensor thrust reflex, increased the sensitivity for evocation of running movements in the hindlimbs and increased the magnitude of the crossed extensor and ipsilateral flexor reflexes above that observed following either nalorphine or morphine. These signs in the intact and spinal dog resemble abstinence signs observed in dogs chronically addicted to morphine and indicates that physical dependence can be consistently produced following a single infusion of morphine.

In elucidating the physiological factors responsible for signs of physical dependence, rapidly changing homeostatic levels seem to be of great importance. The *cerveaú isolé* cat has been used to assess the effects of morphine, nalorphine, and morphine followed by nalorphine on the relationship between serum CO_2, serum pH, respiratory rate, and pulse rate. The *cerveaú isolé* preparation has been used in this study to eliminate the effect of the hypothalamic thermoregulatory center on the respiratory mechanism.

Administration of morphine (20 mg/kg) to the feline *cerveaú isolé* preparation depressed respiratory rate (e.g., RR 9 to 10), caused hypercapnia and increased serum pH slightly. Administration of nalorphine (10 mg/kg) following morphine (20 mg/kg) produces a marked tachypnea (e.g., RR 10 to 35) which gradually diminishes to a modest increase in respiratory rate (2 or 3 breaths per minute over the control level), hypocapnia, and a slight but significant serum alkalosis.

The marked tachypnea observed when nalorphine was administered after morphine was accompanied by a tachycardia. These changes are part of the acute abstinence syndrome and can be explained by the fact that morphine depresses homeostatic level and creates a state in which the equilibrium-concentration of serum CO_2 is higher than in the control state. Administration of nalorphine rapidly shifts the homeostatic level back to the control level. The high serum levels of CO_2 now act as a potent stimulant to the respiratory and cardio-accelerator centers with resultant tachypnea and tachycardia.

Conditioning Factors in Addiction and Habituation (Relapse)

The general theory and basic postulates around which this project was designed have been described elsewhere, and in some detail in previous annual reports. Briefly the theory states that, in part at least, relapse to addicting drugs represents a complex of responses which are conditioned to environmental stimuli (both external and internal) that were associated with periodic reduction of abstinence distress consequent to addiction. It is postulated that once physical dependence on a given drug is established, each dose of the drug serves to reinforce whatever behavior is instrumental in bringing about further administration of the drug, i.e., behavior which results in obtaining the drug and thereby reduces objective and subjective signs of abstinence from the drug increases in probability of occurrence after each "successful" obtaining of drug as a result of the behavior. Furthermore it is postulated that the strength of such "primary" reinforcement, as well as the strength of "secondary" reinforcement by environmental stimuli regularly associated with the former, varies directly with the "effort" or "hustling" inherent in the performance of the instrumental responses. The complexity of primary and higher orders of reinforcement may be expected to greatly obscure the basis for inveterate recidivisms in man. It seemed reasonable to assume however that primary reinforcement of the sort described, as well as relatively simple secondary reinforcements, might well be used with animals to test hypotheses derived from the basic postulates.

Rats were used in previously described work, and are currently being utilized to obtain fundamental information before proceeding to use of primates. Studies are progressing on the two major and interesting aspects of the project. Pharmacological, and initial conditioning investigations of the actions of morphine and a more potent, faster acting opioid (the benzamidazole derivative known as I–G–2). The purposes of these studies are to test preference or aversive behavior to orally administered I–G–2 in nonaddicted rats, and in rats addicted by daily injections of morphine from which opioids are withheld for various periods of time; to develop valid and reliable methods of measuring tolerance to, and abstinence from the above drugs; to determine the relative reinforcing value of ingested I–G–2 on the probability of pressing a Skinner-bar during cycles of addiction and abstinence from opioids.

In studies initiated previously it was shown that reliable evidence of physical dependence on morphine could be obtained only when the daily subcutaneous dose was maintained at 200 mg/kg. While this drug is a standard in many studies on the opioids, it is difficult to administer orally (or otherwise instrumentally) to rats, and by this route in rats lacks the fast onset of action which is essential for effective conditioning procedures. Thus in most of the conditioning and preference studies undertaken to date animals were maintained on 200 mg/kg of morphine subcutaneously daily, and consumption of I–G–2 and instrumental acts of obtaining it were used as the dependent variables. Studies completed or initiated during the year are described below.

One objective of the previous and current year was to determine whether rats could discriminate I–G–2 from water by taste (the work would be much simplified if it were shown that rats could not detect the substance over a wide range of concentrations that might be employed in subsequent investigations). In one study addicted and nonaddicted animals were deprived of water for 22 hours daily and were then permitted, through the use of appropriate "start" and "goal" cages, a choice of drinking either water or a 5-mcg/cc solution of I–G–2. Without training, neither of the groups showed preference nor aversion for either of the solutions. In a second phase of this study distinctive cues were paired with I–G–2 and presented in the same way as above with

water, in randomized right or left order. Both addicted and nonaddicted groups established a clear preference for I–G–2 with no significant differences occurring between groups as measured by amount of solution drunk. During extinction of the selective response to I–G–2, only nonsignificant trends were found. However in subsequent reinforcement and extinction trials in these addicted and nonaddicted rats, it was found that the addicted animals responded to short-term reinforcement, whereas the controls did not. Further studies for delineating preference and taste thresholds of I–G–2 in pressing a Skinner-bar, show that water deprived rats develop no aversion to ascending concentrations of 5, 10, 20, 30, 40, 50 or 60 mcg/cc. Thus these studies clearly indicated that I–G–2 is very appropriate for present purposes insofar as nondiscrimination by taste is involved. In addition, these studies provided data showing that the effects of I–G–2 are similar to those of morphine, since the effects occurred within 4 to 9 minutes after commencement of drinking and partial evidence of cross tolerance between morphine and I–G–2. Addiction has been maintained in these rats by morphine injection (as mentioned, this is inconvenient and hazardous to animals). Studies are now started in which "direct addition" to I–G–2 is being attempted by allowing rats free access to the liquid in their home cage instead of water. Thus far the concentrations have been 5 and 10 mcg/cc. The rats apparently maintain themselves well on these concentrations. They show most of the signs of morphine addiction, and upon administration of nalorphine the few animals thus far tested exhibit mild abstinence signs similar to those found after morphine withdrawal. The I–G–2 concentrations will be increased to the point at which strong abstinence signs occur. Studies were also begun on instrumentation and procedures for obtaining greater validity and reliability in measuring the abstinence syndrome. This pharmacological work will consist of developing and applying apparatus for measuring precisely temperature changes, cardiac rate, oxygen consumption, and the recording of observational data during addiction and abstinence. Data thus gathered, in addition to being informative *per se*, will be used to determine time of peak abstinence, and thus the optimal point for use of I–G–2 as a reward or reinforcer in the behavioral studies.

Training or conditioning was an integral part of one of the above mentioned studies in which animals chose between drinking I–G–2 or water. Some indication of "relapse" to the drug was found. In another instrumental situation, addicted and control rats were conditioned to press a Skinner-bar for either I–G–2 or water under 22-hour water deprivation or 24-hour water satiation. Differential stimuli in the form of visual and tactile cues were presented with both liquids. After considerable training, reinforced bar-pressing of addicted animals exceeded that of nonaddicted rats on water-deprivation days irrespective of the liquid, whereas on water-satiation days the rates of addicted animals were significantly higher only when I–G–2 was the liquid obtained by bar-pressing. During tests in which the stabilization dose of morphine was withheld, statistically significant intergroup differences in abstinence signs were found with peaking at the 72d hour. In testing for "relapse," after all animals had been maintained on water only for 23 days, during which time morphine was withheld from the addicted animals, overall bar-pressing rates were found to be higher in the addicted animals. These trends appeared to be strong although not statistically significant. The latter appeared to be due to the small number of animals, several having died as a result of the morphine injections during maintained addiction.

In addition to the continuing studies mentioned, further conditioning investigations are progressing which use the Skinner-box, refined experimental design, and larger groups of both control and addicted rats. These experiments will supplement the preference and "relapse" data found previously. During the coming year a "rat-rotor" will be used by means of which different schedules of reinforcement by I–G–2 for bar-pressing can be automatically controlled. If rats can be maintained at a sufficiently high addiction level on orally administered I–G–2, the rat-rotor will be utilized for "self-addiction" purposes as well as for scheduling different periods of deprivation and integrating these with different reinforcement programs. It is anticipated that these experiments will provide information basic to the efficient initiation of studies using

primates as subjects in work on conditioning factors in addiction and relapse.

Mental Set

This procedure, described in some detail in previous reports, may be regarded as a method for quantifying "attention." Extending this concept somewhat, the procedure as previously interpreted measures response "set" (readiness or disposition to respond). The relevance of the procedure for studying schizophrenia and for the actions of drugs on behavior depends on the findings of earlier investigators who found that certain indices of reaction time are among the most valid indicants of schizophrenia as compared with other mental illnesses. As described in previous reports, the procedure is a modification of that used by Huston and Singer (Arch. Neurol. Psychiat. 53:365–369, 1945) for measuring auditory-manual reaction times. These are measured following visual "warning" signals at different foreperiods (the time between the warning signal and the signal to press a key) which are scheduled in "regular" and "irregular" orders. In previous years it was found that LSD–25, morphine, and pentobarbital produced changes in response set of nonpsychotic postaddicts which were similar qualitatively but not quantitatively to those found in schizophrenic patients under no-drug conditions. The data on the above drugs, as well as on amphetamine, were transformed to insure homogeneity of variance, analyzed, and compared. Since that time groups of schizophrenics have been tested, and during the past year the effects of "fatigue" on set have been studied in both the addict and schizophrenic groups. The design used to isolate fatigue effects, which may influence reaction time in both groups toward the "schizophrenic pattern," consisted of: (1) Comparing the first and the last nine trials for each foreperiod series of the regular order; (2) comparing data gathered when the 2.0 second preceded the 3.5 foreperiod with data obtained when these periods were reversed; and (3) comparing reaction times found in the regular procedure when it was given first and when it was given after the irregular procedure. The data have multiplied to such an extent that analysis would be overwhelming if attempted manually. They have therefore been collated for processing by the Computing Center of the University of Kentucky (IBM–650). When the analyses are completed they will provide more clearly defined comparisons of "mental set" of schizophrenics and that of postaddicts under the above drugs, and the refined data will, at least in part, determine the extent to which the defect in set found in schizophrenia is due to alteration in the order of "warning" stimuli and to fatigue. Future work will depend in large part on the direction and significance uncovered by the computations.

NATIONAL INSTITUTE OF NEUROLOGICAL DISEASES AND BLINDNESS[1]

INTRODUCTION

The report contained herein represents the first Annual Report of the reconstituted National Institute of Neurological Diseases and Blindness. As of this reporting period, the Institute now has been organized so that the Intramural program contains an integrated Basic and Clinical Unit which is administratively self-sufficient. The accomplishment of this has been the result of much thought on the part of all concerned including the individual scientists, the program directors, and the Institute directors throughout the framework of the entire National Institutes of Health. Through this period of transition, advice has been deliberately sought and gratefully received from the Board of Scientific Counselors and from many scientists both in the United States and abroad.

The Institute at the present time consists of eight integrated laboratories and branches. These are the Laboratories of Neuroanatomical Sciences, Neurophysiology, Biophysics and a single basic Section of Neurochemistry. The branches are as in the previous reports: Medical Neurology, Ophthalmology, Surgical Neurology, and Electroencephalography. Such integration of the basic and clinical programs of the Institute and separation from the basic programs of Mental Health have not been without some concomitant changes in personnel. These will be reviewed in the appropriate units.

The administration of the Intramural program has been placed under the purview of a newly created associate director for Intramural Research. This is the first time the Institute has had a scientific director. Under the administrative reconstitution of the Institute, all scientific programing will fall under the administrative purview of this director.

[1] Prepared by G. Milton Shy, M.D., Associate Director of Intramural Research.

The position of clinical director now will have the overall review, coordination, and administration of all research of the Institute pertaining to patient care or utilization of patient facilities. Dr. Maitland Baldwin has been appointed the clinical director of the Institute. Personnel changes have been minimal in the Clinical Unit with the loss of but one senior scientist, Dr. Michelangelo Fuortes, who returned to Milan, Italy to take over directorship of a new Neurophysiological Unit.

Many changes, however, have occurred in the basic laboratories of the Institute. Dr. William Windle is now the assistant director of the National Institute of Neurological Diseases and Blindness. Dr. Sanford L. Palay has been appointed chief of the Laboratory of Neuroanatomical Sciences. The Laboratory of Neurophysiology will remain a joint laboratory between the two Institutes with the Section on Spinal Cord Physiology under Dr. Karl Frank and the Section on Special Senses under Dr. Ichiji Tasaki being assigned to the Neurological Institute. At the time of the availability of the new basic research building, these two sections will constitute the nucleus of the new Laboratory of Neurophysiology of the National Institute of Neurological Diseases and Blindness. The Laboratory of Biophysics has remained essentially unchanged. The Laboratory of Neurochemistry was split with Physical Chemistry going to the National Institute of Mental Health. Lipid Chemistry under Dr. Roscoe Brady is now assigned to the National Institute of Neurological Diseases and Blindness. It is planned that this section will be combined with the other chemical units within the Institute to form a new Laboratory of Neurochemistry. The appointment of the laboratory chief of this new laboratory will be undertaken in the first 6 months of the next reporting year.

The scientific personnel of the Basic and Clini-

cal Units have joint research meetings each Monday morning involving all sections. On the second Monday morning of the month, one given section reviews for all other section and laboratory chiefs the current and anticipated research undertaken in such a section.

New facilities have also become available to the Institute in this reporting year. A building for basic research to be shared by the laboratories of the National Institute of Neurological Diseases and Blindness and the laboratories of the National Institute of Mental Health has now entered the planning stages. The completion of the initial plans for all laboratories which will fill this building have now been handed to the engineers of the Research Planning Branch of the National Institutes of Health. The new surgical wing is nearing completion, and it is visualized that surgery will start in this unit as of July, next year.

The planning of such facilities has taken much time on the part of laboratory and branch chiefs involved. It has been felt by all concerned that this is an unique opportunity to be able to plan with thoroughness and foresight a research facility with such an imaginary scope as to attempt to foresee the future trends of neurological research. In such discussions, the Board of Scientific Counselors and many scientists at the National Institutes of Health, throughout the United States, and abroad have given much of their time and understanding to this program. All laboratories and branches would appear to be progressively pushing their research frontiers towards the molecular level. Thus, the main changes will be in genetic structure and chemistry, ultrastructure, molecular chemistry, communication networks, theoretical mathematics, X-ray crystallography, enzymatic chemistry, and the biophysics of artificial membranes.

Many of these studies will be incorporated into present laboratories, but it appears to be the consensus of opinion of the scientists here and of the consultants that two new laboratories will be established within the new research facilities. These would be the Laboratories of Molecular Biology and of Neuropathology, the latter using newer techniques of ultrastructure, histochemistry, tissue culture, and immunochemistry. It is anticipated that at least one of these laboratories, i.e., Molecular Biology will be started in the coming year even though it means crowding of present facilities. Two areas of interest would be that of enzyme chemistry and genetic biophysics. Through the aid of the Dental Institute, six modules have become available to start a modest program in these areas.

The promise of a well-integrated program directed towards the further understanding of the normal and abnormal functioning nervous system is perhaps brighter than any other reporting period since the initiation of the Institute. The specific research accomplishments and projections of the individual sections may be seen on the following report which was derived from the reports of the clinical director, the laboratory chiefs, the branch chiefs, and the section chiefs as well as individual surveys of every project report.

Medical Neurology Branch

The Medical Neurology Branch has continued its efforts to investigate: (1) The basic mechanisms responsible for the variety of neuromuscular disorders; (2) the detection of cerebral neoplasias; and (3) the basic metabolic abnormalities in cerebral function. Toward this end, a variety of neurophysiological techniques have been used ranging from microelectrode recordings of intracellular potentials to the physiology of the motor unit, the utilization of labeled isotopes to further understand regeneration and intermediary metabolism, the use of pharmacological agents and their synthesis, the utilization of more sophisticated radiological techniques, the use of histopathological techniques and tissue culture, and the utilization of enzyme chemistry and immunochemistry. Toward this end, 423 patients were admitted and 6,722 patient days were recorded. The average patient stay was 15.9 days, a decrease of 10 over the previous year. The number of patients so admitted was almost double that of the previous year, 373 consultations and 245 out-patients were seen. Of 423 patients, 188 suffered from some type of neuromuscular disorder.

Neuromuscular Disorders

In familial periodic paralysis, it was found: (1) Muscle potassium and sodium are not significantly elevated in familial periodic paralysis

during attacks; (2) there is no significant change in the resting potential of paralyzed fibers; (3) during attacks, the membrane of the fiber does not conduct a propagated potential when stimulated directly; (4) the hyperpolarization apparently cannot be indicated as a cause of the paresis; (5) there is no direct relationship between the level of potassium of the serum and the attack; (6) an abnormality of glucose tolerance curve was present in all patients studied; (7) triamcinolone causes an exacerbation of the disease, and glucagon and epinephrine induced weakness in the resting patient; (8) aldosterone excretion, per se, was not an etiological agent of this disorder; (9) a fall in basal metabolic rate is accompanied by an exacerbation of the patient's symptoms and this is independent of TSH secretion; (10) during attacks there is an ingress of intracellular water, as seen by both the light and electron microscopes and measured chemically; (11) such flocculations of fluid are membrane enclosed; (12) such membranes appear to surround dilated endoplasmic reticulum; (13) a characteristic change in the mitochondria is also suggested by the electron microscope; and (14) it is suggested that granules which are present inside such vacuoles may represent the accumulation of nondiffusible intermediates in glycogen anabolism.

Incubation studies of muscle from patients with muscular dystrophy in which an attempt was made to incorporate tritiated thymidine into regenerating muscle resulted in no successful labeling. This could be because the mitotic interval exceeded the perfusion time or that mitotic division cannot occur in adult striated muscle. To clarify this, chronic osmotic infusion of tritiated thymidine has been carried out over a 3-month interval in the rat. These studies are not completed as yet.

In the late onset myopathies, two distinct programs are emerging. One in combination with the Institute of Arthritis and Metabolic Diseases has demonstrated a group of dysproteinemic myopathies associated with Sjogren's syndrome. Such patients show a typical myopathic picture clinically with gamma globulins up to 10 gm/100 cc in the serum and plasma cell infiltrations into the muscle. A study in combination with the Cancer Institute has just been initiated into the carcinomatous myopathies which, particularly in the male over the age of 55, account for approximately 80 percent of symmetrical muscular weakness of proximal muscles.

The study upon the family previously described in this Institute with a new disorder termed "Central Core Disease" of muscle was carried on by Dr. Franz Seitelberger, director of the Institute of Neurology in Vienna and visiting scientist in the Section of Neuropathology, and Dr. Theodor Wanko from the Ophthalmology Branch. These investigators have demonstrated an abnormal content of a mucopolysaccharide within the central core. There is a decrease of lipid content in the central core and this in turn is confirmed by an electron microscopy study. No mitochondria were found in the central core. A reconfirmation of the original study was found on electronmicroscopic analysis in that it was shown that the fibrils of the central core can contract differently than the remaining normal fibril.

In myasthenia gravis, studies are continuing in the clinical administration of deoxydemethyl lycoramine hydrochloride, and this now consists of a series of eight myasthenic patients. This drug is a quaternary ammonium compound with initial pharmacology worked out in the Section of Applied Pharmacology under Dr. Richard Irwin, and is derived like the Russian drug, galanthamine, from the narcissus plant. It is apparent in these eight patients that this drug is a potent anticholinesterase compound with the therapeutic level of 4.0 μg/kg. There appears to be a wider safety factor between the therapeutic level and the toxic level of this drug. The duration of action of this compound appears equal to or greater than that of pyridostigmine. It is planned to rapidly extend these studies to a larger number of patients; and if continued success is noted, then the tertiary compound will be synthesized for oral medication.

Three hundred and forty-three electrophysiological studies of muscle abnormalities were completed. It now appears that the electromyogram can detect not only the difference between myopathy and neuropathy but also between inflammatory myopathies and other myopathies, and between peripheral neuropathy and anterior horn cell disease. Nerve conduction studies have shown themselves useful in peripheral neuropathy, and repetitive nerve stimulation studies are carried out in abnormal fatigue states such as sarcoid,

carcinomatous myopathy, and myasthenia gravis. Complimentary work on the contractile and noncontractile protein from biopsies removed from now over 300 muscular cases are being carried out in the Section of Neurochemistry of this Branch by Dr. Beni Horvath, and Dr. Karl G. Henriksson. With the arrival of Dr. W. King Engel as associate neurologist, a study has been initiated into the histochemistry of skeletal muscle as far as the distribution of several known enzymes, and tissue culture studies are to be initiated both in patients and animal material. The original project undertaken by Dr. Engel is toward the histochemical localization of myoglobin within the muscle of animal and man.

Dr. Horvath and Dr. Henriksson have continued their studies with contractile and noncontractile proteins in muscular dystrophy patients. These studies are roughly divided into two groups. One is an immunological study utilizing Clam Tropomyosin A and the second is concerned with the extraction and fractional analysis of muscle protein from biopsies now obtained from over 300 cases of patients with neuromuscular disorders.

The first study is based upon the unusual immunological properties of structural muscle proteins which are reported in the previous report (cf. 59–NINDB–7(c)). The time course of antibody titers in rabbits following administration of Clam Tropomyosin A has now been followed. Injections given every other day for 2 weeks result in titers comparable to the highest reported in the literature for any other antigens. However, such titers would decline rapidly to low levels, with a half-life of approximately one week. Months after a titer was maximal, repeated injections of Tropomyosin A cause a rise in the titer. An attempt was made to maintain a titer by injections at repeated weekly intervals. The titer achieved and the time course of the rise corresponded to the theoretical expectation from the response of a single injection and the assumption of a single exponential decline of the titer with a half-life of approximately one week. Such a titer, however, could not be maintained but fell at about the same rate it did after single or closely spaced injections.

Rabbits exposed to closely spaced injections occasionally showed weight loss and death within a few months after the injections. But rabbits injected at weekly or monthly intervals regularly exhibited weight loss and death. Such animals were autopsied and regularly showed a glomerulonephritis. However, few, if any, muscle lesions were seen in such material. It would appear that muscular necrosis from an autoimmune reaction in muscle from these experiments, at least, is not as regularly seen as are nerve lesions after nerve tissue injections as seen in the studies of Adam, *et al.*

In their second study on the extraction and fractional analysis of muscle proteins, Dr. Horvath and Dr. Henriksson have taken advantage of the observation of Helander that muscle extracted with 1.1 potassium iodide (KI) extracts virtually all the proteins of the muscle. Muscle biopsies of as small a size as 0.017 grams to 1 gram may be utilized and with triplicate determinations as routinely done, there is the standard error of only about 0.5 mg. of nitrogen per gram tissue or 2 to 5 percent and that a 95 percent confidence limit for the regression lines calculated from the normal points vary from 0.2 to 1.5 mg. nitrogen per gram. The muscle is taken out and a homogenate is prepared from frozen tissue. This, in turn, is extracted with 0.3 cc. sodium hydroxide in the cold. From this may be determined the total nitrogen. This in turn may be then fractionated subsequently into the nonprotein and protein nitrogen. The protein nitrogen in turn, by the utilization of 0.05 sodium hydroxide, may be divided into the noncollagenous and the collagenous nitrogen. This, in turn, by the utilization of potassium iodide may be divided into two groups. The nonsoluble portion is practically negligible. The potassium iodide soluble protein may again be divided into two groups by the utilization of potassium chloride. Once again, one group is practically negligible, the other group which is not precipitated by the potassium chloride may again be broken into two groups by the utilization of H_2O. That which is water-ssoluble, contain myoglobulin, mitochondria and other noncontractile proteins. That which is not water-soluble contains actomyosin. Utilizing such techniques, these investigators have demonstrated that the normal amount of actomyosin is present for the protein content in five different types of myopathic patients. They are now working on the water soluble fraction, i.e., the noncontractile proteins and there is some

indication that this fraction may be reduced in certain neuromascular disorders. This is of interest in that it would demonstrate the protein abnormality of muscle may not be in the contractile fibrils but in the noncontractile proteins of muscles.

The Section of Neuropharmacology has continued its studies in reference to humoral mechanisms at the myoneural junction and at the parasympathetic end-organs as well as a study on the localization of muscle fibers in a single motor unit. Dr. Richard L. Irwin has continued his work on the pharmacology of several new compounds which are derivatives of the alkaloid galanthamine. These substances have been found (as noted in the past Annual Report) to inhibit muscle, brain, and plasma cholinesterase and to increase the contraction of skeletal muscle. Of the numerous compounds tested in this laboratory, three carried an intrinsic interest to the disorder myasthenia gravis. These were: (1) Deoxydemethyl lycoramine methiodide; (2) the dimethyl carbamate of deoxydemethyl lycoramine hydrochloride; and (3) the dimethyl carbamate of deoxydemethyl lycoramine methiodide. Deoxydemethyl lycoramine methiodide has greater cholinesterase inhibiting potency than edrophonium and differs from the carbamate compounds in having a shorter duration of action. The methiodide of the dimethyl carbamate group had the greatest potency of any of the compounds tested. Compared to neostigmine, muscle potentiation is greater and the duration of action is longer. However, toxicity after parenteral administration of this compound, as judged by LD_{50} in animals, is greater than that of neostigmine. The toxicity after oral administration, however, was less, indicating poor absorption. Atropine protected against doses which are lethal in 100 percent of the animals. The hydrochloride of the dimethyl carbamate group had in general the same activity as the methiodide salt. However, the hydrochloride had a better absorption following oral administration. The potency of the hydrochloride in producing muscle potentiation is only ¼ that of the methiodide, but it is still greater than physostigmine. The hydrochloride form had a delayed onset of action with approximately 30 minutes being required for maximal effect. The duration was similar to that of neostigmine. The hydrochloride has about one-tenth the toxicity after parenteral administration as did the methiodide and is only about ¼ as toxic as physostigmine. It is the methiodide of the dimethyl carbamate form which has been utilized in clinical trials described above. A study now is in progress to determine the absorption across the blood-brain-barrier to see how much inhibition of the esterases occurs in the brain.

Many investigators in the past have postulated that excess parasympathetic stimulation of exocrine glands may be one of the mechanisms responsible for the high electrolyte and polysaccharide content of secretion in the disorder of cystic fibrosis of the pancreas in children. Direct evidence in support of this postulate, however, is lacking. Techniques developed in Dr. Richard L. Irwin's laboratory for bio-assay using the clam heart, and where feasible eserinized frog rectus muscle, were used to analyze sweat from patients with and without cystic fibrosis of the pancreas. Such patients were sweated thermally in plastic bags by the method of Di Sant Agnese for a timed period. The sweat volume, pH, sodium, and potassium were measured. Samples were acidified and used immediately or frozen to preserve activity. Dr. Edward L. Eyerman in Dr. Irwin's laboratory then determined the cholinergic activity due to a choline ester, presumably acetylcholine, and found high amounts in all but one of the 12 patients with cystic fibrosis of the pancreas in contrast to the activity obtained from noncystic samples. These investigators have established the fact that this is a choline ester rather than choline itself. The average activity of the 12 cystic patients expressed as acetylcholine is 0.075 μg/ml. of sweat. A similar value for 12 patients without cystic fibrosis is 0.0054 μg/ml. Three cystic patients gave extremely high values averaging 0.22 μg/ml. The highest value for a noncystic patient was 0.015 μg/ml., thus it would appear that these investigators have established that a choline ester is responsible for or associated with, the abnormal sweat patterns of mucoviscidosis.

It is now recognized and recently emphasized by Nachmanson that the majority of chemicals which alter biological functions do so by interaction with cellular macromolecular components. Such cellular components are commonly referred to as receptor sites and these have been shown

to have a high degree of specificity. However, almost nothing is known as to the chemical composition of such sites either as to which cellular macromolecule is involved or what molecular or submolecular force is responsible for such interaction. Many drugs have their action at levels too low to be determined chemically and hence, must be determined by bio-assay. Such a bio-assay procedure may be used to determine whether an active substance has reacted with a macromolecule (i.e., at the receptor site). Such a study has now been initiated with certain glycolipids in which it has been determined from preliminary results that such glycolipids apparently sequester some di-quaternary compounds which have marked pharmacological effects. The biological activity of monoquaternary compounds, however, does not appear to be changed by the presence of such glycolipids. The significance of this program to the future is great, in that an increased understanding of how drugs combine with cellular macromolecules may make predictable the development of new therapeutic agents and lead to a fuller understanding of cell function in neurological disorders.

Utilizing single axon preparations, Dr. Forbes Norris in Dr. Richard Irwin's laboratory, after electrical stimulation of such an axon, explored the remaining innervated muscle by means of glass micropipette electrodes. The muscle fibers supplied by such an axon were located through evoked intracellular depolarizing potentials. With this technique, Dr. Norris could then map a muscle in cross-section showing the exact location of the muscle fibers in a given motor unit. This study indicated that singly or in small groups, muscle fibers of a motor unit are widely dispersed in the muscle. The method could have selected axons of large diameter (therefore supplying larger numbers of muscle fibers than smaller axons with smaller motor units). There is still some evidence that the motor units' size is related to function, and changes such as suggested by Wohlfart of "Motor Unit Territory" could reflect a functional rather than anatomic change.

Dr. Giovanni DiChiro is continuing his work on the radiological study of the soft tissues in different muscle diseases using high kv. ranges. The light emission of intensifying screen located in the back of the X-ray cassette is blocked with a black piece of paper placed between the screen and the X-ray film. The films obtained by such a technique are reproduced with Logetronic equipment to enhance the contrast.

The purpose of this study is to determine changes of soft tissues of the limbs in patients with different neuromuscular diseases, in particular, adipose infiltration.

Genetic Studies

As noted above, chromosomal counts are being carried on in certain neuromuscular disorders in which there is a high probability of such an abnormality. In addition to this, however, the Institute has recruited a genetic biophysicist, Dr. Donald Cummings. This investigator was recruited in anticipation of the activiation of a Laboratory of Molecular Biology in the first half of the next reporting year. This investigator is now initiating studies to determine *in vivo* the molecular weight of bacterial and viral Desoxyribonucleic Acid (DNA). In this study, the T–2 phage will be utilized as well as the genetic material from the T–2 phage as it is extruded from the tail core into the host cell. E-coli DNA will be obtained by utilizing F+ and F– cells and causing the F+ cells to mate with the cell wall material from the F– cells, thus liberating the DNA from the F+ cells and leaving the rest of the cell intact. This extracted DNA will then be studied by a variety of physical methods, in particular, ultracentrifugation, utilizing the cesum chloride density gradient method. The molecular weight of this native DNA can then be determined from the density of the DNA and the shape of the flotation band.

Brain Tumor Studies

Over 690 scans utilizing radioactive iodinated albumin with the collimation device designed in this Institute has now been accomplished. The present accuracy of confirmed positives is approximately 83 percent. More knowledge has now been obtained as to the type of tumors from such scans, in particular, cystic astrocytomas, meningiomas, and metastatic tumors.

Dr. Giovanni DiChiro has finished his study comparing RISA encephalography and conventional neurological methods and comes to the conclusion that RISA encephalography has its main accuracy in determining the extent of cer-

tain lesions and in detection of multiple lesions such as multiple metastasis and multiple meningiomas; it is not as accurate as conventional radiology for determining the nature of different lesions, i.e., the type of tumor. Tumors missed by normal radiological methods have been detected and conversely tumors missed by RISA encephalography have been found. Dr. DiChiro comes to the unavoidable conclusion that RISA encephalography should be used together with other neurological methods and not in lieu of.

Dr. DiChiro is also carrying out scanning utilizing other radioactive labels for detection of brain tumors, in particular radioiodinated antibodies to fibrinogen. Dr. DiChiro's study suggests that sarcomatous lesions and vascular lesions of the brain may be detected more accurately with antifibrinogen than with RISA. He plans on continuing this study.

Dr. DiChiro has also finished an *Atlas of Fine Encephalographic Anatomy of the Brain* utilizing fractional air encephalograms. This Atlas of detailed normal pneumoencephalographic anatomy has been accepted for publication by Charles C. Thomas and Company.

Dr. DiChiro has finished his original studies in reference to the radiological criteria for the interrelation of the sella turcica and the pituitary in the coronal plane. This study is to be published in the American Journal of Roentgenology. He comes to the conclusion that in over 90 percent of the cases it is possible to reproduce roentgenographically the width (third dimension) of the sella turcica in conventional X-ray studies of the skull. This combined with laminagraphy may establish the width of the sella turcica in 100 percent of the cases. The adding of the third dimension of the sella turcica is the only true way in which the volume may be so determined. By using postmortem material, Dr. DiChiro has proved that the width of the sella, as determined by X-ray, corresponds to the actual width of the pituitary gland and is probably the only true measurement of the pituitary, in that the length of the pituitary does not correspond to the corresponding length of the sella.

Dr. DiChiro is also continuing his study on distribution of the major superficial veins draining the two cerebral hemispheres. His study primarily concerns the three main superficial venous channels. These are the vein of Trolard, the Sylvian vein, and the vein of Labbé. He finds the vein of Trolard more predominant on the right hemisphere and that of Labbé more predominant on the left. He has attempted to correlate this with the Wada test for hemispherical dominance and cortical stimulation at the time of operation and finds usually that in a right-handed person that the left vein of Labbé is predominate. In over 70 percent of the cases, the superior sagittal sinus and the sinus rectus enter into both the right and left transverse sinus. In a few cases, the deep veins enter predominately to the left transverse sinus, and the right transverse sinus takes the superficial venous drainage from the cerebral hemispheres.

Epilepsy and Cerebral Metabolism

Studies by Dr. Bushnell Smith and Dr. Darwin Prockop have continued on the effect of a monoamine oxidase inhibitors on centrencephalic seizures. These investigators concluded from their initial studies using double-blind procedures that the monoamine oxidase inhibitors are not of value in the treatment of centrencephalic seizures. However, tangent observations during their investigation may prove of value in the understanding of such patients. Patients with centrencephalic seizures receiving monoamine oxidase inhibitors present with hyperreflexia, clonus, and confused states at levels where normal patients would exhibit no toxicity. To see where in the tryptophane metabolism this might lie, these investigators removed such patients from therapy and gave loading doses of tryptophane. The patients again presented with the same symptoms with this normal body metabolite. Hence, the centrencephalic patient appears to have some type of naturally occurring block in tryptophane metabolism. It is uncertain at this time whether this would lie at the monoamine oxidase level or at the decarbyloxase level and further studies using 5-hydroxytryptamine ingestion are now being undertaken.

Studies on cerebral metabolism are also continuing in the Section of Neurochemistry by Drs. Donald Tower, Guy McKhann and John Wherrett. These investigators are investigating the metabolism of free and protein-bound amino acids in normal and epileptogenic cerebral tissues. They have found that 40 percent of the

total substrate oxidized by the Krebs cycle passes by way of the γ-aminobutyric acid and have confirmed this *in vivo* in mice using pyruvate 3–C^{14}.

The factors which regulate the channeling of the substrate by way of γ-aminobutyric acid or the alternate succinyl-CoA pathway were investigated using cerebral cortex mitochondria incubated with γ-aminobutyric acid 1–C^{14} with simultaneous measurement of the oxygen consumption and carbon dioxide evolution. In this study, they found that there was a reciprocal relationship of γ-aminobutyric acid utilization and α-ketoglutarate concentration present. If arsenite was added (this inhibits α-ketoglutarate oxidative decarboxylation), then γ-aminobutyric acid utilization is enhanced instead of depressed by high α-ketoglutarate.

If mitochondrial permeability is altered, however, then such a depression of γ-aminobutyric acid utilization by high concentrations of α-ketoglutarate was less than if intact mitochondria was used. When lower amounts of α-ketoglutarate were used, then the addition of oxidized DPN to such swollen mitochondria greatly enhanced the γ-aminobutyric acid utilization. This was not seen with intact mitochondria which are relatively impermeable to DPN.

These investigators are also continuing their studies in free and protein-bound glutamate and asparatate metabolism utilizing C^{14}-labelled amino acids. The substrate is incubated and then the isolated protein is redissolved and reprecipitated to constant specific activity before hydrolysis. Amino acids of the free pool and protein hydrolysate are separated chromatographically and counted. Other samples are incubated with N^{15} ammonium chloride and the various ammonia fractions liberated enzymatically (or by acid), and assayed spectrophotometrically.

These investigators found a dry protein residue of 8.4 (±0.5 percent) of the original wet weight in 36 samples of cerebral cortex. The total amide nitrogen was estimated to be 56 μM/100 mg of dry protein of which 80–90 percent were attributable to glutaminyl plus asparaginyl residues. The total glutamate (glutamic acid + glutamine), was about 80 μM/100 mg. dry protein. Of this, 44 percent was glutamine in samples immobilized immediately after excision, whereas in those slices prepared for incubation in bicarbonate-saline-glucose, only 37 percent was represented by glutamine. Since total glutamate does not change, it was concluded that deamidation of protein glutamine to glutamic acid with liberated amide ammonia appearing free in the tissues had occurred. This is consistent with other studies indicating protein glutamate as a major source of endogenous cerebral ammonia.

The total aspartate (aspartic acid + asparagine) was about 60 μM/100 mg. dry protein. Fifty percent was asparagine under all conditions listed above so that no apparent deamidation of protein asparagine could be detected.

Cortical slices which were incubated in C^{14}-labelled glutamic acid or glutamine showed incorporation of the labelled amino acid into cerebral proteins *in vitro*. This is of the same order of magnitude as that found *in vivo* by Waelsch. When incubated, however, with 40 mM malonate, such slices showed a decrease of protein-bound glutamine from the control values. Such differences were of border-line significance but still may reflect a transfer of protein-glutamine amide ammonia to support additional accumulation of free glutamic acid from α-keto-glutarate under these conditions. When cortical slices were incubated with 10mM ammonium chloride (associated with abnormally high levels of free-pool glutamine and also with intracellular concentration of free ammonia ions), there was a significant increase of protein glutamine at the expense of protein glutamic acid. This was not seen in the white matter and could be abolished from the cortex by intoxication with methioine sulfoximine (blocks free-pool glutamine synthesis). This suggested that the ammonium chloride effect is primarily via free glutamine (i.e., transamidation from free pool glutamine to protein-bound glutamic acid). When N^{15}-labelled ammonium chloride was used the Atoms percent excess of the amide nitrogen from protein-bound glutamine was 18 percent of that for free pool glutamine whereas the C^{14} activity of the protein glutamine was less than 0.1 percent of free pool glutamine specific activity. This indicates then that the entire free glutamine moiety was not directly incorporated into protein.

In their attempts to isolate protein fractions from total mixed proteins, these investigators continued their studies on a model protein, guinea pig serum asparaginase. They were able to obtain complete purification on a DEAE cellulose

chromatographic column using partially purified fractions. The enzyme finally obtained represented only 1 percent of the total protein nitrogen applied. It had a specific activity over 2,000 μM per hour per mg. protein nitrogen. This low protein concentration has prevented the usual studies of purity by electrophoresis and ultracentrifugation.

As noted above, the effect of ammonium chloride on protein glutamine amide appears to occur in the neuron. These investigators are now attempting by differential centrifugation and subsequent chromatography to determine what cellular subunits contain the various proteins. This is done in combination with the electromicroscopy unit of the Ophthalmologic Branch.

Dr. Donald Tower, Dr. Bushnell Smith, and Mr. Edmund Peters have continued their studies on the electrolyte and energy metabolism in normal and epileptogenic cerebral cortex *in vitro* utilizing the methods outlined in the previous report. Studying the effects of exogenous ammonia ions on electrolytes and fluid spaces, these investigators found that when their slices were incubated with 5–10 mM ammonium chloride, that the ammonia ions were found to compete with potassium for inward transport into the slice cells. Such a competition held at least for external ammonia concentrations of 5–10 μEq/ml., and external potassium of 5–30 μEq/ml. The free-ammonia space within the incubated slices under such conditions exceeds 100 percent. This clearly indicated concentration of ammonia ions within the cell against the concentration gradient. An equivalence of intracellular potassium was displaced by the ammonia. No effect, however, on the transport of sodium was noted by the ammonia ions. These investigators felt such effects to be equally present in neurons and glial cells since the same findings were present in white matter as in cerebral cortex. Simultaneously, the non-chloride space of the neurons but not of the glial cells decreased significantly when cortical slices were incubated with the 5–10 μEq/ml. of ammonia, the average value being only 80 percent of normal controls, as reported in other projects. A significant fraction of the total ammonium ion transported into the neuronal cells is bound as the amide nitrogen of free and protein-bound glutamine. The extra glutamine so formed is produced by the amidation of glutamic acid and hence involves an equivalent loss of "fixed" intracellular anion. Consequent to this loss of the intracellular anion, a decrease of fluid volume, i.e., extrusion of H_2O, would be expected and is consistent with the decrease of neuronal nonchloride space observed. Under the same conditions oxygen consumption of both cortical and subcortical white matter was significantly depressed (an average of 16 percent). In addition, cortical slices exhibited a doubling of aerobic glycolysis and an equivalent elevation of glucose consumption. Samples from methionine sulfoximine treated animals behaved in an identical fashion indicating that these effects of exogenous ammonia ions are distinct from the effects on the glutamine synthesizing system. These investigators then carried their studies to subcellular structures, in particular, cerebral cortex mitochondria, and arrived at the conclusion that the effects of the ammonia ion under conditions in which pyruvate or α-ketoglutarate were substrates, appeared to be a direct interference with the oxidative decarboxylation of pyruvate and α-ketoglutarate, both of which utilize similar mechanisms and co-factors. The exact nature of such a block remains to be elucidated.

Electron microscopic studies seem to bear out initially the chemical conclusions in that the preliminary data indicate that those slices incubated with 10 mM NH_4Cl exhibit greater astrocytic swelling (therefore possibly a larger chloride space), swelling of the endoplasmic reticulum and possibly loss of fibrillar components of the astrocytes.

Dr. Michael Sporn and Dr. Charles Dingman in Dr. Donald Tower's laboratory have initiated a study on the isolation fractions of cellular subparticles for studies on the characterization and metabolism of brain nucleic acids (RNA, DNA). Desoxyribonucleic Acid and RNA determinations from the whole brain and on each subcellular fraction are carried out by an adaptation of the method of Scott, et al. The samples were precipitated at 0°, centrifuged with 0.4 perchloric acid (PCA) and washed repeatedly with 0.2 N perchloric acid. The lipids were extracted twice with absolute ethanol, twice with chloroform-methanol and twice with methanol. The RNA was then extracted by hydrolysis with in NaOH at room temperature for one hour followed by precipitation of solubilized protein and DNA

with hydrochloric acid. Desoxyribonucleic Acid was extracted by 1.6 normal PCA at 60 C for 7 minutes. Both R- and D-mononucleotides are measured individually by ultraviolet absorption at 260 m. Total rat brain RNA under these conditions was 1.36 (±0.03) mg./gram wet weight and the DNA was 1.39 (±0.06) mg./gram wet weight. These investigators plan extensions of these studies to the isolation of mitochondrial and microsomal fractions by differential and density gradient centrifugation. The purity will be assessed by appropriate specific biochemical characteristics plus electron microscopic morphological controls. The isolation of microsomal RNA will then be undertaken and compared with similarly isolated liver microsomal RNA for various characteristics.

Degenerative Disorders of the Central Nervous System

The finding of a patient on the ward with typical Friedreich's ataxia and hypogammaglobulinemia allowed a unique opportunity to carry out a series of investigations to further understand the formation of excess proportions of gamma globulin in the cerebrospinal fluid protein of such patients. A number of previous cases of Friedreich's ataxia have been studied at NINDB which have shown elevated CSF gamma globulin. The initial studies of the serum and CSF protein by paper-electrophoresis, while the patient was on bimonthly injections of gamma globulin, i.e., 10 ml. intramuscularly, demonstrated that the serum gamma globulin was 3.1 percent of the total protein whereas the normal is 16.6 percent. The CSF gamma globulin, however, was 23.5 percent of the total protein whereas the normal is 10 percent. Quantitatively, as expected, the serum was calculated to contain 186 mg/100 ml. compared to a CSF content of but 5.5 mg. Thus, it was felt no conclusion could be drawn regarding these altered percentages. The gamma globulin therapy was therefore stopped for 6 months. The patient was reexamined and found to have a serum gamma globulin of only 4.4 percent, i.e., 273 mg/100 ml. The CSF gamma globulin was 2.5 percent, i.e., 0.8 mg/100 ml. This indicated immediately that the gamma globulin observed in excess proportion in the spinal fluid was not formed in the central nervous system since the CSF gamma globulin fell upon removal of exogenous gamma globulin. The patient was then injected with 40 microcuries of I^{131} labelled gamma globulin and the subsequent activity of serum and CSF gamma globulin was followed at frequent intervals. In both the serum and CSF the total activity of the sample was compared with the electrophoretic pattern (in the case of CSF after dialysis). The findings can be summarized as follows: labelled serum gamma globulin decreased after injection with a biological half-life of approximately 13.5 days; corrected for I^{131} decay. The plot, however, suggested two components, one with a half-life of 4.5 days and the other of about 45 days. The significance of two such decay rates is not clear. It may be that the more rapid half-life reflects tissue distribution while the slower represents true catabolism. There was, however, a definite passage of the labelled gamma globulin into the cerebrospinal fluid and such activity became maximal approximately 7 days after injection. Subsequently, the CSF activity declined at a rate roughly parallel to the serum, although it was our impression that the CSF rate was slower, suggesting to some degree sequestration in the CSF compartment or a slower removal therefrom. Because of the uncertainties concerning the exact concentration of gamma globulin in the serum and CSF at lower levels, it is impossible to express the specific activities with any degree of certainty. It is therefore not certain whether the labelled CSF gamma globulin ever reached equilibrium with the serum gamma globulin. When expressed in counts per minute per milligram total protein, the activity ratio of CSF to serum never exceeded 0.435. These studies can be contrasted with Fishman's data using I^{131} labelled albumin, injected into normal dogs where the CSF activity was evident within one hour and the equilibrium time for CSF and serum S.A. (cpm/mgm total protein) was about 20 hours with the serum half-life of 6.3 days and CSF half-life of 8 days.

Electroencephalography and Clinical Neurophysiology Branch

The Branch of Electroencephalography and Clinical Neurophysiology differs in essence from the other branches and laboratories in the Institute in that it carries a heavy service responsi-

bility. A total of 1,844 electroencephalograms were performed during the past year, indicating that although the patient population of the Clinical Center has reached its ultimate level, the utilization of such techniques as this has continued to increase. A comparison to other hospitals across the country has been pointed out by the chief of this unit. It would appear that the service activities of this Branch belong in the upper-half as far as the monthly average of EEG's done in large hospital services throughout this country is concerned. Such hospitals usually have at least two or three electroencephalographers reporting. Here we have but one, and he is also supervising four research projects. It is obvious with the expanded utilization of this service that a second electroencephalographer must be recruited.

The research activities of the unit embrace both human and animal studies to further elucidate both normal and epileptogenic neuronal functions with particular reference to the genesis of seizures, the interaction of cortical and subcortical nuclear aggregates, the effect of expanding lesions upon the electroencephalogram, the direct recording of electrocorticograms, the electric manifestations of the cortical and subcortical substrata of involuntary movements, and the transcortical mechanisms in the study of the morphology of the "slow" electrical potentials evoked in the visual cortex by stimulation of the contralateral homologous regions.

Dr. Ajmone Marsan is continuing his studies on the understanding of the patho-physiological substrata of convulsive seizures, utilizing a combination of approaches, from routine serial EEG's during wakefulness, drowsiness, and sleep, from direct recording of the electrical activity of the exposed cortex during surgery, from direct recording of the cortical and subcortical structures in patients with chronically implanted electrodes, by eliciting seizures with systemic chemical or local repetitive electrical stimulations and finally by critical analysis of experimental data. He and Dr. John Van Buren are observing patients with temporal lobe seizures by autonomic recordings of blood pressure, pulse, skin resistance, esophageal peristalsis, gastric motility and respiration. Many such autonomic changes appeared without electrographic correlates. On the other hand, frequently no vegetative changes were seen to accompany the onset of focal ictal activation either on the surface or the depth of the temporal lobe, although such autonomic changes may occur later as the activity increased in extent and amplitude. A sudden generalized loss of voltage or flattening of the record formed a constant warning of an imminent clinical seizure activity. In a study of the interaction of the component parts of epileptic neuronal aggregates utilizing both macro and micro electrodes, Dr. Ajmone Marsan analyzed the processes responsible for the paroxysmal cyclical firing (inter-ictal) and for the organized repetitive self-sustained (ictal) activity of such epileptical neuronal aggregates, and for the transition from one to the other. He came to the conclusion that more than just simply quantitative differences characterized such two types of activities. This author suggests paroxysmal cyclic discharges could theoretically take place in the isolated neuron, but that the self-sustained (i.e., ictal) activity could occur only in neuronal aggregates.

Controversy has existed since the middle 1940's, when Jasper and Hunter first described a paroxysmal symmetrical spike-and-wave type of discharge from stimulation of the anterior thalamus, as to whether patients with acquired lesions in such an area could in fact present as "petit mal" epilepsy. However, reports in the literature have been found of tumors in this area which are accompanied by suggestive patterns reminiscent of those seen in "petit mal epilepsy." Dr. Ajmone Marsan and Dr. William Lewis present the clinical-pathological and electrographic findings on two patients in whom the electroencephalogram demonstrated an almost typical or highly suggestive pattern of "petit mal epilepsy." One such patient had a large metastasis of one cerebellar hemisphere pressing upon the brainstem, and the other had "petit mal seizures" and generalized convulsions together with a large astrocytoma in the right temporal lobe extending toward the thalamus. They then reviewed all similar cases of brain tumors in the literature associated with the bilateral 3/sec. wave-and-spike complex. Such cases are so rare that the question of a coincidental association of two unrelated phenomena could well be raised. These investigators therefore undertook a statistical analysis which would show that if chance alone were operating, such an association would be

48.2 of 1,000,000 cases. Thus, in the three published series totalling 1,071 brain tumors, with EEG correlation, the expected frequency of 3/sec. spike-and-wave would be that of 0.0517 case, but the actually obtained frequency was six cases. This discrepancy is highly significant and the authors feel that this is evidence that the two phenomena are indeed related and not coincidentally present.

The Branch of Electroencephalography has also carried out an electroencephalographic analysis of metastatic tumors of the brain. Ninety-three patients with intracranial metastases were utilized in this study, all of which were verified at autopsy. In the case of intercerebral lesions, a high degree of correlation was found between the EEG and neurological findings. A few neurologically silent metastases were detected by EEG and, conversely, a few with positive neurological signs were missed by the EEG. By using strict and uniform electrographic criteria, the most significant factor appeared to be the absolute size of the metastatic lesion. A diameter of 2 cm. represented the borderline size for a lesion which would be detected by both neurologic and EEG examination (presence of focal delta activity).

Dr. John Van Buren and Dr. Ajmone Marsan have continued their data collection on patients with chronically implanted electrodes. The numerous records from nine patients are now being analyzed, 14 to 42 electrodes having been chronically implanted in each patient. Such electrodes have been placed within the thalamus, the basal ganglia, and in close proximity to either of these structures as well as subdurally. Such placements permit a direct, simultaneous recording of the electrical activity and a correlation of the superficial electrodes to those of different levels of depth. In addition to this, a technique of local stimulation with brief electrical impulses while recording the evoked potentials has produced promising preliminary results as to the preferential functional inter-relationship between various structures in the human brain. Correlation of the depth electrodes to the subdural electrodes appears likely to yield interesting findings which might be of use indirectly in routine EEG diagnosis as well as clarifying the role of such deep nuclei to both spontaneous and epileptic activity of the cortex.

Dr. Arturo Morillo and Dr. Ajmone Marsan are continuing their studies on the cortical control of sensory inputs. In such experiments, "cerveau isole" cats are used. Tungsten microelectrodes are stereotaxically oriented within the brain of the cat to record unitary potentials in the dorsal lateral geniculate nucleus. These were activated by light and/or by electrical stimulus of the optic tract. The elicitable response in the ipsilateral visual cortex by callosal impulses generated by electrical stimulation of the contralateral cortex was used as a "physiological" conditioning stimulus. These investigators found evoked potentials at the level of the lateral geniculate nucleus following stimulation of the contralateral visual cortex. Such efferent effects (i.e., cortical geniculate), are very likely related to the callosal response and consist of (1) activation of single unitary elements of the lateral geniculate body or (2) modification of the pattern of their firing when the latter is elicited by test photic or optic tract stimulation. Such a conditioning influence by callosal pathways could be inhibitory or facilitatory and could be shown for relatively short as well as for fairly long intervals between the conditioning and test reflex suggesting both multisynaptic and shorter pathways. In view of this specific experimental design these investigators have convincingly demonstrated that such effects are not dependent upon antidromic phenomena and that there are indeed cortical-geniculate fibers. They now intend to use collosal responses in the visual cortext of the cat to study the morphology of the "slow" electrical potentials evoked in the visual cortex by stimulation of the contralateral homologous visual cortex. Combined with such surface recordings from large electrodes, these investigators will study the temporal distribution of unit activity similarly elicited and the relationship of such to the gross surface response. Such interrelationships between the gross and unitary callosal phenomena and between the visual response elicited in the same cortical area by photic or optic tract stimulation will be studied in addition. At the present time this study is in the formative stage.

Ophthalmology Branch

The Ophthalmology Branch reports on 34 separate projects reflecting an integrated program

of basic and clinical work. Such studies include basic and applied approaches to visual processes, studies of intraocular pressure and its abnormalities, studies of refraction abnormalities, effects of radiation and pharmacological agents on the lens of the eye, the various abnormalities of the uveal tract, and on the neoplasias of the eye. For such studies, 151 patients were admitted for 8,297 patient days with an average stay per patient of 54.9 days. Once again, the integration of the various units of this Branch representing their varied disciplines highlights this report. Dr. Michelangelo Fuortes, chief of the Section on Ophthalmology Physiology has returned to Italy to undertake the formation of a Department of Neurophysiology at Milan. His loss will be keenly felt within the Institute. Two senior visiting scientists contributed much to the understanding of visual processes at the retinal level. These were Dr. Katherine Tansley, of the Institute of Ophthalmology in London and Dr. William A. Rushton from Cambridge University. Dr. Peter Gouras has returned to the Branch as a permanent senior investigator.

Visual Physiology of Retinal Elements

Dr. Fuortes continued his studies before his departure utilizing microelectrodes for the intracellular recording of retina elements of the Limulus. In condition of dark adaptation, Dr. Fuortes found depolarizing transients from such cells following the application of weak lights (of the order of 10^4 quanta per second). A statistical analysis of the relationship between the frequency of appearance of such transient potentials and light intensity was performed by Dr. Fuortes, and the results obtained were consistent with the hypothesis that absorption of one quantum may be sufficient for the generation of one transient. Another alternative, however, which may mean that an equal amount of light was sufficient for the release of a given package of chemical transmitter. In a study of the time course of dark adaptation, Dr. Fuortes used both light stimuli and depolarizing pulses directly through the microelectrodes. He found that the response to the depolarizing current recovered within a few seconds from the termination of the impulse but that the response from the testing light pulses recovered but slowly. He then studied the responses to such light and current pulses before, during, and after application of background illuminations of moderate intensity. He found that background illumination increased the response to currents but decreased the response to light. After application of a strong light which is then suddenly decreased to a lower intensity, the nerve cell recovers a large membrane potential but later depolarizes progressively to a steady value. A strong discharge of impulses which was present during the strong illumination is first abolished but later starts again at moderate intensity. Intracellular measurements indicated that the electrical conductance is decreased during the transient hyperpolarizing phase. If light is increased progressively so that the logarith of light intensity is a linear function of time, a transient depolarization may still occur when a certain light intensity is reached (probably true only with gradual changes). The light intensity at which such a response becomes nonlinear is not greatly affected by the rate of change of illumination. Whereas light will always evoke depolarization in the normal cell, if the cell is hyperpolarized by means of a steady current the effect of light may be reversed, i.e., become more hyperpolarized. This finding is relatively new when compared to other excitable cells and it is hoped that further studies may give more insight into such a mechanism.

Dr. W. A. H. Rushton brought to the Institute his elegant apparatus for the measurement of regeneration of rhodopsin in the human eye. The method consists of measuring the difference in the amount of light reflected back through the pupil from the fundus oculi before and after bleaching rhodopsin. The technique employs two beams of light traveling identical optical paths into the eye but oppositely polarized. One beam (516 mμ) is maximally absorbed by rhodopsin and the other (deep red) is not absorbed. When an analyzer is moved in their common path, the total light transmitted at any time can be represented by $r \sin^2 nt$ and $g \cos^2 nt$ where r and g are the intensities of the red and green beams respectively. Suitable adjustment of the intensity of one beam can make the output as well as the light reflected from the fundus constant over time. A change in the concentration of rhodopsin after a bright bleaching light will disturb the balance which can be regained by readjustment of the neutral wedge, representing a

double density change in retinal rhodopsin. The light reflected is measured by a photomultiplier, this output sent through a phase sensitive rectifier which compares it to another polarized signal changing at an identical rate; integrates the multiplied product (which is zero for all signals not changing at the rate the analyzer rotates and considered to be noise) and the final signal is measured by a galvanometer. Dark adaptation is measured by Gunkel's modification of the Goldmann-Weeker's adaptometer.

Using this apparatus, Dr. Rushton found that there appeared to be a critical point of 90 percent rhodopsin regeneration at which the rods begin to make their contribution to the threshold for light detection of the so-called rod-cone "kinck" in the dark adaptation curve. Since both rod curves of dark adaptation obtained by the different bleaching lights are superposable by lateral displacement along the time and since rhodopsin regeneration curves are likewise superposable on themselves, the coincidence between rhodopsin concentration and rod threshold demonstrable for only the final 10 percent of rhodopsin regeneration will therefore coincide through the entire regeneration curve. This is additionally supported by the argument from the kinetics of this reaction. The regeneration of rhodopsin is estimated to follow the rate of a first order reaction indicating that the retinene supply is maintained relatively constant during the process. Since the final 10 percent of regeneration occurs where the demands on retinene are the least, then the curve should be most accurately extrapolatable at this point.

Dr. Rushton comes to the conclusion that there is a fixed relation between the scotopic thresholds and the fractionation of opsin uncombined with retinene and that this relation is mathematically defined by combining psychophysical functions on dark adaptation obtained from others and represents, hence, rhodopsin regeneration. The expression is $I/I_D = ay/y_o$ which shows that the logarithm of threshold is directly related to the fraction of opsin (y) uncombined with retinene; and therefore, rhodopsin concentration.

Using such an apparatus, Dr. Rushton then measured rhodopsin and dark adaptation in a subject with deficient cone vision (photanope). Dr. Rushton concluded that a photanope had about the normal amount of rhodopsin in the retina at 12° temporal to the fovea and that it regenerated at the normal rate. Any attempts made to measure visual pigments in the photanope fovea were marred, however, by irregular fixation, and within 2° of the centrally fixated field neither rhodopsin nor cone pigment could be detected.

In dark adaptation studies, however, it was demonstrated that in contrast to the normal subject, the photanope was not able to distinguish between yellow (580 mu) and green (520 mu) and presented a smooth rod curve. The cone threshold, however, had a castellated appearance, as these photoreceptors were three times more sensitive to yellow than to green. No Stiles-Crawford effect was detected in the rod curve, whereas such an effect, although of low magnitude was demonstrated in the cone curve.

The situation in a rod monochromat permitted the plotting of the rod dark adaptation curve also above the level of the cone threshold. It could be determined, therefore, whether there is an inhibitory effect of rods by cones. In comparring the dark adaptation curve of the normal with that of the photanope, there was no indication of an inhibition of the rods by the cones, but suggested that the two visual receptors follow the independent mechanisms.

In a study dealing with the relation between rhodopsin and threshold in this patient, it was confirmed that the log $1/1_o$ in dark adaptation is proportional to the fraction of opsin uncombined with retinine. It does not, however, necessarily throw doubt on the tenability of Wald's compartment hypothesis of light adaptation as suggested by Dr. Rushton.

Along with Dr. M. G. F. Fuortes, Dr. Rushton also investigated the increment thresholds in a subject deficient in cone vision. He found such a subject apparently to have normal rod function and the rod increment threshold curve in the parafovea agreed with the results reported by other investigators as rod saturation was reached at 1,000 trolands. At this high illumination, increment perception was abolished and the patient lost the sense for visual contrasts.

Cone vision was restricted to the neighborhood of the fovea. The spectral sensitivity at this site was about normal and there was a Stiles-Crawford effect. The cone organization was not

normal, however, because the threshold was extremely high, color vision almost absent, and visual acuity of the patient was low. On the basis of these observations, the existence of a cone-rod inhibition in the normal was questioned by these investigators.

As indicated in the previous annual report, the finding that diurnal squirrels have a pure cone retina which is maximally sensitive to the shorter wave-lengths, made these animals ideal for the study of spectral sensitivity curves. It was in the use of such animals that Dr. Katherine Tansley devoted her primary efforts aided by Dr. Richard M. Copenhaver and Dr. Ralph D. Gunkel during her stay at the Institute.

Electroretinograms were recorded from these squirrels in response to a light stimulus provided by a Xenon arc lamp and double interference filters. The experiments were always conducted under both light and dark adapted conditions, and the stimuli tested through the whole spectral range. Sensitivity curves were plotted using the electroretinograms as the criteria. The retina of all seven species of squirrels examined showed the characteristics of a cone retina. The sensitivity curves were all found to be double humped, with maxima at 535 and 490 mu. While such humps were occasionally the same height more often than not, one or the other was markedly higher. A statistical analysis of the electrophysiological data revealed a mean hump or curve combining all the species examined. This curve was usually symmetrical with the two humps of equal magnitude. In addition, she noted a small but distinct reflexion between 450 and 460 mu. When the eye was adapted to color, the general sensitivity of the retina was depressed, but the results were calculated as the percentage of the maxima and it was found that blue adaptation depressed the hump at 490–500 mu while green to orange depressed it at 535 mu. The depression of the blue peak by blue light and the green peak by green light suggested the existence of two photosensitive mechanisms of different spectral sensitivities.

In view of recent work on photosensitive pigments (Dartnall 1960), Dr. Tansley postulated that the 535 mu peak is the result of interaction of some type between two photosensitive substances with an absorption maxima at 502 and 480 mu and that the 480 pigment is responsible for the peak in this region. If the two pigments were formed one from the other, as it is believed, this would explain the apparent rivalry of the mechanisms responsible for the two peaks, since the more of one pigment present, the less of the other. The off response of the electroretinogram was studied by Dr. Tansley since in the pure cone retina, a marked off-effect is a characteristic feature. She found that the intensity-response curves for the various parts of the squirrel electroretinogram showed that areas with increasing high intensity stimuli, (i.e., the a-wave and b-waves, increased in amplitude). The off-effect was then often depressed. During the development of the normal off-effect, a second superimposed a-wave was much enhanced while a second superimposed b-wave was depressed. The a-wave would return to its normal value about 100 microseconds after the off-effect while the b-wave took 200 microseconds when the off-effect was depressed by a strong stimulus, enhancement of the second a-wave was much reduced and sometimes delayed. The reactivation of the second b-wave was very much delayed. This strongly supports Granit's original supposition as to the nature of the off-effect in post-excitatory inhibition.

During dark adaptation, the off-effect of the red squirrel electroretinogram decreases in amplitude whereas in ground squirrels it increases. Flicker fusion frequencies for the red squirrel is considerably lower than in other squirrels. Histological examination of the red squirrel retina shows it to have a less complex inner nuclear layer and fewer ganglion cells. Therefore, Dr. Tansley suggested that faster responses of the ground squirrel which resulted in a high flicker fusion are due to more efficient production of inhibitions and that this is associated with the remarkable development of the inner nuclear layer in this species. She suggests that interaction of P–III and P–II may be more important for the development of the off-effect in the red squirrel while in the ground squirrel the action of P–II of the off-effect may play a great part.

Dr. Sjoerd L. Bonting has turned his primary interests to the study of the biochemical, morphological, and functional events in the developing visual receptor cells, and used as his model, the DBA mouse from birth to maturity.

His objective was to establish the development and distribution of enzymes in the retina and particularly retinene reductase and retinene isomerase in the eyes of the same animals from birth to maturity, and to attempt to correlate whatever findings might occur with the morphological development of the visual cell as studied by light microscopy and electron microscopy, as well as with electroretinography in collaboration with Dr. Peter Gouras, and finally, to investigate such items for C3H mice and rats with hereditary blindness, as well as with rats treated with iodoacetate and glutamate. To do this, the Lowry-Bessey microcuvette had to be used and tissues weighed on the Lowry balance. In a normal albino rat, rhodopsin appeared at about the seventh day and the concentration increased rapidly and almost linearly to the 21st day and then became constant until maturity. The absolute amount of rhodopsin continued to increase, but after the 21st day, at a slower rate. The rhodopsin concentration and lengths of the whole rod as well as the outer segments were plotted against age. From the zero to the 14th day, there was a striking parallelism between the rhodopsin concentration and the outer segment length. The increase of the latter, however, becomes very slow after the 14th day, at which age rhodopsin concentration has reached only half of its maximal value. The electroretinogram in these rats first appeared at the 10th day, after which the amplitude increased rapidly and almost linearly until the 21st day. After this it, in turn, remained about constant.

In C3H mice which became blind due to hereditary degeneration of the rods between the 10th and 16th day after birth, a normal development of rhodopsin until the 10th day is found. Then the rhodopsin concentration begins to decrease and reaches virtually zero between the 16th and 19th day. In rats receiving injections of 3 X 20 mg/kg. iodoacetate over a 30-hour period in the eighth week of life, the rhodopsin will decrease by 40 percent without morphological changes being visible in the light microscope.

Dr. Bonting has initiated his preliminary studies on the enzyme retinene reductase from the rat and from the cow. The histochemical staining technique with tetrazolium was found to be unsuitable because it indicated "diaphorase" activity rather than retinene reductase activity. Chemical assays in retinal homogenates with ethylalcohol as a substrate following either DPN reduction or oxidation spectrophotometrically were unsuccessful thus far because of low activities and the occurrence of nonspecific and even nonenzymatic oxidation-reductions. The parallelism between rhodopsin concentration and the electroretinogram is an important finding and is in agreement with the recent work by Dowling and Wald on vitamin A-deficient rats.

Similar toxicological studies were carried out by Dr. Ottiwell Jones on patients receiving the monoamine oxidase inhibitor, JB 516. Patients were receiving this drug for hypertension. Such patients were periodically studied for visual acuity, and biomicroscopic, funduscopic, and visual field examination, and color testing were carried out. Tissue was available from one such patient. Of the eight patients studied, all eight developed a decreased visual acuity and a red-green color defect. Two patients developed central scotomata in addition. Six patients recovered after cessation of the drug. The two patients with the central scotomata did not recover normal vision and one of the two patients died and lesions were found in each optic tract just anterior to the lateral ganiculate bodies. This finding is of interest in relation to Dr. Bushnell Smith's investigations (see Medical Neurology Branch) and color vision testing in centrencephalic patients will be initiated.

Dr. Peter Gouras is continuing his electrophysiological studies of the retina of the vertebrate using amphibians in which he is recording with both intra- and extra-cellular microelectrode responses of the ganglion cell layer of the retina. The initial objective of this study is to determine dependency of the ganglion cell discharges on the rate of change of photic stimuli. Stimulus presentation is the unique part of this study and it has varied rate dependencies having slow, continuous changes obtainable by rotating analyzers in polarized light by using sinusoidal wedges or glow modulator tubes. Dr. Gouras believes the ganglion cells of the amphibian retinae have the properties of "differentiators." By this, he means that any photic stimulus can be substituted for any other differing in chromaticity without the ganglion cells detecting the change if the substitution is slow enough.

He found that ganglion cells which discharge to a flickering stimulus varying as the function $\cos^2$ wt. (wt is the rotation of a polaroid in radians/second) trace different curves to different wave lengths when the flicker fusion frequency is plotted against intensity. Long wave length stimuli produce steeper curves than short wave lengths suggesting that the rod function (scoptic) and the cone function (photopic) mechanisms interact on a single ganglion cell; that the long wave lengths are more effective in determining critical flicker fusion at high intensities and the short waves, critical flicker fusion at low intensities in a manner analogous to the Purkinje shift. A surprising result obtained was at either low intensities or low flicker rates the curve may turn back on itself so that fusion results. Thus, fusion was obtained not only when the stimulus was too fast but also when it was too slow.

To clarify signal-to-noise ratio in determination of the electroretinograms, Dr. Gouras has turned to electronic computers. At the present time, he has a study under way which includes 10 patients with pigmentary degeneration, all of whom had "extinguished" electroretinograms on the conventional recording system. All patients, however, had responses, including some with only 5° central fields. He found a direct relationship between the functioning retina as estimated by perimetry and the amount of light necessary producing a constant amplitude electroretinogram. Spectral sensitivity curves determined on such patients by this technique revealed a predominantly photopic curve in patients with functioning macular retinae and scotopic in those with more rod vision. This relationship between the retinal area and the electroretinogram threshold in retinitis pigmentosa, Dr. Gouras feels weakens the Bruch's membrane short circuiting theory proposed to explain the absent electrical response in retinitis pigmentosa patients with only moderate retinal pathology. In addition, Dr. Gouras has been recording the occipital evoked response during each such study. He has demonstrated a depression of the evoked response in a subject with migranious scotoma. Dr. Gouras is also investigating the electroretinograms in animals used in the study and development of rhodopsin by Dr. Bonting.

Finally, Dr. O. W. Jones and Dr. Ralph Gunkel are carrying on functional studies in retinal anomalies and diseases using electroretinography, adaptometry, and perimetric light sense studies. This is a continuation of the study of the previous year utilizing the Goldmann adaptometer and the "retinal profile," on Dr. Gunkel's modification of the adaptometer. Electroretinography was performed on every patient utilizing the contact lens electrode and the Grass 8-channel electroencephalograph. This is largely a correlative study and could be compared to the electromyographic studies now undertaken in the Branch of Medical Neurology. Here again, the results of such studies made it possible to give the correct diagnosis and prognosis in the majority of such cases. A few cases of achromatopsia were examined and significant electroretinographic data were obtained to correlate with the bleaching and regeneration experiments done by Dr. Rushton.

Glaucoma and Aqueous Formation

Doctors Sjoerd Bonting and Kenneth A. Simon are undertaking a study of membrane ATP-ase and aqueous formation. Following the studies of Skou, who demonstrated in the crab nerve that this enzyme is closely connected with sodium and potassium transport across membrane, these investigators found ATP-ase activity in the following tissues of the cat: cerebral white matter, gray matter, the optic nerve, the choroid plexus, ciliary body, kidney cortex, medulla and papilla. Such ATP-ase activity could be inhibited by the omission of the potassium ion or the addition of calcium ion or ouabain. Ouabain concentration at which 50 percent inhibition occurred in the ciliary body was found to be 5×10^{-7}M, which is close to that previously reported for the erythrocyte membrane ATP-ase. Diamox in 10^{-4}M concentration fully inhibited the membrane ATP-ase of the ciliary body and choroid plexus. These findings suggest that ATP-ase may play an important role in aqueous formation.

Dr. Frank Macri is continuing his studies on various pharmacological agents affecting the intraocular pressure and their relationship to the venous pressure and arterial pressure within the eye. He has found that epinephrine and arterenol in concentrations of 10^{-12} produce a rise of iris artery pressure and a slight decrease of per-

fusate flow. When these agents are utilized in doses of 10^{-6}, only, a fall in iris artery pressure flow was greatly reduced. Doses between the two generally produced a biphasic response beginning with a rise in the intraarterial pressure and then proceeded to fall below the control levels. He proposes the following mechanism for such agents: at low doses there is a constriction of the radial arteries of the iris and at high doses, a constriction also of the iris artery. In preliminary studies, Dr. Macri found that low doses of acetylcholine at a concentration of 10^{-12} lowered the iris artery pressure and increased the perfusate flow. He feels that this action is explainable on the basis of dilatation of the radial arteries of the iris. In studies with Diamox, doses of 5 mg/liter which is approximately equal to the human dose, he found that this drug produced a rise of iris artery pressure and a decrease of perfusate flow. This effect is compatible with a constriction of the radial arteries of the iris.

In previous studies on Diamox, the thesis was presented that carbonic anhydrase inhibitors and the sympathomimetic amines reduced the intraocular pressure by diminishing the secretion of aqueous humor. In the present studies, however, apparently at least part of the lowering of intraocular pressure induced by such agents, could be ascribed to a constriction of the iris arteries. Therefore, Dr. Macri turned his primary attention to the role of the vasculature in maintenance of intraocular pressure. He measured the intraocular pressure and the vascular pressure in the various parts of the eye and body. Correlations were made between the intraocular pressure and vascular pressures to show the relative dependence or independence of these various systems.

In cat, he found by simultaneous measurements, that the pressures in the anterior ciliary vein and the vortex veins were identical. He felt this to be explained by the anastomotic communications between the two systems as demonstrated by the cast method discussed in the previous report. In a series of 39 cats, simultaneous measurement of the normal intraocular pressure and "head-on" venous pressure was made at a time when the two variables had been constant for at least 15 minutes. The intraocular pressure was then plotted as a function of the venous pressure; the regression was linear and highly significant and could be expressed by the equation, the IOP = 0.82 VP + 6.51. In such experiments, the measurement of the venous pressure necessitated a total occlusion of the vein and cast, therefore some doubt on the validity of the findings. To meet such criticisms, Dr. Macri reinvestigated this relationship by the use of microcannulae inserted through the wall of the vein upstream so that the vessel remained patent. Again, a highly significant regression was obtained which was almost identical and could be expressed by the same equation. That the total occlusion of one vein in the first series of experiments had no obvious effect on the IOP/VP relationship but produced a mean rise of 22 mm. of mercury in the intraocular pressure was suggestive evidence that the venous pressure may be a dominant factor in the maintenance of intraocular pressure.

To study this further, another series of experiments were undertaken. Using intact eyes of anesthetized cats, the venous pressure again was measured "head-on." Acute changes of blood pressure were produced by the administration of various agents which in turn produced acute changes in both intraocular pressure and venous pressure. They were plotted against each other and the curves of 12 such experiments were grouped and could be expressed by the equation now IOP = 0.90 VP + 3.42. Using enucleated and arterially perfused eyes, a series of 45 eyes were examined. Again, a highly significant linear regression was found between the intraocular pressure and the "head-on" venous pressure which could be expressed by the formula IOP = 0.77 VP + 7.43. Finally, a series of eleven eyes were perfused as above but the intraocular pressure and venous pressure were varied in each eye by raising and lowering the perfusion pressure. The mean equation obtained at this time was IOP = 0.89 VP + 3.58. Therefore, it became apparent that the different procedures used to produce a primary effect on the vasculature of the eye all produced a relationship of IOP/VP similar to that found in "normal eyes." When aqueous humor volume was changed, it was found that IOP/VP relationship could be ascribed by the equation IOP = 1.74 VP − 18.87. This equation varied significantly from those obtained in the 39 cat series.

Dr. Macri concluded that since the changes of venous pressure produced values of "K" in relationship IOP = K VP + C similar to that obtained in "normal" eyes and mice. Changing the volume of aqueous humor resulted in significantly different "K" values; the intraocular pressure of the eye is dependent in a large part on the venous pressure.

Since no relationship had been found at steady states between intraocular pressure and systemic blood pressure by these experiments and accepted clinical experience, yet it is well-known that the acute changes of blood pressure produce parallel changes of eye pressure; Dr. Macri postulated that some slowly compensating mechanism existed to explain the lack of correlation of the intraocular with the systemic blood pressure at steady states. The two most likely possibilities he felt for this would be alteration of aqueous humor dynamics, i.e., the outflow and inflow, or vascular pressure dissociations occurring somewhere between the heart and the exit veins of the eye. This latter possibility was explored. Correlation measurements between the systemic blood pressure and ophthalmic artery pressures were made specifically between pressures of the ophthalmic artery and iris artery and between the iris artery and intraocular pressures. It was thought that a dissociation of pressure within any of such pairs might indicate the control of one vessel of such a pair. Dr. Macri found a every high correlation between the femoral artery pressure and the ophthalmic artery pressure and concluded that the ophthalmic artery pressure varies continuously with the general blood pressure of the animal. No correlation, however, could be found between the pressures of the ophthalmic and the iris artery under the conditions of the experiment. In contrast to this, a highly significant correlation was obtained between the iris artery and the intraocular pressure.

He concluded, therefore, that the pressure in the iris artery is not associated with general blood pressure and dissociation occurs at the level between the iris artery and the ophthalmic artery. The mechanism of such dissociation at this time is unknown. The equation relating the intraocular pressure to the iris artery pressure was IOP = 0.82. IAP – 4.90. Since the slopes of this equation and the equation of intraocular pressure to venous pressure are the same, he concludes that there is a 1:1 relationship between the venous pressure and iris artery pressure. Dr. Macri now plans to see if similar correlations hold for higher mammalian species.

Dr. Scullica with Dr. Macri studied the flow pattern in the ciliary process of albino rabbits. In albino rabbits, the ciliary processes extend upon the posterior surface of the iris and go beyond the circulus arteriosus. Because of the lack of pigment, the blood vessels of the ciliary processes are visible and the flow of red cells can be easily observed and studied. Eyes were, therefore, enucleated and the artery cannulated with a hypodermic needle and a solution of Krebs-Ringer solution, horse serum, and red blood cells were perfused through the arterial system. These investigators found the blood supply of such processes to be composed of a variable number of vessels which have their origin from the arteries of the iris and the ciliary body. Such vessels entered the base of the process at various points and branched either into a larger number of small vessels or into a small number of larger vessels. On the dorsal portion of the ciliary processes was one large vessel which began at the tip to join the choroid. At the beginning of the arterial perfusion, one can see the red cells coming from the radial branches of the iris artery into the anteriormost artery of the process and then proceed from there to the large vessel at the dorsum and hence to the choroid. Immediately afterwards in the majority of such eyes, the flow was observed in other arterioles and capillaries which goes to the same dorsal vessel. The dorsal vessel has the characteristic of a vein and has been called by these investigators the marginal or dorsal vein. Occasionally, the anterior portion of the processes is shunted from the flow and in these cases, the main circulation of the blood cells proceed through an arterial vessel which goes to the middle or posterior part of the ciliary processes and to an ancillary dilated capillary bed which then again communicates directly with the marginal vein. This marginal vein at the level of the orbiculus, divided into two branches, one superiorly, and one inferiorly. The superior one then went on into a capillary net which lay between the choroidal and iridiociliary vessels. Inferior vessels proceed underneath the ciliary processes and

joins the choroidal veins. Thus, the flow can be seen to proceed from the marginal vein to either or both of these vessels. The anterior portion of the ciliary processes had a second mechanism of flow, which was but rarely seen, made up of a large vessel which originated from the capillaries and proceeded under the processes to the choroidal vessels.

Dr. Edward Okun and Dr. Ludwig von Sallmann have continued their clinical glaucoma study in an attempt to evaluate suspected known cases of glaucoma and to find better means of diagnosis and control, as well as to further investigate the disturbed intraocular fluid dynamics in glaucoma. Just as tonography gives an indication of the ease with which fluid leaves the eyes, the inflow studies give an indication of the rate which the fluid entered the eye; 1,794 patient days were utilized in this study. Extensive glaucoma workups were performed on each patient which include tonometry, applanation tonometry, biomicroscopy, gonioscopy, and repeated tonography with and without provocative tests. In addition, the visual fields were carefully plotted using the Goldmann perimeter and the Gunkel tangent screen. This is still in the initial phase and reliable studies are as yet not completed. It appears, however, even initially that tonography when properly performed has proven to be of great value in conjunction with dilatation provocative testing for narrow angle glaucoma. Studies are underway at the present time in cases of borderline glaucoma for the positive water drinking provocative tests. Applanation tonometry, as seen in these initial studies, may be fairly sensitive means of measuring inflow.

Corneal Endothelium

Enzymatic zonulolysis is now widely used for cataract extraction. During the surgical procedure, injuries to the endothelium of the cornea occur. An investigation was initiated by Dr. Hückel to see whether alpha chymotrypsin interfered with the endothelial wound healing. The number of cells undergoing mitosis is taken as an indication of such an effect. When animals were killed 24 hours after the injury to the cornea, the number of dividing cells were slightly less in the eye treated with alpha chymotrypsin. This difference disappeared at a 48-hour interval. Local instillation of 1-epinephrine also depressed mitotic activity. This investigator concluded that in young, healthy rabbits the depressant effect of chymotrypsin on cell division is of low magnitude and transient in nature. The use of this enzyme in cataract surgery cannot be considered as a hazard by delaying endothelial regeneration.

Dr. Ludwig von Sallmann has continued his investigation as to the soundness of the generally held concept that mitosis does not take place in the endothelium of the cornea. Using his elegant technique of flat mounts which are required to contain the whole cell population, corneae were processed with the Feulgen reaction and mitotic counts obtained from both eyes of more than 60 animals. In contrast to the generally held opinion that the mitotic division in the endothelium is present only shortly after birth, Dr. von Sallmann found that the mitotic activity actually exists in the endothelium of growing animals. The dividing cells were more numerous in the peripheral zone of the cornea than in the center and that their number decreases with age. The mitotic index was calculated by counting the cell nuclei per microscopic field and taking planimetric measurements of the whole area of the mount. Such mitotic activity, however, could not explain the increase of the size of the cornea by cell division alone and increase in cell size and flattening of the cell body greatly contribute to the growth process. Diurnal variations were demonstrated by early morning and afternoon counts. The ratio of these two counts increased with age. Dr. von Sallmann concluded that after growth had stopped, the corneal endothelium behaves as a non-renewal area where dividing cells in fully grown animals are few or missing. Such findings places the corneal endothelium in the group of tissues of which the vascular endothelium and the endothelium of the peritoneal cavity are also examples.

In his studies on incorporation of tritiated thymidine in the corneal endothelium of the rabbits, Dr. von Sallmann used the osmotic pump to allow chronic administration of chemical agents into the anterior chamber at a very slow rate of speed, i.e., 0.5 ml/24 hours, by using fine polyethylene tubes, measuring from 100–300 microns in diameter. This study is in a preliminary stage, but it has been shown that the number of labelled nuclei seemed to exceed the

number of cells which were calculated to pass through mitosis during a day; and that the labelled nuclei were abundant in areas which appeared to have been injured but the incorporation of the isotope was also seen a great distance from such an injury. In the normal endothelium, labelling seems also to take place in the central part of the cornea where dividing cells are usually thought to be absent as noted in the previous study.

Lens Structure, Physiology, and Abnormalities

Dr. Robert Resnik is continuing his studies on the proteins of the lens, in particular, alpha F and alpha G. In his studies of the physical constituents of crystalline of the lens, Dr. Resnik has found that Alpha F and Alpha G crystalline transform into particles of lower molecular weight. This is indicated by the appearance of slower sedimenting material in the ultracentrifuge patterns. The molecular weight of the native and fully transformed alpha G crystalline as determined by the approach to equilibrium methods in the ultracentrifuge are 1,086,000 for the native variety, and 514,000 for the transformed variety. The sedimentation coefficient of 0.29 percent solution of alpha G crystalline in 0.05 M phosphate buffer at pH 6.8 is 20.1 X 10^{-13}. The diffusion coefficient of the same sample is 2.0 X 10^{-7}. These values will give a molecular weight then of 940,000. A 0.41 solution of fully transformed alpha G crystalline gives sedimentation and diffusion values of 14.6 X 10^{-13} and 2.7 X 10^{-7} respectively. These parameters give a calculated molecular weight of 509,000. Such a pH induced decrease in the molecular weight of alpha G crystalline is irreversible. Dr. Resnik feels this obviates Bon's hypothesis of a change in shape without a change in size. The charge of the molecule of pH 8.7 does not appear to be altered since boundary electrophoresis does not reveal the two forms of alpha G crystalline when known mixtures were used.

Dr. Theodor Wanko is continuing electron microscopic study on tissues of the eye, in particular, the lens epithelium and lens fibers after cataractogenic agents have been administered, as well as examination of human cataractous lenses obtained immediately after operation. He is also studying the morphology of the ciliary epithelium with the electron microscope as well as the human conjunctiva. His studies on the lens are a continuation of last year's report on the suture areas in the cortex of the normal mature rat and rabbit lens. It is noted in the vicinity of the suture, the regular array of the lens fibers membranes gradualy changes to a system of many protruding cell portions interposed and interidigitated between similar projections of the adjacent cells. In such areas of the lens, specialized contact areas of cell boundaries classified as demosomes are present. Such interidigitations of cell surfaces may preserve the morphological integrity of the cell during the process of accommodation.

In experimental cataracts, Dr. Wanko examined the myleran cataracts at 2, 4, and 6 weeks, as well as the mimosine cataracts at 5, 7, and 14 days. He now has eight human senile cataract lenses. The normally homogeneous lens capsule occasionally exhibits (in the senile cataract) an interposition of dense granular material in a regular arrangement. In the epithelium as well as in the cortical lens fibers, structures resembling fragmented or disintegrated cellular constituents may be seen.

Nine conjunctival specimens from Sjogren's syndrome have been obtained. Studies are now in progress on this. As noted above, Dr. Wanko's laboratory is also active in delineating the subcellular particles used in the DNA and RNA determinations of the Section of Neurochemistry and is participating in the elucidation of many neuromuscular disorders as noted in the Branch of Medical Neurology.

Dr. von Sallmann and Dr. Howard J. Curtis of the Brookhaven National Laboratory are studying the effects of high energy proton microbeams, simulating cosmic primaries on the mouse lens. It has been assumed that the biological effect of cosmic ray particles result from the dense cluster of secondary protons surrounding the cosmic ray alpha particle. Such a cluster is said to create a core of ionization about 25 mu in diameter. Beams of 25 micron diameter and larger were obtained with a 22.5 MEV deutron beam from the 60 in. cyclotron of the Brookhaven National Laboratory. The beam was directed through round apertures 25, 75, and 1,000 microns placed in front of the target area. The beam was well collimated to a depth

of 1.5 mm. Thus, the peak of the Bragg curve was expected to be found at a circumscribed site of the equatorial lens cortex. Doses administered were from 1,012 to 33,000 rads. The animals were studied biomicroscopically in bi-weekly intervals and killed at varying times for the histological examination. Cytological examination of flat mounts of the epithelium was also carried out in a small portion of the series which, at the present time, comprises the eyes of 130 mice. Thirty-two mice were set aside to study the late effects. Opacities were visible in the biomicroscope resembling fine round dots or elongated irregular shapes in the middle superior sector of the posterior cortex in a small group of the mice exposed to the 25 micron beam of 33,000 rads, 20 weeks after radiation. The lesions did not seem to progress in the following months. Radiation with a 75 micron beam at a dose level of 5,200 to 13,000 rads produced signs of radiation injury much more frequently and at a shorter latency period. Lower doses caused minute changes five to six months after radiation. With the 1 mm. aperture, doses of 5,200 rads produced fine opacities in 20-22 weeks. Subsequently, the number of these opacities increased and in several instances, were accompanied by a diffuse cortical opacification around the posterior pole. Similar signs of radiation damage were observed in doses from 1,012 to 2,600 rads. The histology of such radiated eyes did not always parallel the results of the biomicroscopic studies. In the group irradiated with the 1 mm. aperture, migrated and degenerated bow nuclei and swollen fibers were seen as early as 4 weeks after exposure to 4,700 rads or more. At the 25 microbeam, such changes occurred at higher dosages, i.e., 13,000 or 33,000 at the 12 to 14 week interval. At six-month intervals, irradiation with 1,012 rads through the 1 mm. aperture induced cataractous changes in all animals. The use of the lens as against other tissues is important in this study in that the lens is avascular. Thus, in similar studies on the brain, the Brookhaven group came to the conclusion that the larger radiation area of the brain was dependent in part upon vascular disturbances. However, the lens being avascular removes the effects of such tissue damage. In the observation of mouse lens, it can be assumed that the biological effect of the simulated cosmic primaries are of a low order for this organ. The question of recovery from minimal radiogenic lesions in the lens and restoration of the lens to normal still needs further study.

The finding that 17 of 44, i.e., 39 percent of patients, with rheumatoid arthritis or prolonged corticosteroid therapy had posterior subcapsular cataracts and that 19 patients untreated with corticosteroids did not have these cataracts led Dr. von Sallmann and Dr. Oglesby to investigate this phenomena. They found that the occurrence of these cataracts was correlated with the dose and duration of the corticosteroid therapy and not with the age of the patient, the severity of the disease, or therapy received. Although approximately 50 percent of the patients receiving chronic corticosteroids have abnormal glucose tolerance curves, there was no concomitant finding that cataracts were present more frequently in those than with abnormal curves than those without. Patients with other collagen diseases treated with corticosteroids have also been observed to develop such cataracts. The ultimate outcome of the cataractous process is still undetermined until such patients have been followed for longer periods of time. One such patient has come to post-mortem, and in this patient, the primary finding was that of swelling of the lens fibers underneath the capsule and that no migration of nuclei were noted as is seen in radiation cataracts described above. Thus, the radiation received by such patients with chronic rheumatoid disease does not appear to be, at least in this case, a cause of the cataract.

To elucidate this further, corticosteroids were given chronically to 35 male rats of the Sprague-Dawley strain, in which 15, 10, or 20 micrograms of Decadron were given to such rats. Control animals were injected similarly and food intake, weight gains, and so forth, were checked periodically. The major findings were a decreased weight gain of the differentially treated animals when compared with each other and controls, and this indicated the effectiveness of the steroid injections. The retardation of body growth was greatest in the animals receiving the highest dose of the corticosteroid. No biomicroscopic signs of cataract formation was found. The mitotic counts in the lens epithelium in the treated eyes were of the same magnitude as those of controls. The alleged depressant effect

of steroid therapy on cell division could not be demonstrated on the lens epithelium. Skin abcesses reported to occur at the site of the injection were not found and the suggestion that these complications caused the decrease of weight gain could therefore not be substantiated. It was concluded by these investigators that the chronic administration of high dosages of corticosteroids continued for four months does not affect the lens of the young growing rat.

Refraction Anomalies

In his summary of the Ophthalmology Branch, Dr. Ludwig von Sallmann has drawn special attention to the new conceptual approach of Dr. Gerald van Alphen to refraction anomalies. In this study which has now taken approximately three years, Dr. van Alphen by mathematical treatment of the data provided in the literature of correlation of optical elements, shows that the present theories (Steiger) as to the mechanisms operative in emmetropia and ametropia do not, in fact, provide an answer to the fundamental mechanisms at fault. Dr. van Alphen went on to show that the links between the optical elements could be best explained under the assumption that the choroid itself behaves as a sheath muscle, which is able to counteract in part, at least, the intraocular pressure. He then went on to measure the tension and tension changes of the choroid as well as the effectiveness of the elasticity of the sclera and the response of both to pharmacological agents. Parasympathetic stimulation results in contraction of the ciliary body-choroid unit. In experiments on isolated strips of this tissue, acetylcholine also brings about a contraction, the extent of which depends upon the tension of the preparation. Adrenergic compounds, however, produce a relaxation. The results of these experiments provide experimental data to strengthen the statical analysis of the correlation of the optical elements of the human eye, and they in addition leave the inference that the study of the relation between the muscle sheath and the pathology of intraocular pressures should also be undertaken.

Uveal Tract Inflammations

One of the largest studies of the Ophthalmology Branch and one which has been followed almost since the concepton of this Branch has been a study of ocular toxoplasmosis. A large number of patients have been continually referred to the National Institutes of Health with definitely active uveitis, a majority of which were resistant to previous therapy. Of such patients, 43 percent showed a complete disappearance of activity of the lesion after treatment with Daraprim (pyrimethamine) and sulfonamides.

Patients with uveitis responding best to such treatment as pointed out in previous reports, have the disease before the age of 20 and the uveitis is unilateral and is not exclusively anterior. There was no correlation between the level of the dye test and the therapeutic responses.

Dr. von Sallmann and his colleagues feel that the toxoplasma skin test appears to be an excellent screening test for antitoxoplasma antibodies and was positive in 95 percent of patients with positive toxoplasma dye tests and who had not had steroid therapy immediately before skin testing. Only one patient of the 200 controls had a positive toxoplasma skin test and this was an atopic patient who responded to all antigens. In their recent series of patients, however, these investigators find that the toxoplasma skin test was negative in several instances in patients with a high toxoplasma dye test titer. Patients who do not tolerate therapy well and respond with a severe depression of the platelet count will be treated with Vitamin B-12 and folic acid.

Dr. Theodor Wanko is doing an electron microscopic study of the protozoon *Toxoplasma gondii.* Dr. Wanko points out that initially toxoplasma cysts were collected by a micromanipulatory procedure from homogenized infected mouse brain. Such isolated cysts studied in the initial phase of this project are providing some information as to the presence and gross arrangement of organisms inside the cell wall but this had to be abandoned since the isolation of the cysts from their natural environment prevented the simultaneous observation of the surrounding tissue. In the micromanipulation, only the largest cysts were selected and no information on cysts development could be expected. Therefore, Dr. Wanko turned to the brain tissue itself and demonstrated therein that the organisms were encapsulated by an opaque layer within which vesicular and membraneous elements could be resolved. Within the cyst itself, the space between the cell boundaries of an individual organism

and its partner was filled with an homogeneous material which is in continuity with the capsule and is of similar opacity. The observations at present suggest that such opaque material between the organisms was derived from the organisms whereas the membrane and vesicular profiles in the cyst wall may correspond to remnants of the host cell. Individual organisms have been seen in brain capillaries and intracellularly in the brain.

The cellular organization of the organism in the cyst is characterized by the following constituents: nucleus with a nucleolus; mitochondria, Golgi complex, endoplasmic reticulum, toxonemata emanating from the conoid, a submembraneous fibrillary apparatus, vacuoles, and a continuous cell membrane.

Neurosurgery Branch

The efforts of the Branch of Surgical Neurology were directed toward the further understanding of the normal physiology of superficial and deep nuclei of the human brain and the abnormalities of the same, associated with abnormal cerebral discharges, directed particularly to the temporal lobe. This study, as in previous years, was a joint effort involving primarily the neurosurgeon, the electroencephalographer, the radiologist, the pathologist, and the psychologist. This was accompanied by fundamental studies at a cellular level. The coordination of this team was under the direction of the Branch Chief. The branch in addition, has now launched a major program in the study of the basic mechanisms underlying involuntary movements, as well as surgical procedures towards the alleviation of the same. Detailed studies in the vascular permeability of cerebral vessels and cerebral edema have been carried out using immunohistological techniques. Studies on the functional anatomy and pathology of the human visual system have continued. The effects of deep hypothermia in higher primates, and radio-frequency wave effects in the brain of primates were investigated. Abnormalities detected on and about the perinatal period have been studied.

Toward this end, 234 patients have been admitted during the past year. Three hundred and nine visits to the Outpatient Department were recorded and there were 73 consultations. One hundred and eighteen major surgical procedures were done of which 41 were epileptics, 22 had intracerebral tumors, 20 suffered from involuntary movements for which stereotaxic procedures were performed and 35 operated on for miscellaneous lesions requiring surgical treatment. The branch prepared 23 reports for publication.

Epilepsy and Functional Anatomy of the Brain

During the reporting year, under the supervision of the Branch Chief, 130 patients with cerebral seizures were studied in the operating room and on the ward. Of these, 70 were localized to one or both temporal lobes. Studies were concerned with behavior, language, and response to high and low sodium diets, and response to various anti-convulsants. Eleven of such patients were also studied by Dr. Van Buren with depth electrode techniques. The Branch Chief at this time summarizes the entire experience over the years by the Surgical Branch with focal seizures and notes that now over 300 such patients are recorded. One hundred and seventy-two temporal lobectomies were included in this group and the results of 88 such cases have been reviewed by Dr. Donald Maccubbin and the Branch Chief. Such cases were followed for periods of one to eight years. Coincidentally, with this study, Dr. Robert Savard of the Social Service Unit of the Clinical Center completed a social study of the postoperative effects in 33 cases now 5 years after operation. Of the 88 cases, 38 percent were seizure-free while 75 percent were considerably improved. Dr. Savard's study showed clearly an improvement in both family and community relationships as well as economic usefulness.

In a review of the surgical anatomy of the temporal lobe, Dr. Maitland Baldwin came to the conclusion that removal of the lateral cortex alone does not relieve the seizures but such a removal must include mesial structures, but paradoxically one can exclude the pes hippocampus. If this latter structure is removed then serious disturbances in recognition and recollection may follow. The excision of the amygdalae did not seem to produce discernible affects, but did seem necessary for the irradication of seizures in most cases.

The psychoses related to temporal lobe seizures were studied by Dr. Philip Ferris and Dr. Bald-

win in 25 cases characterized by overt-psychotic episodes. The majority of such episodes consists of dissociation and bizarre language accompanied by hallucinations. These investigators came to the conclusion that persons with epileptic disease of the temporal lobe are prone to a schizophrenicklike psychosis and such a psychosis is an exaggeration of the inter-ictal behavior in all cases and could be precipitated or exaggerated by withdrawal of anti-convulsants. They did not note such psychosis when other parts of the brain were involved in abnormal seizure patterns. The removal of the affected lobe would lead to relief of the psychotic episodes.

In cooperation with the nursing staff, under Mrs. Mary Thompson, over 6,000 spontaneous ictal seizures have now been recorded by photographic means. An exhibition of this study has been prepared for the American Medical Association.

The effects of high and low sodium diet on seizure frequency has also been studied and it would appear that electrolyte regulation in so far as can be done by diet, plays an important therapeutic role. In the laboratory, utilizing higher primates, (i.e., chimpanzees) the study of penicillin-induced lesions continues. Once a penicillin-induced seizure activity is maintained in an area approximately 1 cm. square on the cortex of the chimpanzee and monitored locally, the injection of 10 percent sodium chloride by vein, will cause the intermittent firing in such an area to become continuous and subsequently spread and the disturbance becomes diffuse. If such a lesion is allowed to become inactive so that the discrete focal spikes are no longer recorded, it may, in turn, be reactivated within 30 minutes after the intravenous injection of 10 percent sodium chloride. If the animal is made hypotensive, no change of any type is found in the firing of the lesion. This lesion is being studied as far as vascular permeability by Dr. Igor Klatzo and Dr. Richard Otenasek, by means of tagged-albumin and ordinary fluorescent techniques. Functional representation within the temporal lobe of man and higher primates has continued utilizing electrical stimulation and recording of the human, chimpanzee, and monkey temporal lobes during operative exposure, and indirectly by depth electrodes and scalp recordings. Ablation experiments of the same lobes in higher primates and anatomical studies of the whole brain after temporal lobe excisions are continuing. In addition to this during the past year, studies on the parietal cortex have been added. In one preparation, the parietal cortex has been ablated on both sides and paradoxically, no parietal syndrome was noted. This preparation will now have an ablation of the adjacent temporal cortex as there is some evidence that in a large amount of conjectures that the temporal and adjacent portions of the parietal cortex act together and that this large association area is, in fact, a complete functional unit. The removal of the dominant hemisphere in the chimpanzee does not in this study interfere with communication, however, in the case of 172 temporal lobe excisions in man, Dr. Baldwin and his colleagues indicated changes in affect, language; in some cases sexual function. It is no interest that Dr. Savard's studies seem to show that speech improves after right-sided temporal lobectomy. The Branch now reports some 532 temporal lobe stimulations in the human and again are collecting secondary sensory responses. The number of so-called psychical responses from surface stimulation seemed to be a rare event. Nonetheless, there are 60 such responses in the series at this time. Motor responses have been found from the postcentral area as well as precentral. The relationship suspected between amygdaloid stimulation and autonomic responses have not been confirmed. This group now anticipates the publication of a monograph based in 172 cases of right or left temporal lobe excision and 532 responses to stimulation of temporal region. Social studies, operative results, electroencephalographic and electrocorticographic tracings, depth stimulation, and anatomical connections, as well as Dr. Dekaban's data on the embryological development will also be included. Once again, the utilization of a wide variety of hallucinagens has demonstrated in the chimpanzee whose temporal lobes have been removed, that no responses to such drugs were noted, whereas intact animals did react. Of such drugs, psilocin was of particular interest because it produces hallucinations in humans without panic or perceptual disorganization which characterizes the effects of lysergic acid. The tryptomine derivatives were also utilized in this study.

Dr. Herbert Lansdell, in his studies of patients

with temporal lobe disease, has arrived at the conclusion that the temporal lobes on the basis of lobectomized patients, do not seem to be especially involved in the types of tasks which are included traditionally in the standard intelligence tests. The temporal lobes seemed to be more involved in maintaining more subtle functions, as detected by verbal and perceptual tests, particularly, in what might be termed the "judgment of the well-adjusted person." After lobectomy, he notes a slight decline in the patient's abilities in this regard.

Dr. John Van Buren is continuing his study on the psychological anatomical correlations in man as well as an evaluation of the effects of new methods for treatment of tumors of the central nervous system. In his study, depth electrodes were implanted in patients subject to seizures of the temporal lobe. Such electrodes were placed in the mesial surface of the temporal lobe and controlled pneumographically. Such electrodes were left in place one to two weeks and the effects of depth stimulation were observed clinically, photographically, and with a polygraph apparatus for determining the autonomic responses. This latter apparatus records blood pressure, skin temperature, electrocardiograms, skin resistance, plethysmograms, esophageal and gastric motility and finally respirations. Dr. Van Buren's studies seem to indicate that clinical automatism was found particularly in or near the amygdaloid nucleus and the hippocampus. Autonomic effects noted by Dr. Van Buren included hypertension, tachycardia, fall of skin resistance, esophageal peristalsis and inhibition of respiration or expiratory apnea. Emotional responses were few and fear was noted in but two apprehensive patients but not in those previously at ease. In one patient, laughter was elicited with stimulation and was reproduced as part of the automatism.

Autonomic changes, aside from those accompanying automatism, were relatively few and were usually accompanied by a subjective sensation within the viscera and occasionally, a poorly localized sensation of somatic nature. The epigastric sensation was the most frequent.

Along with Dr. Ajmone Marsan, Dr. Van Buren studied a series of 21 ictal episodes associated with metrazol injection in patients with seizures presenting with characteristic visceral phenomena. Here the same stereotype response of the autonomic nervous system was elicited. Variations of the pattern lay predominantly in the failure of a given change to appear or in the sequence of the changes. Most of the aurae were reported by the patients when no significant electrographic activation had occurred and many autonomic changes appeared without electroencephalographic correlates. No autonomic or clinical change was seen to accompany the onset of focal ictal activation either on the surface or in the depth of the temporal lobe, although such changes may appear later as the activity increased in extent and amplitude.

Stimulation of frontal regions might or might not be accompanied by autonomic changes. Thus, most of the changes of the autonomic system appeared against a background of gradually increasing generalized activation with or without a preponderance in the temporal region.

In 11 patients, the orbito-temporal cortex was explored while such patients were undergoing pituitary surgery for metastatic cancer. Constant responses were obtained from the anterior uncus at its junction with the hippocampal gyrus. Here blood pressure would fall. Coincident with this would be a bradycardia. Respiration was inhibited or arrested. This could occur at any stage in the respiratory cycle. Esophageal contractions which appeared could well be independent of the laryngeal component of swallowing. The similarity of this complex to that of stimulation of the superior laryngeal nerves and vagi in lower animals was noted by this observer.

Dr. Van Buren is also continuing his studies on the functional anatomy and pathology of the human visual system in which he now has eleven human cases with normal visual fields and normal retina; one normal monkey and one normal chimpanzee. He has eight human cases and one monkey with lesions of the optic radiation; five monkeys with lesions of the optic tract; seven patients with lesions of the chiasma; two monkeys with the unilateral section of the optic nerve; and one patient with amblyopia exanopsia.

Dr. Van Buren has now started reconstruction studies of the topographical anatomy of the retinal ganglion cell layer. He has found that in the normal retina such an arrangement of

cells is not circular around the macula but forms an elongated oval with its long axis horizontal. The thinner the ganglion cell layer of a given retina, the greater is the ratio of the long axis to the short axis of the oval. He notes that although the optic nerve head appears circular, the actual opening in the ganglion cell layer is an oval with its long axis vertical. In addition, he notes that those ganglion cells over 15 micra tend to have their highest population at the intermediate margin of the more densely populated portions of the ganglion cell layer, i.e., over one cell thick. In the case of a craniopharyngioma, a maximum ganglion cell thickness in the parafoveal area of four cells permitted a visual acuity of 20/20 in one eye; in the other eye with a maximum parafoveal cell layer of three cells thick, the visual acuity was only 20/100. Since the distance between the blind spot and the macula was known both in the degrees of the visual angle and in millimeters of retinal surface, the visual fields and the anatomical plots of the ganglion cell anatomy can be superimposed on the same chart, and Dr. Van Buren noted that while isopters of 5/2000 or larger could be noted by the patient outside the portion of the ganglion cell layer more than one cell thick. This patient had a striking bitemporal visual field defect and there was an outstanding degeneration of the retina in both blind fields. In a patient with an occipital metastatic lesion, there was a demonstrable degeneration of the larger ganglion cells in the blind quadrant and a depression of the margins of the ganglion cell plots in this region. In other words, the margins were closer to the fovea in the blind area than elsewhere. In an initial survey of a monkey retina in an animal with an occipital lobectomy four years before killing again, this transneuronal degeneration of the retina of retrograde character was distinct as demonstrated by the retention of the ganglion cell layer of only two cell thickness as opposed to the normal of seven to eight ganglion cells on the intact side. Thus, it seems clear that Dr. Van Buren has demonstrated that transneuronal degeneration in the visual system not previously reported.

Dr. Choh-luh Li is continuing his study as to the function of the cerebral cortex by studying unitary systems using microelectrodes. Interaction of unitary systems by way of collosal response was utilized by this investigator in a study similar to that reported by Dr. Ajmone Marsan. For example, if a cell became depolarized and excited following a trans-collosal volley, a cell contiguous to such excited cell might become hyperpolarized in response to the same stimulation. This phenomena was noted in the motor area and the somatosensory area and the visual cortex. Dr. Li concluded that this could serve as direct evidence for the existence of an inhibitory interneuron in the cerebral cortex and further suggested that the inhibition of responses of certain cortical neurons to an electrical stimulus is due to either a depression of the facilitory interneurons or an activation of inhibitory interneurons. Likewise, he postulates that the inhibition of certain cortical neurons to a given pharmacological agent may be determined by such an agent's capacity for either depressing the facilitatory interneuron or activating the inhibitory interneuron.

Dr. Li has also done a study of unitary potentials measured by evoked responses from the striate cortex of cats, following a single electrical stimulus applied to the lateral geniculate body. This response was simultaneously registered by intracellular electrodes and by surface recording electrodes. He noted that the outstanding feature of such experiments was the hyperpolarization following the initial depolarization and excitation of the cortical cell membrane. On the basis of his previous observations, he suggests that the stimulation activated excitatory fibers as well as inhibitory fibers, projecting from the lateral geniculate to the visual cortex. He feels that the inhibitory mechanism in visual function may be more important in somatosensory function since hyperpolarization was relatively rare in the latter. He also notes unitary responses could be coincident with the time of small deflections in the surface-positive waves and that this would seem to rule out the argument that small deflections are due to the arrival of impulses along geniculo-striate fibers.

Dr. Li then turned his attention to the rhythmical oscillations in electrical potentials which can be recorded from the cerebral cortex. He tests the theory of general thought that this activity is generated from dendrites. He found intracellular dendritic potentials difficult to re-

cord and this would be anticipated from the studies of Frank, et al., in which it was shown that the positive spike of the dendritic potential is of such a small area that only the large negative spikes associated with motor neuron could be found. Dr. Li found that prolonged repolarization following the discharge of a surface-negative wave which has so far been thought to be due to activities of dendrites in the superficial layers of the cortex was always accompanied by depolarization and excitation of cortical neurons. Prolonged repolarization following a discharge of a surface-negative wave has been known as the "superficial response of Adrian." Surface positive waves or "deep response of Adrian" which has so far been believed to be due to the activity of cortical neurons and deep dendrites was found to occur in the absence of neuronal activity recorded from intracellular microelectrodes.

Dr. Li now turned his attention to intracellular recording at a cortical level accompanied again by a surface recording electrode. He found the basic mechanism of single cell discharge in the cerebral cortex to be very similar to that in the single muscle fiber. Thus, there appeared to be an intrinsic mechanism and an extrinsic mechanism. The intrinsic mechanism was manifested by rhythmic oscillations of the cell membrane and the extrinsic mechanism by random small potentials. In the epileptic cortical neuron, there was a decrease in the resting potential and an increase in the rhythmical oscillating potential. To understand this further, Dr. Li and Dr. Klatzo are undertaking physiological intracellular recordings in tissue culture material. The technical difficulties are such at this time that they must be resolved before further studies are done.

Involuntary Movements

With the completion of his basic studies on the stereotaxic device designed by himself, Dr. Van Buren has now entered into an intensive study of the altered physiology and therapy of involuntary movements. In doing such, he is joined by Dr. Ajmone Marsan, Dr. Hertz, Dr. Lansdell, and Dr. Cornman. Studies undertaken on such patients are: the recording of reported sensory results of stimulation; oscillographic recording of evoked activity at the cortical surfaces from stimulation of the basal ganglia; oscillographic recording of areas of spread electrical impulses from stimulation of the basal ganglia; recording of autonomic effects of such stimulation; EEG recording of activity in depth after waking, sleep, and drug-induced sleep; EEG recording of the effect of lesions in the depth upon electrical activity from the scalp. Concomitant with this will be an evaluation of the endocrinological effects of lesions within the basal ganglia and analysis of the psychological effects of such lesions as well as the practical physiological effects of therapeutic ablations as of such ganglia. To date, eight patients have been subject to depth electrode placements in the basal ganglia. Such patients are diagnosed as choreoathetotics, dystonics, Parkinson's syndromes, etc. Multiple port depth electrodes were placed in the thalamus or pallidum in such patients, as well as extradural electrode over the frontal cortex. These investigators now have studied 98 points in the anterior inferior thalamus, the genu of the internal capsule, both segments of the pallidum, putamen, and caudate nucleus. It is of interest that sensory responses have been obtained in nearly all patients. Practically all of these were referred contralaterally to the side stimulated. If the placement is near the optic tract, visual sensations contralaterally or straight ahead may be evoked. But more rarely, (i.e., 2 patients) formed objects have been seen. Somatic sensations have consisted of tingling, tickling, or warmth referred to various portions of the contralateral body. It would appear that brief stimuli within the medial segment of the pallidum evoke a wide distribution in the thalamus, while conduction of stimuli originating in the inferior portion of the ventralis lateralis of the thalamus is found only to the mesial section of the globus pallidus.

Stimulation and Ablation of Central Nervous System By Radio-Frequency Energy

Dr. Baldwin, Col. Bach, and their associates have continued their studies on the effects of radio-frequency energy waves on the primate brain. This apparatus has now been adapted so as to study the effects on the chimpanzee as well as the monkey, and a new system has been devised in combination with the Fort Knox Microwave Laboratory so that the effects of radio-

frequency on serum protein targets can also be observed. Dr. Baldwin now feels that there is a relationship between the frequency of the output and the clinical or chemical effect. Thus, the chimpanzee responds to a frequency of 240; whereas in the monkey, the frequency range is 388. Electrophoretic changes have been produced in vitro in serum albumin in the range of 30 mc. and harmonics of this frequency. He and Col. Bach feel that the dissociation constant of the solvent media play an important part in the protein changes during radiation. Thus, protein in distilled water is not effected while protein in buffered saline was effected. The effect of radiation on the whole animal was inadvertently demonstrated with the first application of the new kilowatt transmitter to the chimpanzee in that this animal died in less than a second. The changes in surviving animals have been carefully studied by Dr. Klatzo and blood-brain-barrier changes continue to occur around the aqueduct in the upper part of the fourth ventricle. Pathological changes are characterized by tigrolysis localized in the pons and medulla. These investigators found the previous aspiration of CSF protects the animal from the more severe clinical effects. The reasons for this at the present time are unknown.

Deep Hypothermia

Now that the majority of severe intracranial operative procedures are carried out under hypothermia, this Branch has continued its long-term investigation as to the effects of hypothermia in both dogs and higher primates. The study was carried out by Doctors Baldwin, Galindo, and Adamkiewicz. Doctors Galindo and Bucknam have now applied the technique of deep hypothermia to more than 100 dogs. Working in conjunction with Dr. Baldwin, they have shown that esophageal temperatures in the range of 4 to 7° C. can be obtained and maintained without cessation of extracorporeal circulation. They have applied similar techniques to higher primates and it is now clear, at least in the chimpanzee, this will provide a relatively safe background for intracranial procedures. One frontal lobotomy was carried out at a 4° C. in a chimpanzee whose circulation had been arrested for 45 minutes. The esophageal temperature at the time of this operation was 7°. At this point circulatory arrest was begun and continued in the temperature range of 4–7°C. and the animal recovered. These investigators found that blocking the sympathetic system would reduce or entirely prevent the chance of cardiac fibrillation. Selective cooling of the heart may also be done so as to decrease the time required for cardiac arrest. During such a procedure, the brain as might be expected is markedly changed and it appears white and flattened. The vessels usually of moderate to minimal size are now invisible on the surface and the larger vessels appear as fine lines. Dissection at this time is relatively easy and the brain separates along the anatomical lines with great ease. The utilization of such low hypothermia in human procedures is the ultimate aim of this investigation.

Vascular Permeability in Cerebral Edema

Dr. Klatzo and his section have continued studies on the vascular permeability of the brain in a variety of situations utilizing intravenous fluorescein labeled serum proteins. He and Dr. Webb Haymaker of the Armed Forces Institute of Pathology have now studied the brains of 63 rats who were exposed to alpha particles at a surface dose of 6,000 rad from a 60 inch cyclotron of Berkeley, Calif. The cyclotron aperture through which the alpha particles passed was 14 mm. and was of sufficient size to allow radiation of most of the dorsal surface of the cerebellum and cerebrum bilaterally. Following such radiation, the rats were sacrificed at various time intervals. Prior to the sacrifice, the majority of animals were injected with fluorescein labeled serum proteins (FLSP). The major findings of these investigators on such radiated animals was that the primary noxious effect of radiation appears within 24 hours, at a depth band corresponding in intensity to the Bragg curve (see Ophthalmological studies on the lens in radiation). The primary damage within the Bragg zone is exemplified by pyknotic nuclear changes and the appearance of PAS droplets. The first vascular permeability changes appeared at 48 hours after radiation and were demonstrated by the microscopic leakage of FLSP from the capillaries, as well as by a perivascular reaction of the astrocytes in the zone of radiation damage. Extravascular leakage of proteins could be demonstrated microscopically as late as

36 days after radiation, whereas gross injury to the blood brain barrier, as tested with sodium fluorescein, is not detectable after the 18th day. Similar studies were carried out by Dr. Klatzo in primates utilized in the radio-frequency energy study. In animals sacrificed within a few minutes after exposure to the lobe excision "low position," changes in the permeability of the blood brain barrier may be found in the pons, medulla, and extending to the white matter of the cerebellar hemispheres. Occasionally, such changes are found lower and involve the dorsal areas of the cervical spinal cord. Histological changes in the acute experiments were confined to striking changes in the large segmental neurons of the pons, medulla, and dentate nucleus of the cerebellum.

In chronic experiments utilizing the radio-frequency energy, Dr. Klatzo found that only a few animals showed changes in the blood brain barrier and these changes were localized in the dorsal portion of the cervical cord. They consisted in severe tissue destruction, mainly in the posterior columns. In such areas, small cystic cavities could be seen. A few such cavities were found in the medulla and pons. The neurons in the vicinity were well preserved.

Dr. Klatzo is continuing, also, his study on pinocytosis (drinking in by cells) in nervous tissue. His observations *in vitro*, utilizing tissue cultures of neural cells, demonstrated that such an uptake of FLSP by pinocytosis was correlated with the characteristics of the individual cell type, thus it was more active in the microglial macrophages. An uptake of FLSP was also present in the astrocytes, oligodendrocytes, and Schwann cells. Pinocytosis was not observed in nerve cells on the other hand, but was vigorous in the perineuronal satellites. *In vivo*, the uptake of FLSP by glial cells in an area of edema and in the subarachnoid space showed a striking resemblance to that observed in the tissue culture suggesting that the same mechanism is involved. These investigators feel that their observations emphasized the importance of glial cells as metabolic intermediary structures.

Utilizing the same techniques of fluorescein labelled serum protein (FLSP), these investigators studied the blood brain barrier and vascular responses to allergic encephalomyelitis. They found in animals sacrificed after the occurrence of symptoms, there could be seen fluorescent perivascular exudates without inflammatory cells in the Virchow-Robin spaces. They also found other areas in which there was an intense inflammatory cell response without any evidence of increased permeability of FLSP. Occasionally, such exudates could also be observed within the stroma of the choroid plexus. In some areas, there was a wide parenchymatous FLSP spreading similar to that seen in the edema reported above. Such areas again were observed in the white matter and occasionally were devoid of any inflammatory cells. Animals were sacrificed later in the disease at the time of demyelination and intense astrocytic proliferation and no increased permeability to FLSP was found. In one severely affected animal, FLSP was found in the subpial astrocytes. In a further study of cerebral edema, Dr. Klatzo and Dr. Miquel injected the FLSP intravenously after precipitating localized edema in the white matter by a cooled metal plate reported in the previous Annual Report. The FLSP was also injected into the ventricles and into the subarachnoid space. Again they found a wide distribution of the FLSP throughout the white matter in the first hours after cold injury. Distribution of this was much larger than the area of the injury and again limited to the white matter. Fluorescence tended to disappear as time progressed; and FLSP was then found within the neuroglial cells. Such inclusions within the cells were strikingly similar to the pinocytotic inclusions observed in the tissue culture cells. After 48 hours, two distinct routes of transport of the FLSP could be recognized; one towards the pia and the other towards the ependyma. The microglial cells appeared to be the main transporters of the material. Following intraventricular or subarachnoid injection of FLSP, no intraparenchymal invasion was observed in animals provided no signs of increased intracranial pressure were present. In animals which tolerated this procedure poorly and developed increased intracranial pressure, a diffuse green fluorescence was again observed in the superficial band of brain tissue facing the pial and ependymal surfaces.

Perinatal Abnormalities

Dr. Anatole S. Dekaban is continuing his study in both animal and human material of ab-

normalities occurring on or about the perinatal period. He has concluded his Atlas of the normal mouse brain at 11 age horizons. He has gone on to produce malformations by x-radiation of such mice. Ninety-eight litters were obtained from irradiated mothers. Approximately 10 percent of these had major abnormalities, about 25 percent minor abnormalities, and the rest were free of detectable pathology.

In his study of pregnant women and their offspring at the Walter Reed Army Hospital and the National Naval Medical Center, 4,156 pregnancies and the products thereof were studied in great detail. From such studies of these pregnancies, he found 28 severe abnormalities. Thirteen of these were from complicated pregnancies and five were found in the controls. Of the moderate abnormalities, 50 were found in complicated pregnancies but fifteen in the controls. In the mild abnormalities, 87 of 96 were found in complicated pregnancies.

Congenital malformations, retardation of development, and epileptic seizures constituted the most serious abnormalities encountered. Malformations were the cause of 18 fetal deaths, neonatal or infantile deaths, and a cause of a serious handicap in the 26 surviving offspring.

Dr. Dekeban studied intensively 15 brains from infants who died in the acute phase following cerebral birth injury. The hemorrhagic lesions he found were grouped by him into six categories: (1) The hemorrhage of massive nature associated with the tear of the tentorium or falx; (2) massive, focal subarachnoid hemorrhages associated with hemorrhagic lesions in the brain; (3) intraventricular hemorrhages; (4) hemorrhagic infarctions; (5) direct, traumatic bruising of a part of the brain; and (6) minor, extravasations of blood in the subarachnoid space. In a study of the infant's occiput at birth and a surviving 8-year old child, Dr. Dekeban drew the plausible hypothesis that rapid extraction of the fetus or prolonged difficult birth in certain positions causes great elongation of the occiput. This, in turn, may result in consequent stretching and compression of the posterior cerebral arteries causing infarction of the occipital lobes. He feels that this may give some insight into the pathogenesis of symmetrical occipital ulegyria.

This section studied the outcome of 235 pregnancies in diabetic and prediabetic women and matched these with 249 controlled pregnancies. In the diabetic sample, there were 29.9 percent abortions and previable deaths. In the prediabetic group, there were 20.5 percent such deaths while in the normal control there was but 12.4 percent. Stillbirths presented a frequency of 11.5 percent for diabetic mothers, 5.1 percent for prediabetic mothers and 1.2 percent in the normal controls. Neonatal deaths amounted to 8.3 percent of all pregnancies in diabetic women, 1.3 percent in prediabetics and 3.6 percent in the normal controls. There were six abnormal surviving offspring in a sample of 157 diabetic pregnancies which amounted to a total of 3.8 percent. In a normal control sample of 249 pregnancies, there was only one abnormal surviving offspring. The abnormalities detected in such surviving offspring of diabetic mothers included mental deficiency, congenital malformations, birth injury, and seizures. Of those offspring with congenital malformations, two presented with mental deficiency and one with epilepsy. Of those infants born dead, eight had a pulmonary hyaline membrance disease, three had congenital malformations, two had cerebral birth injury, and no gross lesions were found in the remaining three.

In Dr. Dekeban's investigation of etiology, pathology and clinical manifestations of "cerebral palsy," he came to the conclusion that epilepsy of idiopathic type can occur in infants under 1 year of age and frequent daily attacks of "petit mal" occurring under 1 year of age can cause significant and irreversible mental retardation.

Finally, this Section is initiating a study of chromosome abnormalities in patients with congenital malformations, and will be joined in this study by Dr. Michael Bender of the Oak Ridge National Laboratories.

Laboratory of Neurochemistry

The Laboratory of Neurochemistry's combined program has now been split. The reporting on the Section of Lipid Chemistry under Dr. Roscoe O. Brady is now included in this Annual Report. Dr. Brady has left the section for a year of further training in California, and Dr. Bernard Agranoff is now stationed in Michi-

gan for the coming year. The laboratory will within the next 6 months be incorporated into the other sections of Neurochemistry of the Neurological Institute and a new laboratory will be formed. In this reporting period, Dr. Eberhard Trams has submitted an acting chief summary of the functions of the Section of Lipid Chemistry. The section reports on nine projects during this reporting period, five of which are directly concerned with biosynthesis of lipids of importance in the central nervous system and of the myelin sheath. The Neurological Institute has maintained in the past and will continue to maintain and expand this activity, in that brain structure is so dependent upon an understanding of lipids and their normal and abnormal metabolism that such studies are unique almost to this Institute. The biosynthetic studies reported by these investigators concern sphingolipids, cholesterol, fatty acids, brain gangliosides, and certain aromatic compounds, particularly the formation of methylsalicylate. The elucidation of the synthesis of gluco- and galacto-cerebrosides (sphingosine lipids) is of importance in the understanding of the lipid storage diseases which may affect the central nervous system, particularly Gaucher's disease and Niemann-Pick disease. The formation of the glycosidic linkage, the formation of the N-acyl linkage of spingosine, and the various possibilities for the formation of long-chain fatty acids in the ceramide portion of cerebrosides are studied in this laboratory with the conventional analytical techniques. Such a study utilized the spleens of patients with Gaucher's disease or Niemann-Pick's disease, and *in situ* formation of the cerebrosides in the spleen was demonstrated. It was concluded, however, by these investigators that the rate of cerebroside formation in Gaucher's disease was not sufficiently elevated to warrant the conclusion that the biochemical lesion in this particular type of lipid storage disease was due to an increased rate in lipid synthesis. These investigators now propose, therefore, to investigate the catabolism of such lipids in patients effected by the disease.

The large amount of cholesterol in the brain and its low metabolic turnover coupled with the fact that it is largely unesterified might indicate a structural function for brain cholesterol and an important role for a synthetic pathway. Dr. Agranoff continued this study on the biosynthesis of cholesterol in collaboration with Professor Lynen; he utilized C^{14}-labelled precursors which were incubated with tissue preparations and the reaction products were isolated and identified. Pathways previously elucidated in yeast were confirmed in animal tissues. He found the condensing system to make a 30-carbon chain from six 5-carbon precursors and termed this system "Terpene Synthetase." This name was chosen presumably to emphasize the polymeric nature of the product. Dr. Agranoff feels that a chemical resemblance between isopentenyl pyrophosphate iosmerase enzyme-substrate compound and penicillin was found suggesting to him at least an analogous relationship between terpene formation and the formation of penicillin in micro-organisms.

In his summary, Dr. Trams emphasizes that *in vivo* synthesis of the N-acyl bond in the ceramide portion of sphingolipids is as yet poorly understood. The fatty acid portion in some sphingolipids is represented by fatty acids which have a chain length of more than 18 carbon atoms and the origin of such long-chain fatty acids is unknown. This section undertook an investigation of the origin and activation of such long chain fatty acids, i.e., C 20, since they have found that this seems to fit well into the already established program on the biosynthesis of fatty acids. In such a study, carbon or tritiated labelled precursors were used as substrates for rat brain and rat liver enzymes. Gas chromatographic analysis was then performed for the identification of the reaction products. Conventional fractionation procedures were employed to prepare the requisite enzymes. Previous studies from this laboratory have demonstrated that Malonyl coenzyme A was one of the key intermediates in fatty acid biosynthesis and this has now been confirmed by several other investigators and the Associate Director would like to indicate a recent review of this in the British Medical Bulletin of the current year on insulin metabolism. Dr. Brady and Dr. Trams have described a method for the chemical synthesis of carbon labelled Malonyl CoA. This and other labelled precursors were utilized in the further study of the reactions necessary for the formation of palmitic or stearic acids. Their observations indicated the enzymes involved were of the sulfhydryl type

and that the juxtaposed R–SH groups within the enzyme were an integral part of its active site. In an extremely nice demonstration, these investigators showed that the synthesis of palmitic acid is accomplished by the condensation of one mole of acetyl CoA with seven moles of Malonyl CoA. The search for intermediary products that might be produced during elongation of the carbon chain was non-rewarding, so these investigators concluded that the long-chain fatty acids were formed directly on the enzyme surface without liberation of intermediary products. Further steps to accomplish such were done with the use of tritium labelled triphosphopyridine nucleotide. The observation of the incorporation of tritium into the beta position only, to form palmitic acid was considered by them to be a significant finding, and thought to support the mechanism of fatty acids synthesis postulated previously in this laboratory. These investigators now hope to extend these studies to fatty acids containing up to 24 carbon atoms. Their preliminary studies have demonstrated that such acids are not necessarily synthesized by the same mechanism.

Dr. Roscoe Brady has undertaken further investigations on the enzymatic carboxylation of acetyl CoA. The objective is learning the mechanism of the biosynthetic pathway for the formation of malonyl CoA and related compounds. As pointed out before, malonyl CoA was the key intermediate in fatty acid synthesis. These enzymes were isolated from liver and heart which catalyzed the carboxylations in the presence of ATP and bicarbonate, and were shown to be biotin dependent. Dr. Brady further showed that the pyruvate or phosphoenol pyruvate would stimulate the carboxylation reaction and that manganese was required. Carboxylation of propionyl CoA proceeded at a much faster rate than the reaction with acetyl CoA and the requirements for the two reactions differed in some respects. However, no clean separation of the propionyl from the acetyl CoA carboxylase was achieved. Further investigations will be undertaken to attempt to separate these two carboxylases.

In the disorders of the nervous system known as the amaurotic idiocies, it has been found that gangliosides accumulate in the central nervous system of children so affected. The study on the structure and biosynthesis of brain gangliosides was hence undertaken in this laboratory. A new method was developed for the isolation and purification of the gangliosides from brain. Highly purified preparations were analyzed by standard procedures for their component building blocks. In agreement with Klenk these investigators established that the gangliosides was composed of sphingosine, fatty acids, glucose, galactose, N-acetyl galactosamine and N-acetyl neuraminic acid, however, no simple repeating unit could be isolated. Sedimentation studies indicated that the polymer was not bound through co-valent linkages; that it was indeed an aggregate in aqueous solution. Enzymatic degradation revealed substances in many ways similar to the so-called cytosides. Isotopically labelled precursors in the ganglioside showed that the total synthesis and turnover of such material was very slow.

Dr. Eberhard G. Trams, Dr. Robert A. Resnik, and Dr. Richard L. Irwin, the latter two from the Branch of Ophthalmology and the Branch of Medical Neurology have collaborated in a study to see if such gangliosides could be used in an attempt to identify certain macromolecular glycolipids as cell receptors in neural tissue. In such a study, gangliosides were subject to simultaneous chemical and physical analysis in order to identify the shape, size, and charge of the molecules. The interaction of these materials with quaternary amines and other central nervous system active drugs was studied by bio-assay on isolated organs and in whole animals. The initial studies of these investigators indicate that the gangliosides seem to be able to antagonize the effects of certain of the quaternary ammonium compounds. An investigation regarding the possible role of these and related materials as cell receptors sites has hence been initiated. The possibility that glycolipids are able to sequester a number of quarternary ammonium compounds such as curare and thus apparently exert a blocking action of some of these compounds in biological systems has important implications. Attention to this has recently been directed in the public press through the work of Nachmannson of Columbia University. Doctors Roscoe O. Brady, W. Siegelman, and M. D. Lane continued their investigations on the mechanism of formation of aromatic com-

pounds, the objective being to discover the enzymatic pathway of the formation of methylsalicylate which arises apparently through the condensation of 2-carbon fragments derived from acetyl-CoA. Standard enzymological methods as well as tracer techniques were used. It appears that labelled acetate is a preferential precursor for the biological formation of compounds containing benzene rings. In plants at least, a biological route exists for the benzene ring formation other than that by way of sedoheptulose, quinic, and shikimic acids. These investigators feel that they have established a preferential conversion of labelled acetate to 6-methyl salicylic acid *in vitro*. Insight into such mechanisms could give an indication as to the pathway of formation of certain quinones such as vitamin K and coenzyme Q. This project will be discontinued until Dr. Roscoe O. Brady's return from California. Dr. C. S. Spyropoulos of the Laboratory of Neurophysiology and Dr. Roscoe O. Brady reported in 1959 that oxidating agents were capable of causing a decrease of the resting potential of a nerve fiber with a concomitant spontaneous continuing firing. This suggested to these investigators that the membrane of living nerve tissue normally contains a component in the reduced state. In efforts to determine whether or not redox reactions participated in excitation and impulse conduction, the effects of conenzyme Q and derivatives thereof were investigated on chronically stimulated unmyelinated nerve fibers. The project is in the preliminary stage and will be continued upon Dr. Brady's return from California. Finally, Dr. B. W. Agranoff with Dr. Harry Eagle of the National Institute of Allergy and Infectious Diseases undertook an investigation of the function of inositol as a vitamin. Inositol, labelled with radioactive tracers, was incubated in the presence of cells grown in culture and the radioactive products analyzed for inositol content. The concentration of inositol was determined by microbiological and chemical methods and these investigators found inositol-dependent mammalian cell types appear to require inositol for the production of structural phosphatidyl inositol. Inositol was also found in a bound form in protein and nucleic acid fractions. In addition, however, certain cell types were able to synthesize inositol to support growth in deficient cell types when the two culture vessels were separated by a dialysis membrane. This present study indicated that particular somatic cell types may possibly furnish constituents for other cells.

Laboratory of Neurophysiology

With the reorganization of the Neurological Institute, it was the decision of the program directors that the Laboratory of Neurophysiology should allow area sections to continue to act as a single entity under the present laboratory chief, Dr. Wade Marshall. However, those areas in the province of each of the two Institutes were clearly demarcated with regard to space, budget, and personnel. The sections of Spinal Cord Physiology, and Special Senses, thus fall in the scope of this annual report. The laboratory chief, Dr. Marshall, will make his report concerning the integrated laboratory to both directors of each of the two Institutes. Upon completion of the new facilities, the two sections, Spinal Cord Physiology and Special Senses Physiology, will form the nucleus of a new Laboratory of Neurophysiology within the National Institute of Neurological Diseases and Blindness.

Three broad areas of research endeavor are represented in these two sections. First, the major efforts of both sections has been used to further understand the basic mechanisms underlying the generation of impulses and the mechanism of nerve excitation using diversified techniques dependent upon the section's approach to the problem. Second, using the auditory pathways, investigations were undertaken to understand integrative mechanisms of cellular substations utilizing bilateral stimulation combined with microelectrode records within such substations. The third study has just been initiated and is directed towards possible chronic changes that might occur in single cells of the central nervous system associated with altered sensory input, i.e., cellular changes possibly associated with learning.

Impulses and Nerve Excitation

Dr. Ichiji Tasaki and Dr. C. S. Spyropoulos have continued their studies concerning the basic mechanisms of nerve excitation, including impulse generation, conduction and their conse-

quences. The highly productive work of this section over the past years has now been incorporated into a review which includes all the concepts on excitation arrived at by these investigators. This has been submitted and is to be published in *The Physiological Reviews.* During the current year, these investigators have turned largely to the radioactive isotopes to further elucidate the mechanism of nerve excitation, and in this study have used multiple intracellular wire electrodes, intracellular injections of isotopes, and perfusion of the intracellular compartment. In such studies, the squid giant axon was used. By means of gamma-ray spectrometry, simultaneous measurements of radioactive sodium and potassium were made and combined with electrophysiological techniques to study ionic movements in the squid giant axon. Similar radioactive tracer techniques were used with artificial membranes of the cation exchange type. A highly ingenious rotating fractionator was employed for measuring tracer movements during prolonged action potentials. The principles governing the movement of tracers across animate and inanimate membranes, and the relation of such tracers to the stable species were interpreted on the basis of thermodynamics of irreversible processes. These investigators feel there is a justification of such proposed principles as applied to nerve membranes in that similar results were obtained by study of movement of tracers across the cation exchange resin membranes. Radioactive tracers employed were K^{42}, Na^{22}, Ca^{45}, P^{32}, Cl^{36}, S^{35}, tritiated water, Cs^{134} and the various C^{14} organic molecules. In the resting state, the giant squid axon lost intracellular tritiated water with a time constant of 0.5–1.0 minutes, whereas the corresponding figure for the Ca^{45} divalent ion was approximately twenty minutes. When C^{14}-labelled thiourea and urea were measured there was a time constant of 30–100 minutes, whereas for all univalent cations, the time constant was between 5 and 15 hours. For anions examined by these investigators, i.e., chloride, sulfate, and phosphate, and for large neutral molecules such as sucrose and starch, the time constant was greater than 50 hours. These investigators felt that calcium, choline, and phosphate were chemically bound with the axoplasm and possibly also, in part, with the membrane.

If the axon now was repetitively stimulated, the movement of the unbound cationic tracers such as sodium, potassium, cesium, and guanidinium was accelerated; similarly, if calcium and magnesium concentrations in the bathing fluid were reduced or the potassium concentration increased, there was also an increase in the rate of loss of these unbound cationic tracers. None of the neutral or anionic tracers were found to be affected by either repetitive stimulation or by potassium depolarization. Based on such findings, these investigators postulated that the resting squid axon membrane had a fixed negative charge and that the membrane remained negatively charged during its activity.

Using the rotating fractionator, these investigators collected samples of sea water flowing by such a squid giant axon at different intervals during a prolonged action potential. Their preliminary results indicated that both K^{42} and Na^{24} were released from the axon both at the beginning and at the end of the prolonged action potential. There did not appear to be any profound preference of one of these cations over the other. Such collections, however, were at millseconds intervals.

In previous studies from this Section, Dr. C. S. Spyropoulos has demonstrated that the nickel divalent ion will lengthen the action potential. These investigators applied nickel chloride to the toad single node preparation and found it was possible to initiate in an all-or-none manner, an action repolarization after subsequent application of a small amount of K-rich fluid. The application of a calcium divalent rich fluid to the node during plateau of prolonged action potential was found to abolish such an action potential. Similarly, a sudden temperature rise applied during such a plateau was also found capable of abolishing the action potential. These investigators came to the conclusion, based upon these findings, that competition between univalent and divalent cations at the negative sites on the membrane play an essential role in the two stable states of the nerve membrane. Using the squid axon membrane, these investigators reconfirmed the finding that an injection of tetraethylammonium chloride along a long stretch of the axon will prolong the action potential from its normal value of approximately 1 millisecond to as long as 100 milliseconds or more. However, when only a short portion of the axon was

treated with TEA solution, there was only a slight prolongation of the action potential. This was attributed by these investigators to the effect of the electrical current flowing between the normal-untreated and TEA-treated portions of the membrane which brings about a premature termination of such a prolonged action potential. This process of abolition of the action potential was found to propagate along the length of the axon. Hence, these investigators feel that the mechanism of initiation of repetitive firing of impulses at such a boundary was clarified. Similar cationic and anionic studies were carried on with the Nitella cell. The time course of loss of intracellular K^{42} during activity was found to roughly parallel the impedance loss associated with such an action potential. They failed, however, to detect a similar movement of Na^{24} tested by the same technique. These investigators suggest that in this organism, the major portion of intracellular Na^{24} is in a bound form. The influx and efflux of the anion Cl^{36} was investigated in two species of Nitella and it was concluded by these investigators that this anion was not involved in the primary steps of action potential production. In such studies, these investigators were also able to develop a technique for space clamp of the Nitella internode. Using the Aplysia ganglion cells, these investigators demonstrated two stable states in that ganglion cells were found whose membrane potential stabilized periodically, presumably for intrinsic reasons, at the active state for several seconds. Such a potential could be abolished by inhibitory impulses which impinged upon the cell. These investigators found that they could simulate such inhibitory potentials by passing an inward current into the cell. In such studies, these investigators were aided by Dr. Toshihiko Oikawa from Japan, and Dr. Angelique A.-Chalazonitis and Dr. Nicholas Chalazonitis from France.

Simultaneously, in the Section of Spinal Cord Physiology, studies on the generation of impulses of nerve cells were being undertaken by Doctors Karl Frank, Phillip Nelson and Toshihiko Oikawa, a visiting scientist from Japan, in that Section. Within the medulla oblongata of the goldfish is found a pair of giant nerve cells which have been termed the Mauthner cells. These cells consist of two long dendrites and an axon passing down the contralateral side of the spinal cord to the tail muscle of the opposite side. This is a highly specialized nerve cell which lends itself readily to electrical measurement and could provide a model for other less accessible nerve cells. In the study of such cells, intra- and extracellular action potentials were recorded during anti- and orthodromic stimulation. Membrane excitability was measured by direct excitation through a glass micropipette. The relationship of the extracellular potential fields to the cell structure was studied by using marking techniques and histological studies. Antidromic excitation of one cell by stimulation of its axon near the tail produced inhibition of the other Mauthner cell presumably by way of axon collaterals. Repetitive firing of this single cell resulted in multiphasic potential fields which were mapped by these investigators. Reconstruction of the activity of such a cell from the study of these fields indicated that a large monophasic negative spike was associated with firing in the axon cap; the positive-negative potential and monophasic positive potentials fields were concluded by these authors to be the result of a passive depolarization of the cell soma and dendrites respectively. Thus, it appeared that active propagation ended at the axon hillock and the invasion of the soma and dendrites was electrotonically propagated. These investigators, however, did not see in such extracellular fields, an inflection point in the spike potential as is seen in many other nerve cells including spinal cord cells as reported previously by Doctors Karl Frank and Michelangelo Fuortes. Resting and action potentials were measured intracellularly by these investigators; the resting potential of the cell soma being 70 to 80 millivolts. The action potential, however, was but 30 to 40 millivolts showing, therefore, a reduced response consistent with electrotonic propagation.

Similar maps of the external electrical fields produced in the spinal cord by activity of single motor neurons have been carried out by Doctors Karl Frank and Phillip Nelson. The axon of a single motor neuron is isolated and antidromically stimulated. Extracellular potentials produced by this cell at a distance, however, were smaller than the random electrical activity of other cells as well as the background random noise generated by either the microelectrodes or the amplifiers. Using techniques for augmenting signal

to noise ratio, maps have been prepared for both the compound AB-spike and the simple A-spike of the spinal motor neuron. These investigators found that the latencies of the peaks of both the A- and B- components increase with distance from the cell, and that the conduction velocity of both waves is the same within the limits of precision of the experiment. This was at variance with the previous findings of Fatt, who had formerly observed such an increasing latency for the B-spike and had interpreted it as indicating active conduction in the dendrites. However, since the A-spike is probably not conducted in the dendrites and has the same apparent conduction velocity, this interpretation is unjustified. Doctors Frank and Nelson had an alternative explanation: that increasing latency with distance represents decrementing electrotonic spread over the dendritic tree. Thus, they found that peaks of both A and B spikes were nearly synchronous over a region of 5 to 100 microns for the A, and 100 to 200 microns for the B. Although these are both centered about their respective maxima, the two regions are not coextensive, but are displaced by about 200 microns in the dorsal-ventral direction. The B-spike is followed by a positive after potential while the A-spike is not.

Dr. Wilfred Rall in the National Institute of Arthritis and Metabolic Diseases has computed external fields around a single firing nerve cell to be expected in a volume conductor such as the spinal cord using different sets of assumptions. He comes to the conclusion that around such a structure as a motor neuron, predominantly negative signals would be recorded. Correspondingly, positive signals would be confined to very small regions, indeed, around the dendrites. Doctors Frank and Nelson's experimental results verify Dr. Rall's prediction; predominantly negative spikes were recorded. Consideration of these external potential fields suggests that the antidromic spike activity dies out in the soma or bases of the dendrites. Finally, Doctors Rall, Frank, and Nelson conclude that an after positivity, recorded extracellularly, does not necessarily indicate that active conduction has proceeded beyond the nearest active membrane.

Sensory Integrative Mechanisms

In Dr. Karl Frank's section, Dr. Phillip Nelson and Dr. S. D. Erulkar have undertaken a study of sensory integrative mechanisms utilizing the auditory system of the cat. In such a study, clicks and tone envelopes were delivered to the cats' ears. Microelectrodes were then inserted into the inferior colliculus or medial geniculate body and into individual cells of such structures. Both synaptic and spike potentials were recorded as responses to various patterns of auditory stimuli as well as to currents delivered through the micropipettes. These investigators found that both the inferior colliculus and medial geniculate have cells in which both excitatory and inhibitory post-synaptic potentials could be recorded. Polarization of the cell membrane revealed a direct inhibitory process in both collicular and geniculate cells. When the usual burst type response to external stimulation occurred, an inhibitory component to the synaptic activation could be seen usually. Some units in the colliculus could be seen to respond to a click in one ear with an excitatory post-synaptic potential and a click to the other ear would result in an inhibitory post-synaptic potential. By varying the interval of the stimulating source, the interaction of synaptic potentials from the two ears provides a precise mechanism for comparing the time of arrival of a click. AB-spikes like those of motor horn cells were clearly observed in some of the cells penetrated. Potentials similar to those associated with Renshaw firing in the spinal cord have also been seen occasionally. Other studies will be carried out to see if an intranuclear integrative mechanism analogous to the Renshaw loop of the spinal cord can exist at other levels of neural activity.

Electrical Changes Associated with Learning Responses

Learning has been commonly thought to involve primarily anatomical changes in groups of nerve cells or their processes. Accordingly, mapping of cortical areas following conditioned stimuli have occupied much of the time of the Anatomical Laboratory of the University College, London, under Dr. J. Z. Young. Doctors Frank and Nelson suggest that such changes also imply that individual cells could have been physiologically modified even if this only involves changes in their connections. In this study which is projected largely into the future, these in-

vestigators plan to apply prolonged series of stimuli to peripheral nerves on one side only of a chronic spinal cat, in an attempt to produce measurable changes in the spinal reflexes of the two sides. If such reflex changes can be produced, then the preparations will be examined with microelectrodes to determine which cells have been altered and in what way. These investigators plan to initiate such a study using very young cats. In their initial studies, techniques are being explored for use in perinatal animals. These investigators plan to apply repetitive joint flexion as a means of producing a steady unilateral sensory inflow. If such a study demonstrates reflex changes, and subsequently, intracellular changes, these investigators plan to study such material jointly with the Laboratory of Neuroanatomy to further elucidate anatomical substrata of such changes.

Laboratory of Biophysics

The Laboratory of Biophysics is continuing to investigate the ionic movements fundamental to the initiation and subsequent propagation of the nerve impulse.

This, as in the past, is a group of studies developing mostly from the voltage clamp concept and its application to the squid giant axon. These now involve: (1) The rather complex measurements of the ion flows across the membrane without the complications of excitation and propagation; (2) extension of these investigations to a lobster axon; (3) the measurements of radioactive tracer fluxes during the times of the principal ionic current flows across the squid axon membrane; (4) investigations of ionic permeability concepts by mathematical analysis and computations with particular emphasis on nonlinear differential equations; (5) the development of similar mathematical models for passive iron electrodes; and (6) measurements of the effect of stereospecificially tailored aminoalcohol derivatives on the electrical activity of the single node in terms of threshold and action current parameters.

In some of these studies, Dr. K. S. Cole notes the collaborations of the Naval Medical Research Institute; the Biophysics Department of Purdue University; the Physiological Laboratory at Cambridge University; Technische Hochschule, Darmstadt; the Computer Laboratory of the Bureau of Standards, and the Marine Biological Laboratory, Woods Hole. These studies resulted in ten publications of fundamental importance in the understanding of ionic transport in the initiation and transmission of the nerve impulse.

Cognizance is taken of the Laboratory Chief's anxieties as to the continued productivity of this Laboratory within but six modules of space. Upon completion of the new facilities, this laboratory will be expanded to 16 modules. Some studies in physics and physical chemistry and the use of pharmacological and radioactive tracer techniques may then be initiated or extended. The Scientific Director, however, is cognizant that this does not relieve the immediate situation. It is hoped that temporary adequate facilities may be found within the next year. To secure some flexibility within this Laboratory, initial investigations into these possibilities are already underway.

Voltage Clamp Techniques

In a voltage clamp, the ionic current flow is measured after a sudden change of the membrane potential. This potential is measured between the internal micropipette and nearby external reversible electrode and is maintained and controlled as required by an electronic control system. This system computes, produces, and measures the necessary flow of membrane current between other internal and external axon electrodes.

Doctors J. W. Moore, R. E. Taylor, and Cole have reviewed the voltage clamp concept and made critical surveys of the adequacies and inadequacies of its application to the squid axon. Doctors W. K. Chandler and Cole have extended this work by calculations of the stability of the membrane potential and its distribution along an axon. They show the conditions for a small patch to be under uniform control and for a region of a long axon near the potential electrodes to be under adequate control. Mr. L. Binstock and Dr. W. J. Adelman, Jr., have continued development on the original equipment and also produced a simpler more portable version of it. Dr. Moore with Doctors F. Julian and D. E. Goldman, at the Naval Medical Research Institute, are developing methods for measuring individual ion permeabilites of other

nonmyelinated axons. To do this, these investigators are using electronic feedback and/or sucrose gaps to minimize the external leakage and thereby provide an accurate measurement of the membrane potentials without the usual requirement of an internal electrode. Potential control of the membrane with current fed longitudinally also appears feasible. So far these investigators find that the sucrose insulation gaps used with a lobster giant axon are very effective for obtaining membrane potential and current data.

Voltage Clamp Experiments

Doctors Cole and Moore showed that although the Hodgkin-Huxley empirical expression for the potassium ion current is reasonably satisfactory for the usual range of axon phenomena, it is however quite inadequate to express the delay in the onset of this current after even a moderate hyperpolarization of the axon membrane. Doctors Moore and Adelman have completed studies of the internal sodium ion concentration as measured by the sodium potential of the membrane. Doctors Adelman and Taylor are reporting that the leakage current (which appears in addition to the large, slow, rectification currents of sodium and potassium ions under voltage clamp conditions) also showed some rectification—but with a time constant of less than 100 microseconds. This leakage current appears, however, not to be dependent upon an inward movement of sodium, potassium or chloride.

To further understand ionic transport across such membranes, Doctors U. Kishimoto and Adelman used detergents which were either cationic, anionic, or nonionic. All such detergents increased the leakage current of the membrane, cationic being the most powerful, the anionic second, and finally, the nonionic. Only the cationic detergents irreversibly reduced both the resting and the action potentials. Differences among effective concentrations of these three types of detergents suggest that the membrane is negatively charged.

Doctors Taylor and Chandler investigated bridge techniques to determine the high frequency alternating current impedance of the squid axon membrane. From this, Dr. Taylor made an attempt to measure the membrane series resistance and the preliminary data are still undergoing analysis.

Membrane Current Components and Radioactive Fluxes

Dr. Adelman, with Dr. L. J. Mullins and Dr. R. A. Sjodin of Purdue University, utilizing the giant axon of the squid under voltage clamp conditions began a comparison of the membrane current components with the outfluxes of radioactive sodium and potassium ions. Their preliminary results indicated that large current components exhibiting delayed rectification (described previously by Hodgkin and Huxley) could be accounted for by the tracers to within 15 percent for the sodium current, and 30 percent for the potassium current.

A radioactive sodium loaded axon was pulsed for short durations and low frequencies to the approximate value of the normal membrane potential. Outward early membrane current resulted. Corresponding sodium efflux was determined by collecting the effluent radioactive sodium into sodium-"free" external sea water which was rapidly circulated past the axon. A similar study was done with radioactive K^{42} in which axons were pulsed with longer duration pulses and low frequencies.

These investigators conclude that the more satisfactory and more complete experiments will probably provide independent evidence to support the Hodgkin-Huxley interpretation of voltage clamp data.

Mathematical Analysis and Computer Investigations

Doctors R. FitzHugh and Chandler are continuing their analysis of the properties of the Hodgkin-Huxley equations using phase plane concepts and the Berkeley analogue computer, as well as the digital computers of the NIH and the National Bureau of Standards. This is an attempt to combine the Hodgkin-Huxley equation with the cable theory. In doing so, these investigators have demonstrated that as the linear leakage conductance is increased, the velocity of propagation decreases. When such a leakage conductance is greater than 8.9 mmho/cm^2, a propagated action potential is impossible. The curve relating conduction velocity to leakage shows two velocities for each leakage conductance, one of which is probably unstable.

Dr. FitzHugh at the present time is working in the Electrochemical Department at Darm-

stadt, Germany, with Prof. U. F. Franck. They are utilizing mathematical models of passive iron electrodes, and are developing differential equations for electrodes in sulfuric acid and in nitric acid. In sulfuric acid, such equations produce the stable current oscillations obtained experimentally under voltage clamp. Two variables of state were noted in the sulfuric acid iron electrode, the first being the surface area covered by the oxide film, and the second being the pH of the electrode surface which interacts with such an oxidation.

Studying the Ostwald-Lillie model of nerve which is iron in nitric acid, he has found three ariables of state: (1) The surface area covered by the oxide film; (2) the electrode potential; and (3) the nitrite concentration on the electrode surface. The nitrous acid enters into an autocatalytic reaction which contributes a current component of its own having a dynatron characteristic.

Acetylcholinesterase Inhibitors and Nerve Activity

This is an investigation by Mr. E. R. Whitcomb with Dr. S. L. Friess of the Naval Medical Research Institute of the effects of stereospecifically tailored aminoalcohol derivatives on the electrical activity of the single node in terms of threshold and action current parameters. This is another study of approach to the action at macromolecular levels, of the strength and nature of drug-tissue interaction, similar to the study of Dr. Eberhard Trams and Dr. Richard Irwin using gangliosides and anticholinesteras. In this study a single frog sciatic nerve fiber was isolated and mounted for recording from a single node and the node stimulated directly once per second. Time course and amplitude of the threshold and action current of the node are studied as functions of concentration and molecular structure of the blocking compounds added to the Ringer's bathing medium. In this study, both the cis- and trans tertiary benzilate compounds were used. The cis-member was three times more effective than its trans-counterpart in attenuating the action current and increasing the threshold. However, the trans- produced a greater degree of irreversibility as indicated by the amount of recovery of the action and threshold after replacing the drug with Ringer's solution. In the second study, the drugs Tropine-O-chlorophenylacetate and 4-tropine chlorophenylacetate were used. Tropine-O was five times more effective than the 4-tropine in attenuating the action current and increasing its threshold however, the tropine-O also produced a greater degree of irreversibility. The third set of compounds used were tropine diphenylacetate and 4-tropine diphenylacetate. Here the 4-tropine diphenylacetate was five times more effective than the tropine diphenylacetate in attenuating the action current and increasing the threshold. Both produced approximately the same degree of irreversibility. Thus, the order of effectiveness of the entire group was: cis-tertiary benzilate > 4-tropine diphenylacetate > trans-tertiary benzilate > the tropine O-chlorophenylacetate > tropine diphenylacetate >-tropine O-chlorophenylacetate.

Laboratory of Neuroanatomical Sciences

The Laboratory of Neuroanatomical Sciences has undergone many personnel changes. Dr. William F. Windle is now the Assistant Director of the Institute. Dr. Sanford Palay has been appointed the laboratory chief. He will continue as chief of the Section of Neurocytology. The Section of Perinatal Physiology in Puerto Rico has been shifted from the Laboratory of Neuroanatomical Sciences to the office of the assistant director. Dr. Lloyd Guth has been appointed the acting chief of the Section on Development and Regeneration succeeding Dr. Windle in this position. In anticipation of new facilities, the Scientific Director and Dr. Palay will attempt recruitment in the area of experimental embryology within the next 6 months. Investigations in this laboratory include: ultrastructure of neural tissues, coordinated approach of investigations in the special senses, in particular, those observed by the 8th cranial nerve, enzymatic studies of cellular fractions, studies on the interrelationships of neurons and glia, the vascular tree of the brain, studies on artifact versus pathology, and studies on regeneration and reinnervation with emphasis on selectivity of pathways and specificity of nerve terminations.

Ultrastructure of the Nervous System

At the time of this report, Dr. Palay has

completed a study on the first successful electron microscopic examination of secreting neurons, utilizing the nerve cells of the preoptic nucleus of the goldfish. He found such cells to display a marginated Nissl substance, eccentric nucleus, and a central zone containing the Golgi complex mitochondria, neurosecretory droplets, and multivesicular bodies. The secretory material appears in the form of dense droplets of two sizes, 0.1μ and 1μ or larger. He found no intermediate stages between the two sized droplets. Each droplet was enclosed in a single membrane, 60 A thick. It is suggested that the small dropets arise primarily in the cisternae of the Golgi complex, whereas the large droplets originate by gradual transformation of the multivesicular bodies. The distinction between the two types of droplets may indicate, therefore, a different chemical composition and/or different functions.

A comparative study of ependyma in fishes and rats was initiated by Doctors Milton Brightman and Sanford Palay. They found that the ependymal cells in the rat are nearly filled with fine filaments oriented in a roughly spherical fashion about the nucleus. In the goldfish, however, these filaments were infrequent and instead, the cytoplasm was occupied by large numbers of tubules and vesicles. The ciliary apparatus was also different between the two species. In the rat, the basal corpuscle of the cilia were fitted with a bundle of short thin filaments that extended at a slight angle from the corpuscle. In the goldfish, each basal corpuscle was fitted with a pair of long, cross-striated bands consisting of fine filaments and extending from the basal corpuscle to a plane perpendicular to the longitudinal axis of the cilium. Apparently, here they insert in the terminal bars of the lateral cell border.

Studies on myelinated neurons in the 8th nerve ganglia were carried out by Dr. Jack Rosenbluth and Dr. Palay. These studies have now been completed and extended to the corresponding cells in the spiral, and vestibular ganglia of the rat. In this mammal, the myelinated neurons had 5–10 lamellae of myelin, whereas in the goldfish, the sheaths could be 50 or more lamellae thick. Studies have been initiated with Dr. Mary Grillo on the autonomic ganglia in the stomach and intestine, and with Dr. Wolfe on the fine structure of the developing dorsal root ganglia in the chick.

Enzymatic Studies on Cytological Fractions

Dr. Robert Albers and Dr. Larry Embree continued their studies as to the role of gamma aminobutyric acid in the metabolism of the central nervous system. In so doing, attention has been directed to the enzyme, succinic semialdehyde dehydrogenase, which was first described by them in 1959. This enzyme has now been purified from the monkey brain and they are studying its properties. They find the affinity of the enzyme for the substrate considerably exceeds the original estimate and they feel this can account for the extremely small concentration of this enzyme present endogenously in the brain. They feel that several properties of this enzyme in relation to the mechanism of action of aldehyde dehydrogenases, as well as the interaction with other brain metabolites which have neurohumoral associations, are of interest.

Pathology or Artifact

Dr. Jan Cammermeyer is continuing his studies in an attempt to standardize techniques of pathology so as to minimize artifactual contaminants. In a study on volumetric and histologic variations in the spinal cord, he finds uniform and excellent histological results were obtained by the use of a rapid coagulant fixative such as Heidenhain's Susa solution or p-toluene sulfonic acid, provided the autopsy was delayed for four hours. In his discussion of the so-called "dark" neurons, Dr. Cammermeyer comes to the conclusion that these are mostly artifactual and could be avoided by the techniques developed herein. A series of experiments were performed to either avoid or produce such neuronal staining. A hypothesis was formulated by this investigator that these cells are formed by post-mortem severance of delicate attachments between the membranes and their surroundings. The arrangements of cells and vessels of the central nervous system from various mammalian species were also studied extensively. A study of the oligodendrocytes as well as other cells of the central nervous system showed again that proper fixation and delayed autopsy was important. It was found that the size of the oligodendrocytes varied with both the region and the species examined. They

tended to have an exclusive perivascular arrangement near neurons, and perivascular arborizations of both gray and white matter. From these studies, Dr. Cammermeyer concludes that the older concepts about the functional significance of oligodendrocytes may well be refuted. He presents a new hypothesis that these cells form juxtavascular clusters which may be concerned with the intrinsic control of the blood flow to the neurons. In this respect, it is of interest that Dr. Cammermeyer noted that neurons in all regions contact blood vessels for varying lengths.

Regeneration and Reinnervation

The Specificity of End Organs. Dr. Lloyd Guth and Dr. C. J. Bailey and Dr. Karl Frank (Laboratory of Neurophysiology) as well as investigators from Boston University School of Medicine and Columbia University School of Medicine have in the past 2-3 years carried out a fascinating study in which attempts have been made to reinnervate on the one hand, sympathetic nervous system structure by the other sympathetic nerves which ordinarily do not subserve the same function, and in a more difficult situation of using a parasympathetic nerve to reinnervate what is ordinarily a somatic function. In the first such study, nonpupillary sympathetic nerves are utilized to see if they could mediate a pupillary reflex. In this study, transection of the sympathetic rami T1–T3 is carried out to allow collateral sprouting from the T4–T7 sympathetic segments (which ordinarily do not carry pupillary rami) to see if such sprouts could innervate the pupillary postganglionic cells of the superior cervical sympathetic ganglion. In this study, they found that pupillary tonus was restored by the foreign nerve fibers, but that these fibers did not participate in sympathetic pupillary darkness reflexes. In the other study, anastomosis of the proximal recurrent laryngeal nerve and the distal phrenic nerve segment was attempted; and they demonstrated that the recurrent laryngeal nerve could indeed restore function to the denervated hemidiaphragm of the rat. In the monkey, the recurrent laryngeal nerve transmits functionally effective inspiratory action potentials to the denervated hemidiaphragm only during asphyxiation. This has significance in human diseases which are characterized by destruction of phrenic motor neurons. If reinnervation of the diaphragm by the recurrent larygneal nerve could be established, such patients might survive without the assistance of an artificial respirator. The experimental phase of this program with animals has now been completed and the initial clinical aspects are being studied in Boston.

Studies on the regeneration of peripheral nerves have been planned by Doctors Lloyd Guth and Jerald Bernstein to see if such peripheral nerves regenerate selectively to appropriate endings or randomly toward any termination. This is a projected experiment in which the sciatic nerve of the rat will be transected and resutured, and after regeneration is complete, isometric tensions developed in the soleus and plantaris muscles upon stimulation of spinal roots L4 and L5, will be studied. A difference in the proportions of the L4 and L5 fibers innervating such muscles would be evidence that the regeneration was not random. The second approach will be that of the cervical sympathetic trunk of the cat which again will be transected and sutured. After regeneration is complete, the rami of T1 to T7 will be stimulated. If T1 to T3 dilate the pupil, and T4 to T7 constrict the ear vessels, this would be evidence that the regeneration again had not been random. Four other such experiments are anticipated by these investigators, all directed toward the determination of a specificity between site of origin and the end receptor.

Dr. Earl Feringa has also undertaken a study in the regeneration of the peripheral nerve to evaluate nerve grafting procedures as a method of obtaining regeneration across relatively long gaps in peripheral nerves. In this study, a segment of sciatic nerve approximately 2 cm. long was removed from goats and dogs. The gap in the nerve was filled with small, frozen, dried, irradiated nerves and the entire area was wrapped with a Millipore cuff. Functional progress was recorded with motion pictures and conduction across the area determined electrophysiologically. These investigators have found that functional return was noted in three of the four dogs and in two goats. The transmission of the nerve impulses across the graft was found in three dogs and in one goat.

Further attempts toward studying regeneration in the spinal cord of cats and monkeys in which an attempt to facilitate such regeneration

by spinal fusion and inhibition of vertebral mobility, has been undertaken by Dr. Feringa. Successful fusion after the spinal cord was transected, has also been done on 13 monkeys. The effect of these procedures on spinal cord regeneration has not as yet been evaluated.

Dr. Feringa has also initiated a study as to the possible role of auto-immune antigens in cerebral scar formation with a hypothesis that the glial scar which blocks axonal regeneration following central nervous system injury may result from an auto-immune response to the release of brain-antigen following the injury provoked breakdown of the blood-brain-barrier.

Dr. Feringa is also using tritiated-labelled thymidine to determine in spinal cord regeneration whether such axons which are regenerating come from pre-existing neurons or from outgrowth of neuroblasts that have newly differentiated from ependymal cells. In this study, he has injected the tritiated thymidine in newts approximately 12 days after cord transection and has noted labelling of the ependymal cells only. This would indicate that whatever regeneration was present in the newt spinal cord was derived from new neurons in response to spinal cord transection.

Two other projects, one related to the neural pathways of color vision in the fish and one related to gustatory function following heterogeneous reinnervation of the taste buds in the fish, have been initiated by Dr. Bernstein.

Vestibular and Auditory Systems

Under the guidance of Dr. Grant Rasmussen, the investigation in connection to the vestibular and auditory system has continued at all levels, from the peripheral receptor to the cortex. In this study, advantage was taken of the histochemical method of Koelle in which acetylcholinesterase histochemical stains were utilized since efferent fibers possess a much higher degree of this activity than do afferent fibers. With such stains, Dr. Rasmussen and his colleagues have been able to follow efferent fibers of the vestibular and cochlear nerves from the brain to the epithelium of all the receptor organs of the inner ear in the chinchilla and the cat. In the organ of Corti, the efferents were easily traceable to the inner and outer hair cells. Those fibers to the outer hair cells have been observed to bend sharply apicalwards winding in and out the rows of receptor cells. Dr. Rasmussen points out that each fiber could, therefore, make synaptic contact with numerous hair cells, but this remains to be proven by electron microscopic studies of the organ of Corti. He points out, however, that all these fibers are directly related to the efferent bundle, originating in the brain, since all traces of acetylcholinesterase activity disappears seven to ten days after cutting the bundle in the medulla.

Dr. Sanford Palay points out that Dr. C. Smith of St. Louis, has for several years, been studying with the electron microscope, the innervation of the organ of Corti in the normal guinea pig. Such studies thus far show disproportionately large numbers of efferent type endings in respect to the relatively few afferent fibers.

Drs. Rasmussen and Richard Gacek have previously demonstrated the efferent innervation of all the vestibular receptors, as well as the auditory, with the aid of fiber degeneration methods. And, Prof. Gosta Dohlman, a visiting scientist from Lund, Sweden, in Dr. Rasmussen's Laboratory, has demonstrated a high concentration of acetylcholinesterase in some fibers located next to the hair cells of these receptor organs. Once again, the histochemical reaction disappears in all vestibular receptors following transection of the efferent bundle in the medulla. Professor Dohlman and Dr. Rasmussen have found, contrary to previous evidence, that not all of the ascending fibers from the colliculus pass in the brachium to the medial geniculate body. Nor do all such fibers enter the geniculate body or terminate in all parts of it. Many fibers leave the brachium of the inferior colliculus to ascend in the reticular formation of both sides to undetermined destinations. Other fibers course outside of the principal auditory pathway and terminate in the subthalamic nucleus. Most surprising to these investigators was the finding that the auditory fibers of the brachium, which is the principal auditory pathway to the geniculate body, apparently terminate only in the inferior portion of the geniculate body. The dorsal portion of the geniculate body remains strikingly free of fiber and synaptic degeneration following destruction of the inferior colliculus. It would appear, therefore, that the dorsal portion of the medial geniculate body may have an entirely different function.

Dr. Donald Morest is undertaking a study for this particular problem using modified Nauta-Gygax methods for determination of axonal and preterminal degeneration, subsequent to brain lesions in the thalamus and midbrain. These will be combined with synaptic stains, such as those of Glees and Rasmussen. The cellular morphology will be worked out by suitable Golgi methods. Doctors Rasmussen and Walther are studying the ascending and descending connections between the medial geniculate body and the auditory cortex, by placing small lesions in various locations of the medial geniculate body and the auditory cortical areas AI, AII, and EP, the insular and temporal gyri. The resultant fiber and synaptic degeneration is studied by these investigators by Marchi stains, and by the Nauta and Rasmussen synapse preparations. Geniculotemporal projection studies to the auditory cortex are still not complete but the cortical feedback connections of the geniculate body have been completed. It has been demonstrated that the principal part of the medial geniculate body, except for its dorsal portion, receives descending connections from auditory areas AI, AII, and the posterior ectosylvian gyrus (EP), and also from the more recently established auditory areas located in the insular and temporal cortex. A large cell portion of the medial geniculate body which is usually considered as a nonauditory cell group, also receives connections from the classical cortical auditory areas. In addition, such areas project to other auditory nuclei at lower levels of the brainstem, i.e., the nucleus of the inferior colliculus of both sides and the dorsal nucleus of the lateral lemniscus of the same side.

Further studies on the efferent component of the vestibular nerves are being carried out by Doctors Gacek and Rasmussen to determine the manner in which such fibers terminate to locate the exact cells of origin of the efferent vestibular fibers. Techniques used are the Sudan Black technique, previously described by Dr. Rasmussen for the study of Wallerian degeneration of nerves of the petrous bone, combined with serial sections of the vestibular root and all of its peripheral branches, including the receptor epithelium. In addition, Protargol silver, Glees' ammoniacal silver, and the Nauta-Gygax axonal degeneration methods are employed. The method of Brodal based upon the phenomenon of retrograde cell changes is used to demonstrate the cell bodies of the efferent fibers. Elucidation of the ultimate termination of such efferent fibers is necessary to understand how such an efferent system may regulate sensory input as the cochlear efferent bundle does to the cochlea.

Conclusion

This then concludes the annual report of the Intramural program of the National Institute of Neurological Diseases and Blindness. A variety of studies related to basic and applied mechanisms of neural action have enriched the Institute's understanding of the functions of the nervous system. Basic studies may outlive the more readily (and hence, more easily outmoded) applied investigations. However, the interchange of scientific knowledge among all investigators, whether such knowledge has immediate application or derived for the sake of knowledge itself has nevertheless acted to make a stronger unit and a more critical one. Scientists at NIH and laboratories throughout the world have collaborated to make such studies possible. This Institute would like to acknowledge such joint studies:

National Naval Medical Institute (Biophysics)
Physiology Laboratories at Cambridge and Plymouth, England (Biophysics)
Computer Laboratories of National Bureau of Standards (Biophysics)
Walter Reed Medical Center (Ophthalmology)
Department of Biophysics, Purdue University (Biophysics)
Brookhaven National Laboratory (Ophthalmology)
Oak Ridge National Laboratory (Medical Neurology) (Surgical Neurology)
Donner Laboratory, Berekeley, California (Surgical Neurology)
Biophysics Department, Uppsala, Sweden (Biophysics)
Fort Knox Microwave Laboratory (Surgical Neurology)
Departments of Surgery of Boston University, Columbia University (Neuroanatomical Sciences)

Armed Forces Institute of Pathology (Surgical Neurology)
National Naval Medical Center (Surgical Neurology)
Max Planck Institute, Germany (Neurochemistry)
Marine Biological Laboratories, Woods Hole, Massachusetts (Biophysics) (Neurophysiology)
Technische Hochschule, Darmstadt (Biophysics)
National Institute of Arthritis and Metabolic Diseases (Medical Neurology) (Neurophysiology)
National Heart Institute (Medical Neurology) (Pharmacology)
National Institute of Allergy and Infectious Diseases (Ophthalmology)
National Cancer Institute (Medical Neurology)
National Institute of Mental Health (Neurophysiology) (Neurochemistry)
Physiology Department, University of Texas (Neurophysiology)
Walter Reed General Hospital (Surgical Neurology)
U.S. Agricultural Experimental Station, Beltsville, Maryland (Neurochemistry)

Many of these investigations would have been impossible without such free interchange of scientific information and collaborative research with such units.

The clinical director has indicated his appreciation to the Nursing Staff of the Institute, in particular, Miss Margaret Hulburt and her three Chief Nurses, Mrs. Thompson, Miss Soltow and Miss Maccia. The Institute is also indebted to the Office of the Director of the Clinical Center and to the various clinical service units of this Center, in particular, Diagnostic Radiology, Clinical Pathology, and Anatomic Pathology.

NATIONAL INSTITUTE OF DENTAL RESEARCH

INTRODUCTION

In the relatively brief span of 12 years since the establishment of the National Institute of Dental Research, the unfolding pattern of program activities has been characterized, perhaps most significantly, by a redefinition of dentistry's scope of research.

With early emphasis directed toward the development, on a foundation of existing scientific knowledge, of a rational basis for understanding the natural history of the teeth and their supporting structures in health and disease, there rapidly evolved an era of unprecedented productivity. Today's assets may be measured, in part, by the removal of much of the artificial but traditionally structured separation of dental research from the total body of the biological and medical sciences. With this accomplishment has come new breadth, new responsibility, and new meaning to dental research. How better to herald this new era of understanding than note, without a raising of eyebrows, the essentially unchanged direction and significance of a scientist's research program as he moves organizationally to or from one of the other categorical Institutes of the NIH.

Although an expanding horizon of dental research implies an increasing availability of well-trained young scientists, the unfortunate fact is that in spite of increased college enrollments the total number of baccalaureate graduates earning Ph.D. degrees has not increased substantially in recent years. Further manpower shortages are apparent in graduate programs where the M.D. or D.D.S. degree is a prerequisite for advanced training, pathology being a notable example. Nevertheless, the evolution of graduate education in dentistry has been both rapid and gratifying, and the role of the intramural program of the National Institute of Dental Research in stimulating its further progress is significant. For example, during the past year, in the area of biophysics alone, two associates in the NIH Visiting program and six guest workers participated in research activities which provided valuable experience in methods of electron microscopy, electron and X-ray diffraction and microradiography. From the Institute's own permanent staff, two young investigators with exceptional promise were singled out for out-of-service graduate training in the fields of human genetics and oral surgery.

While some concern over recruitment for dental research has been related to the rather widespread adoption of the basic biological sciences by graduate medical education, this acquisition probably represents an organizational identification with schools of medicine and does not reflect the increasing association of science departments with schools of dentistry and, more importantly, with general universities. It is entirely probable that this transition is contributing to a greater flexibility of the graduate student's orientation toward a variety of the health professions, and promises increasing equality of opportunity for the advancement of broader fronts of scientific knowledge in the years ahead.

An indication of the extended horizons and new meaning of dental research is the recent establishment of two important program activities in the Dental Institute. Since enzymology has such obvious implications for all biological processes, the point is perhaps excessively belabored that a knowledge of enzymes is fundamental for an understanding of biological phenomena. Recent technical advances, however, have made it possible to study enzymes more intensively as molecules, and the prospect is thereby opened for understanding the ways in which individual enzyme activities can be altered biologically through mutations and chemically through reactions with pharmacological agents or poisons. It should be emphasized, however, that this is a vision of the future since at the present time the complete amino acid sequence is known for only one enzyme, namely, ribonuclease. But even in this case, no complete model of enzyme action has been described.

In order, therefore, to gain further insight into the essential structures and catalytic mechanisms of enzymes, a Section on Enzyme Chemistry was established in July 1960 under the able direction of Dr. Alan Mehler.

Another newly instituted program activity is concerned with the normal anatomy and physiological actions of the mouth and pharyngeal region, and with distortions of form and function resulting from anomaly or neurological disorder. Inasmuch as the major purpose of this program, to be established as a Section on Oral Pharyngeal Development and Function, is to provide a continuous interaction of studies of the normal developing human, the impaired human, and normal and experimentally impaired animals, the personnel involved will engage in various patterns of combined interests. Under the direction of Dr. James Bosma, these will include the application of available methods of pressure and sound recording, electromyography, cinematography and cinefluorography to functions of respiration, feeding and speech in subjects with orthodontic deformities, neurological impairments of the mouth and pharynx, and anomalous defects such as cleft palate and hypoplasia of the tongue, pharynx, and mandible. The very nature of these impairments dramatically emphasizes the role of the mouth and its adjacent structures in the formulation of speech and in the maintenance of essential life processes of respiration and nutrition. Viewed in such logical perspective, the mouth and adjacent structures is as much a vital organ as the heart, liver or lungs.

Continuing to serve the Dental Institute in matters of long range planning and policy making, the Board of Scientific Counselors met formally on two occasions during 1960. Characteristic of its objective approach to problems was the time devoted at a recent meeting to an evaluation of Board responsibilities, and ways and means by which its mission of service might be more successfully discharged.

In taking note of those areas of research warranting increased emphasis, the Board expressed strong support for plans to (1) better coordinate and identify the diverse physical biology activities by conferring laboratory status on this important program area; (2) reorient the program of the Laboratory of Histology and Pathology toward experimental embryology with emphasis on an approach to developmental biology through methods of molecular analysis rather than along more rigid lines of classical embryology; and (3) establish an Immunology Section in the Laboratory of Microbiology in order to coordinate research in host defenses, whether mediated by specific antibodies or nonspecific mechanisms, with a view to the control of infectious diseases of the mouth and related structures; to study allergic reactions to a variety of local anesthetic agents, antibiotics and prosthetic materials used in everyday dental practice; and to facilitate the required flexibility of immunologic research programs in general.

The anticipated expansion of Institute activities made possible by occupancy of a new laboratory research building makes more urgent than ever the maintenance of an effective safety and sanitation program. Other needs related to increased animal experimentation are the coordination of animal care, special animal breeding for germfree research, and the operation of a diet kitchen and cage washing facility. Furthermore, the Dental Institute will assume, for the first time, full responsibility for its own glassware preparation facility including decontamination, cleaning, wrapping, sterilizing, special preparations, repair and delivery. In order to coordinate these many functions and assure their effective operation, request was made and authorization given to establish a central Research Services Section in the Institute.

Inasmuch as the account of laboratory and clinical research to follow is presented largely within a framework of operational categories, it should be emphasized that this mainly serves the purpose of a simplified and organized presentation and should not deprecate the more important concept of research by programs.

LABORATORY OF BIOCHEMISTRY

Collagen and Related Proteins

In continuing studies of the chemical and structural characteristics of collagen and related proteins, recent findings by Doctors K. A. Piez and M. S. Lewis show that denaturation by heat or urea of collagen in solution results in the formation of at least two subfractions which are

not identical. Although these fractions have been separated by chromatography and analyzed, it is possible that improved separation procedures may effect the resolution of still other components. Parallel crystallographic studies of protein isolated from the enamel of pig embryos, suggest the structure of a keratin although chemical analysis shows a difference in terms of a very high content of proline and a major amino acid component of histidine.

As an example of the increasing attention given to the biochemistry of amino acids and proteins in mammalian cells, collaborative studies with Dr. H. Eagle of the NIAID have shown that even though cystine is synthesized in cell culture from methionine-sulfur and glucose-carbon, it is still necessary to include it in cell culture media. This necessity stems from the observed inability of the cell to synthesize cystine at a sufficient rate to meet the combined demands of metabolic utilization and loss to the medium. Thus, in very large cell populations where this demand can be satisfied by more cells, cystine is no longer required. Such a seemingly simple observation assumes considerably larger proportions when it is recognized that cell culture is still a relatively new tool admirably suited for the study of many metabolic processes.

Prenatal and Dietary Factors in Experimental Dental Caries

The experimental approach to problems of oral disease was considerably advanced this year by studying the effect of prenatal factors on the development of dental caries in the progeny. In these investigations by Dr. C. T. G. King, pregnant rats were rendered acidotic by exposure to an atmosphere of 30 percent carbon dioxide in 70 percent oxygen at different periods of gestation. It is significant that the offspring of animals treated between the 7th and 9th days of pregnancy developed a severity of dental caries (score of 19 $\pm$ 1.8) that was twice that observed in offspring of untreated control animals (score of 9.25 $\pm$ 1.23). On the other hand, acidosis after the 14th day of pregnancy did not alter the caries susceptibility of the offspring.

In other studies by Dr. F. J. McClure on the effect of calcium and phosphate minerals and cereal proteins on experimental rat caries, a number of interesting findings and promising leads may be recorded. These include: (1) An unexpected cariostatic effect of insoluble sodium phytate which presumably exerts its oral activity only after hydrolysis by phytase; (2) an altered mineral composition of teeth related to dietary change in calcium/phosphorus ratios which, however, has no apparent influence on caries susceptibility; and (3) an increased caries activity following the feeding of certain cereals in the absence of any freely available sugar.

Approaching the oral disease problem in another dietary area, Doctors G. R. Martin and H. M. Fullmer studied the role of ascorbic acid in the formation and maintenance of dental structures. Particularly important in their examination of scorbutic guinea pigs was the observation that granules of enamel matrix accumulate in and around those ameloblasts situated adjacent to sites of defective dentinogenesis; thus suggesting that degeneration of odontoblasts in ascorbic acid deficiency is a fundamental cause of structural enamel change and a possible indirect factor in caries susceptibility.

Fluoride Studies

The caries preventive effect of sodium fluoride administered in tablet form was reported this year in a joint publication by Doctors F. A. Arnold, Jr., F. J. McClure, and Mr. C. L. White. In this important study, the dental caries experience of 121 children who ingested 1.0 milligram of fluoride in tablet form every day for an average two-thirds of their lives was comparable to the caries score data previously reported for children of similar ages who drank water containing 1 ppm of fluoride. Although water fluoridation remains a more practical and dependable procedure for caries control, the findings in this study suggest an effective solution in those situations where fluoridation is not convenient.

The use of fluoridated drinking water by large population groups was further investigated by Dr. I. Zipkin with respect to the chemistry of human bone tissue. Over a wide range of skeletal-fluoride concentrations, the percentage of calcium and phosphorus in the bone tissues remained normal as did magnesium, sodium, potassium, and carbon dioxide. Particularly interesting, however, was the consistent slight increase of citrate which accompanied an increase in the

content of fluoride in bone tissues. Additional laboratory studies have confirmed this relation of citrate and fluoride in bones of white rats.

Calcification

The interdependence of the biochemical properties of collagen and the process of mineralization was well demonstrated by a collaborating team of investigators contributing their several specialized skills to a common but complex problem. Observing the fate of intraperitoneal implants of purified undenatured sterile collagen gels, tendon, and decalcified dentin, it was found by Doctors S. E. Mergenhagen, G. R. Martin, A. A. Rizzo, and D. B. Scott that mineralization occurred with hydroxyapatite in crystals parallel to the axis of the collagen fibers, thus resembling normal calcified tissues This information suggested the availability of a model system that would be subject to a variety of controllable experimental conditions, and thus useful for the study of mechanisms controlling biological calcification. As applied in subsequent investigations it was learned that a collagen gel previously dialyzed against sodium chloride solution calcifies much more slowly than that dialyzed against phosphate buffer at the same pH. Additional observations were that: (1) Dentin decalcified in dilute acid seems to remineralize differently from dentin decalcified in ethylenediamine tetraacetate at neutrality; (2) rat tail tendon calcifies promptly when implanted in the abdominal cavity of the donor animal; (3) collagen gel enclosed in dialysis sacs mineralizes as well as when it is freely exposed to the cellular reactions of the recipient animal; and (4) selected human oral bacteria, enclosed in dialysis tubing and implanted intraperitoneally, developed a mineral deposition morphologically indistinguishable from dental calculus.

In another study of mineral metabolism by Dr. R. C. Likins, the deposition of injected strontium^{89} and calcium^{45} was compared in several different calcified tissues of the rat. Based on X-ray diffraction line broadening analysis and on radiochemical studies, it was determined that discrimination against strontium relative to calcium increased with an increase in crystal perfection. This finding of a preferential utilization of calcium over strontium in biological crystallization is in accord with previous *in vitro* observations that the final composition of a series of synthetic hydroxyapatites is related to the degree of crystal perfection, and that there exists a range of size and/or crystal perfection in the crystal structure of various calcified tissues including bone, dentin and enamel. In fact, this range of difference is even found to exist within adjacent regions of a single tissue such as the basal and incisal ends of a rat incisor. It is of interest to hypothesize on the discrimination in metabolism of strontium and calcium as it may relate to a means of effecting a reduction in present and future skeletal burdens of strontium^{90} due to fall out contamination of foods. Such a study is contemplated for the early future.

Enzyme Chemistry

During the past year, Doctors J. E. Folk and E. C. Wolff, in collaboration with Dr. J. A. Gladner of NIAMD, made interesting advances in their study of the pancreatic enzyme carboxypeptidase B. A major development was the discovery of great variation in the nature of the catalytic activity with changes in the metal of the enzyme. Thus, when the zinc originally present as a component of the enzyme is replaced by cadmium or cobalt, the relative activity with peptide substrates compared with ester substrates is respectively increased or abolished. This finding provides the basis for more detailed analysis of the reaction, using the three metal ions and a variety of substrates, that may clarify our understanding of the function of metals as components of enzymes. Such studies will be greatly facilitated by the development of spectrophotometric assay methods for measuring carboxypeptidase activity with both peptide and ester substrates. These methods, using a number of synthetic substrates prepared recently, permit analyses of partial reactions to be made, thus giving additional insight into the mechanisms by which enzymes bind substrates, effect changes in the bound substrates, and release products. An unexpected finding in studies with synthetic peptides was the detection of a dipeptide (instead of an amino acid) liberated from substrates in which a basic amino acid occupied the penultimate, instead of the terminal position.

A related project, in collaboration with Doc-

tors Gladner and K. Laki of NIAMD, has been concerned with the analysis of the proteolytic enzyme thrombin that participates in blood clotting. To date, the enzymatic capabilities of this enzyme have been tested with a number of synthetic substrates; the structures of the peptides liberated from fibrinogen studies; and complete amino acid sequences determined of both peptides split from fibrinogen, including the unusual finding of an acetyl group masking the terminal amino group of the so-called B peptide. Also under investigation is the structure of the enzyme itself.

Another project that is concerned with detailed analysis of an enzyme is the study of aldolase by Dr. Mehler. Aldolase is an essential component of glycolysis and has been known as a pure crystalline preparation for many years. During the past year the nature of this enzyme and its mechanism of action have been investigated by several technics. Direct measurement of an enzyme-substrate complex has been made using ultraviolet spectrophotometry, and the identification of this complex as the catalytically active combination has been supported by kinetic data and by experiments with inhibited enzyme. A kinetic method for assaying the reverse reaction of aldolase has been developed and used for the determination of the reaction rates and affinity for the substrates and analogues. The kinetic assays have been particularly interesting when applied to aldolase slightly modified by proteolytic enzymes, since the altered enzyme exhibits relatively different kinetic properties with various substrates.

The sum of these observations provides the basis for a theory of enzyme action, various aspects of which (e.g., the mechanism of binding of substrate to enzyme, and the role of specific groups of the protein in the catalytic process) can be tested by further experiments. Eventually it is hoped that the theoretical model will be correlated with the chemical structure. To this end, both chemical and enzymatic methods are being applied to the analysis of the primary structure of aldolase. An aspect of substrate binding that has both theoretical and technical value is the protection of aldolase against certain proteolytic enzymes by substrates and analogues of substrates. The structural requirements for this protective action are being determined and are interpreted in terms of the nature of binding and the resultant modification of enzyme structure. The limited proteolysis obtained with one enzyme attacking substrate-protected aldolase yields a peptide and an altered enzyme that are currently being analyzed as part of the study of the primary structure.

One problem of enzymology is to define the metabolic pathways of individual organs and organisms in terms of the enzymes that participate in the overall reactions. Dr. H. Blumenthal has been exploring the phosphatases and diaminases of salivary glands from this point of view, and in collaboration with Dr. T. Shiota has been studying the degradation of galactosamine by oral flora.

LABORATORY OF MICROBIOLOGY

Experimental Infections

Studies on the pathogenesis of experimental infections with oral microorganisms related to periodontal disease were continued during 1960 by Doctors S. E. Mergenhagen, A. A. Rizzo, E. G. Hampp, H. W. Scherp, G. R. Martin, and D. B. Scott. In this productive collaboration attention was concentrated primarily on: (1) The isolation, purification, chemical analysis, immunological reactivity, and physiological effects of bacterial endotoxins; and (2) conditions in physiological processes such as inflammation that might contribute to the progress of periodontal disease by degrading collagen fibers of the periodontium directly or by altering them to increase their digestibility by microbial proteases.

Endotoxins of the classic glucolipid type have now been isolated for the first time in quite pure state by the two-phase phenol-water extraction of Westphal from all of the major microorganisms implicated in periodontal disease; i.e., fusobacteria, selenomonads, bacteroides, veillonellae and the three main types of oral spirochetes. They were not found, however, in oral viridans streptococci and diphtheroids. The toxicity of these substances has been demonstrated in rabbits by: (1) Inflammation and necrosis at the sites of intracutaneous injection of microgram quantities; (2) elicitation of a marked leucocytic

response; (3) elicitation, by dual injections at intervals from 6 to 48 hours, of the dermal Shwartzman reaction of hemorrhagic necrosis and the generalized Shwartzman reaction characterized particularly by bilateral renal cortical necrosis; and (4) provocation of fever in rabbits by intravenous injection of submicrogram quantities. Similar histotoxic effects of these substances have been demonstrated by injection in the oral mucosa of rabbits. As shown by pyrogenicity, the prepared endotoxins are absorbed from these sites about 25 times as effectively as from intracutaneous sites.

In view of the very recent and rapidly growing knowledge of subtle, long-term systemic effects of bacterial endotoxins, the considerable amounts of them calculated to be present in the oral cavity (in the milligram range) might well have much broader significance than their possible contribution to the inflammatory phases of periodontal disease. Coupled with previous studies of Dr. Scherp, which demonstrated hyaluronidase production by oral viridans streptococci, chondroitinase production by oral diphtheroids, proteolytic activity of various oral bacteria, and symbiotic collagenolysis by a mixed oral flora, the more recent studies of endotoxins complete a composite picture of an armamentarium of the oral flora quite sufficient to account for the soft tissue lesions seen in periodontal disease.

Studies by Doctors Mergenhagen and Martin of dermal inflammatory sites, induced by such means as the Arthus and Shwartzman reactions in varying degrees of severity, surprisingly failed to reveal evidence of alteration of collagen at the molecular level in the form of either collagenolytic products or changed distribution of the several soluble fractions of collagen, despite microscopic evidence of disorganization of collagenous elements. However, when collagenolysis was induced *in vivo* with either papain or *Clostridium histolyticum* collagenase, specific hydrolytic products in the form of hydroxyproline-containing peptides were readily demonstrated in the circulation.

In a study aiming to elucidate the mechanism of calculus formation, Doctors A. Howell, Jr., and A. A. Rizzo found by combined clinical and cultural methods that streptococci and veillonellae tend to predominate during the formation of human dental calculus, contrary to the long-held belief in the predominance of filamentous types. Of further significance is the fact that some of these elements calcify when implanted in dialysis sacs in the abdominal cavities of experimental animals. While the specificity of such calcification is still under study, this laboratory approach combined with clinical studies reported in another section, should bring light to the problem of subgingival calculus, which is generally agreed to be the predominant immediate incitant of chronic periodontitis.

Viral Studies

Viral studies directed by Dr. Scherp have achieved a working immunologic scheme for characterizing strains of herpes simplex virus, based on comparison of the kinetics of their neutralization by homologous and heterologous antisera. By making possible quantitative comparison of strains isolated in successive recurrences in a given individual, in reaction either with his own serum taken at appropriate intervals, or with sera from other persons, or with animal antisera, this procedure should elucidate hitherto inaccessible phases of the ecology of this ubiquitous virus.

Microbial Physiology

Since *Borrelia vincentii* is the predominant spirochete in acute necrotizing ulceromembraneous gingivitis, an important objective has been to describe clearly its metabolic activities. In a recently completed study by Dr. T. A. Nevin on the utilization of arginine as a focal nutrilite of this organism, it was shown that the metabolic process was mediated by a dihydrolase system with the production of desmidase, ureidase, and ADP. Ornithine, citrulline, ammonia, and carbon dioxide are produced, while inorganic phosphate is fixed (in both acid-labile and acid-stable compounds) in an amount equivalent to the carbon dioxide liberated. It was shown further that *B. vincentii* metabolizes another important nutrilite (cysteine) to pyruvate, hydrogen sulfide, ammonia, and carbon dioxide; and that an ascitic-fluid growth factor, previously shown to be essential for *B. vincentii*, is a peptide containing tyrosine and a sulfur-containing amino acid. Since oral bacteria producing lactic acid are

probably of crucial importance in the initiation and progression of dental caries, definition of their biosynthetic mechanisms is essential. In continuing studies, Dr. T. Shiota has now demonstrated that the synthesis of folic acid by isolated enzymes of *Lactobacillus plantarum* proceeds via condensation of 2-amino-4-hydroxy-6-hydroxymethyltetrahydropteridine and p-aminobenzoylglutamate to dihydrofolic acid, which is then reduced to tetrahydrofolic acid by a specific reductase with either reduced di- or triphosphopyridine nucleotide as coenzyme.

Systematic Microbiology

In collaboration with Dr. Marion Gilmour, Eastman Dental Dispensary, Rochester, New York, Dr. A. Howell, Jr., completed a definitive appraisal of the disputed taxonomic relationships of the different oral filamentous micro-organisms that previously have been designated as *Leptotrichia buccalis* and have long been considered critically important as a matrix for dental calculus. Based on detailed descriptions of cytology, growth cycles, and metabolic activities, a separation has been recommended into two genera and species, *Leptotrichia buccalis* Trevisan, which has been well described hitherto and appears to belong with the lactic-acid bacteria, and *Bacterionema matruchotii* nov. gen. nov. comb., of which a full account is currently in press.

In the course of a two-year assignment with the National Institute for Research in Dairying, Reading, England, Mr. M. Rogosa collaborated with Dr. M. E. Sharpe to publish an extensively revised classification schema for all species of the genus *Lactobacillus*, based on the authors' investigations of the biochemical reactivities and serological relationships of these species (cf. the well-known Lancefield schema for streptococci). This new schema will probably achieve official status in Bergey's Manual of Determinative Bacteriology, the standard reference in bacterial taxonomy.

Continuing his morphological and biochemical studies of fusobacteria, Dr. R. R. Omata concluded that two of the three presently accepted species, *F. nucleatum* and *F. polymorphum*, were essentially indistinguishable in cultural characteristics and biochemical activities. However, the third species, *F. fusiforme*, differed very distinctly in morphology, did not produce indole and hydrogen sulfide, and had a homofermentative carbohydrate metabolism with lactic acid as the chief product. In these respects it corresponded very closely to *Leptotrichia buccalis* Trevisan.

Germfree Animal Research and Experimental Dental Caries

A year ago, Dr. P. H. Keyes, in collaboration with Dr. R. J. Fitzgerald, provided evidence that dental caries in hamsters is a true infection. Since that time, continuing studies have been providing additional important information on the transmission of the disease through streptococcal inoculation. This has led logically to a greater emphasis on the assay of various agents thought to possess caries inhibiting potential. Examples of the activities pursued to date are: (1) The demonstration by Doctors Fitzgerald and H. V. Jordan that dental caries can be induced in the gnotobiotic rat on a caries potentiating diet by infecting them with a single strain of streptococcus isolated from dental caries in conventional rats; (2) the observation by Doctors Fitzgerald and Keyes that caries developed in the normally caries-free albino hamster when another type of streptococcus, isolated from dental caries in the golden hamster, was implanted in the oral cavity; and (3) the suggestion, in collaborative studies with Dr. Jordan, that the cariogenic streptococci belong to a group that shares characteristics of the enteric and lactic streptococci in that they produce large amounts of lactic acid from common carbohydrates but demonstrate no extracellular proteolytic activity.

Significantly, the cariogenic streptococcus from the rat is without effect on the dentition of the hamster. Likewise, some seemingly identical strains are noncariogenic in the rat. Furthermore, none of the lactobacilli, diphtheroids, antinomycetes, micrococci, fusobacteria, or other types of streptococci tested to date have been cariogenic in gnotobiotic experiments. Should these preliminary observations be confirmed by current investigations in collaboration with Dr. R. H. Larson, presently held concepts that dental caries in humans can be caused by a variety of microorganisms would need reappraisal. If human caries is also found to be caused by a single, or

only a few, specific microorganisms, the problem of its eventual control will be greatly simplified. One obvious possibility, although purely conjectural, would be the development of an anticaries vaccine.

In another interesting study of anticariogenic agents, Doctors Jordan and Fitzgerald extended their findings of a year ago showing sodium metabisulfite to be an effective anticaries agent in experimental animals, with the result that a new class of potential caries-inhibitory agents has been revealed. Some of these aldehyde-trapping compounds are now being demonstrated as effective inhibitors of caries development in experimental animals and in addition exhibit pronounced antibacterial effects *in vivo* and *in vitro*. An added advantage is that some of the more active compounds have a relatively low order of toxicity, suggesting that they would be suitable for preliminary testing in humans.

LABORATORY OF HISTOLOGY AND PATHOLOGY

Biophysical Studies of Calcified Tissues

During the past year biophysical studies have been concerned with various aspects of the structural, physical and chemical properties of calcified tissues, their development and the calcification process itself. Utilizing electron microscopy, electron diffraction, microradiography, and X-ray diffraction and spectroscopy, Doctors D. B. Scott, M. U. Nylen, and J. G. Helmcke collected new data on the ultrastructure and size of the crystallites in mature enamel and on the configurations assumed by both the inorganic and organic constituents within the large prismatic units of the enamel. These findings suggest that the long crystal-like objects characteristic of mature enamel are in reality made up of smaller units, on the order of 300A in diameter, which have developed from separate nuclei and then fused together, usually incorporating small amounts of organic matter at the fusion points. This affords an explanation for the marked morphological variation previously reported and offers a basis for understanding the widely divergent measurements on record.

Additional studies concerned with the organization of the crystalline and fibrillar elements within the prismatic units of fully mature enamel have yielded interesting new information on the cross striations in the prisms which are evident at the optical level. Contrary to the long standing opinion that such configurations are due to differences in the relative amounts of organic and inorganic substances laid down at regular intervals along the prisms, it now appears that the optical phenomenon can be explained on the basis of variations in the arrangement and alinement of the submicroscopic crystalline elements themselves. Thus, in areas which optically appear banded, the ultrastructural components seem to vary only in arrangement and not in quantity or character.

Pathogenesis of Dental Defects

Continuing electromicroscopic studies of odontogenesis by Dr. Nylen support the concept that in the beginning stages of normal tooth development the various odontogenic layers exert a profound influence on each other. The mechanisms of these interactions now seem, however, to be more complex than previously thought. The present results indicate that, in spite of the fact that dentin matrix formation commences prior to enamel matrix formation, mineralization begins in reverse sequence. Thus, calcification starts in the enamel matrix immediately as it is laid down, and it is not until after this initial deposition of mineral that the previously formed predentin layer begins to calcify. Fundamental differences in the calcifiability of the two types of matrix have thus become apparent. The immediate mineralization of enamel matrix suggests that it is calcifiable as formed, whereas the the time lapse between dentin matrix elaboration and its mineralization seems to indicate that other factors may also be involved.

With the increasing knowledge of odontogenesis, it seemed opportune during the past year to initiate exploratory investigations into the ultrastructural manifestations of pathological conditions. In a study of the effect of a rachitogenic diet on tooth development, it was found by Dr. Nylen that cell differentiation and initial matrix formation take place in a normal fashion and sequence. However, although apatite crystals are

deposited immediately in the first formed enamel matrix, no mineralization of the already formed dentin matrix appears to take place. The odontoblasts seem to degenerate and to lose their highly differentiated ultrastructural characteristics. Further formation of predentin is markedly retarded, while enamel matrix continues to be formed and calcified in a normal manner. These preliminary results indicate that the effects of the rachitogenic diet on mineralization are very complex. Since the enamel matrix calcifies normally there is apparently sufficient mineral systemically available and it is therefore felt that the lack of mineralization of the predentin may be the result of an interference with factors which make the matrix calcifiable. From the cytological picture it is suspected that the odontoblasts must play a vital role in the processes involved, and the next step in these studies will be to observe the effects of reversal of the pathological conditions through vitamin D supplementation.

Aside from the obvious implications of these studies in dental pathology they are of considerable importance in the establishment of criteria for the electron microscopic assessment and evaluation of minute intracellular changes. This has as a particular connotation the ultimate association of structure with function. A further outcome of this program activity is the contribution made through a number of inter-laboratory and inter-Institute collaborative projects. These include studies of ectopic calcification, amorphous mineralization systems, formation and properties of collagen, demineralization systems in lower forms, and the ultrastructure of cultured cells and their components under varied conditions.

Enamel Surface Structure and Properties

These investigations have been in progress for a number of years and are directed toward ascertaining the changes in surface structure which occur with age, and the alterations in chemical composition and properties which can be brought about through chemical treatment. This year the refinement of data on the point-to-point alterations in specific tooth surfaces with age has reached a stage at which preliminary formulation of a useful system of age determination has been possible. The first trials of this technique indicate that it will yield accurate enough results to make it of interest in anthropology and medico-legal science, as well as in research on the surface properties of teeth themselves.

The background afforded by these and related studies have made it possible to investigate further the reactions of fluoride compounds on the external tooth surface. In preceding years it was suggested that although the mechanism of action of stannous fluoride may be somewhat different from that of sodium fluoride, a marked protection against acid action is imparted to the enamel surface by aqueous solutions of the tin preparation. Currently, attention is being directed toward the interesting problem of whether or not similar protection can be effected *in vitro* by incorporation of stannous fluoride in a dentifrice. Evidence, to date, indicates that if the tooth is immersed in the dentifrice proper for five minutes, there is created a degree of protection against acid attack that is quite comparable to that achieved with aqueous solutions. On the other hand, if the fluoride-containing dentifrice was applied to the tooth surface by brushing, little or no protection resulted. Investigations along these lines will be continued due to their bearing upon the general problem of the initiation of dental caries at the tooth surface and the protection that may be afforded to tooth surfaces by chemical treatment.

Histochemistry of Connective Tissues

In the course of the past few years, histochemical studies of connective tissue by Dr. H. M. Fullmer have provided interesting new information on the chemical composition and properties of various fibrillar components. An example of particular importance is the further study of the recently discovered oxytalan fibers which indicates that their origin may be in the mucoprotein elements situated between collagen bundles. Other histochemical studies and an exploration of various tissues for the presence of these fibers support the concept that they are most likely an elastic-type tissue in an immature form.

Additional studies by Dr. Fullmer, in collaboration with Dr. L. T. Kurland of NINDB, have demonstrated for the first time that a characteristic dermal connective tissue disorder exists in

a major proportion of patients afflicted with amyotrophic lateral sclerosis; and that the disorder is most likely a primary feature of the disease rather than a sequel to the neurological damage which is most characteristic.

Maternal Influences on Fetal Development

It is only within recent years that any appreciable attention has been directed to *in utero* effects on mouth and tooth development. Certainly, the rather common deformities of harelip and cleft palate have presented a confusing history with respect to their etiology. In some instances they are unmistakably related to defective genetic composition, whereas in others a clearly defined environmental factor exists. Although clinical studies are most essential to elucidate the causes of harelip, cleft palate and other defects of oral development, some of the more significant advances in knowledge have been gained by the experimental method. While too early to report conclusive findings, the recently initiated studies of Dr. F. J. Kendrick and other investigators at the Dental Institute promise to add appreciably to our understanding of congenital oral anomalies. Among the factors being evaluated are blood supply to the uterus and placenta, amniotic sac compression, and loss of amniotic fluid. To date, a high incidence of palatal clefts, limb deformities and resorptions have been observed in the term fetuses of experimental rats as compared with control groups.

EPIDEMIOLOGY AND BIOMETRY BRANCH

Nutritional Factors in Oral Disease

Whereas in previous years, investigations have concentrated primarily upon the problem of dental caries, the relatively recent recognition of the widespread occurrence of periodontal diseases and their relationship to tooth loss and disability have stimulated increased attention to the need for obtaining new data. In the United States, in individuals beyond middle age, these diseases account for the loss of more teeth than does dental caries. However, few reliable epidemiological data are available on population groups in other countries. A particular emphasis has, therefore, been given during the past year to an amplification of the descriptive and determinative characteristics of these diseases.

In collaboration with the Interdepartmental Committee on Nutrition for National Defense, Dental Institute investigators (Doctors A. L. Russell, C. J. Donnelly, E. C. Leatherwood, and N. W. Littleton) have now participated in a number of surveys which may provide significant leads to the possible influence of varying levels of nutrition on oral tissues.

While analysis of findings within and between these study groups (in Alaska, Ethiopia, Peru, Ecuador, Vietnam, Chile, Colombia, and Thailand) is incomplete, preliminary reports indicate that, by United States standards, the prevalence of dental caries is generally low whereas the prevalence and severity of periodontal diseases are correspondingly high. The following summarization suggests several promising leads.

In Alaska, extreme variations in dental caries prevalence was associated with place of residence; i.e., numbers of decayed, missing, and filled permanent teeth decreased with increasing isolation. Although Eskimo males from the principal centers of population had about the same dental caries rates as observed in white males in the continental United States, those from the more remote areas had significantly less decay. Interestingly enough, both high and low levels of caries experience were found in persons subsisting on diets that were high in animal protein, moderate in fat, and low in carbohydrate. In the case of Ethiopians using a traditionally high cereal diet which is low in sugar, low in protein, and high in carbohydrates, a uniformly low level of dental caries was observed. A similar low prevalence of dental caries was found in Vietnamese who subsist on a diet that contains highly milled rice as the staple cereal. A correspondingly low dental caries rate in Ecuadorian adults could presumably be related to optimal or high intakes of fluoride.

In contrast to the relative freedom from dental caries in the observed population groups, the only areas where periodontal disease was significantly less prevalent than in the United States were remote regions of Alaska and certain parts of Ecuador inhabited by primitive Jivaro In-

dians. Elsewhere in Alaska, and generally in Peru, disease levels were comparable with those seen in the United States among people of low socioeconomic status. In Ethiopia, Ecuador, and Vietnam, prevalence levels were much above any so far recorded for the continental United States. In this regard, it is of interest to note that Ethiopians with deficient or low serum ascorbic acid levels had somewhat higher levels of periodontal diseases, whereas in Alaska this relationship was not apparent. Furthermore, in the Eskimo group, the lower prevalence of periodontal diseases could be correlated with a high thiamine excretion and a low level of plasma vitamin A.

So far as analysis of data from these studies has progressed, it appears that nutritional factors exert only a minor influence on the periodontal tissues. On the other hand, the general state of hygiene in the mouth—presence and extent of calculus and debris or their absence—seems invariably related to the condition of the periodontal tissues; i.e., within-group comparisons indicating that periodontal tissues are in a better state of health in individuals whose mouths are relatively free of these deposits.

Familial Factors in Oral Disease

In another approach to the epidemiological study of dental caries and periodontal diseases, an investigation has been in progress to evaluate the etiological significance of familial factors. In selecting population groups for study, the desirability was recognized of having a large number of family units readily available for examination under as nearly comparable environmental conditions as possible. For this purpose, the Adventist summer camps seemed ideally suited. Preliminary findings from this study, conducted on non-Adventists as well as Adventists groups, show that dental caries prevalence is consistently lower in the latter population. However, this tendency does not hold for periodontal diseases. While too early to make any definitive conclusions, current indications are that the periodontal tissues of children tend to reflect the periodontal status of their parents in both Adventists and non-Adventists groups.

CLINICAL INVESTIGATIONS BRANCH

Clinical research during the past year has been concerned chiefly with elucidating the underlying biological processes involved in diseases related to the oral cavity, with finding more successful methods for the prevention and treatment of these diseases, and with contributing to our understanding of disease processes in general. Since the development of most oral diseases is influenced by a multitude of environmental conditions as well as biological factors which involve the entire organism, the scope of these investigations has been necessarily broad.

Dental Caries

The past year marked the initiation of a comprehensive clinical study to evaluate the effect of dibasic calcium phosphate on the dental caries experience of children. The research leading up to this program indicated a significant cariostatic action of this phosphate as well as other more soluble phosphates. For example, in experiments on Sprague-Dawley rats (under the direction of Dr. F. J. McClure), bread prepared with a flour containing 1.0 percent of Na_2HPO_4 resulted in a reduced caries incidence averaging 51 percent and a reduced severity score averaging 83 percent. When $(NH_4)_2HPO_4$ was used, these averages increased even further to 72 and 93 percent respectively. On the other hand, $CaHPO_4$ was found to have no inhibitory effect unless it was supplemented in the bread preparation by dietary salt. This would suggest that the relatively insoluble dibasic calcium phosphate is solubilized by NaCl, thus providing a soluble, readily available, phosphate ion in the oral cavity. Through a common ion effect, which presumably could apply to the calcium ion as well as the phosphate ion, the dissolution of enamel or dentin could then be reduced and so limit the caries decalcification process.

This hypothesis is currently being evaluated by Doctors I. I. Ship, R. C. Likins, I. Zipkin, A. L. Russell, and F. J. McClure in a group of 1,800 American Indian children residing in eight Bureau of Indian Affairs and Mission schools. Four of the schools are supplied with a phosphated bread (2 percent $CaHPO_4$ in the bread flour) and four with a conventional, nontreated bread. Since only the first annual examination

period has been completed by the collaborating Institutes and organizations (NIDR, NIAMD, Division of Indian Health, Bureau of Indian Affairs, South Dakota State College of Agriculture and Pennsylvania State University), it is premature to suggest any evidence of effectiveness. However, it is expected that a sufficient trial period will have elapsed by the end of fiscal 1963 to draw meaningful conclusions.

In another long range study by Dr. R. M. Stephan, a number of possible contributory factors are being evaluated in selected cases of rampant dental caries. While principal consideration has been given to microbic plaque formation, intraoral pH, food retention in occlusal fissures and interproximal areas, salivary flow rates, dietary habits, drinking water, and family history, the predominating features thus far have been overgrowths of microbic plaques on caries susceptible areas, with a predominance of mixed coccal and filamentous forms; low rates of saliva secretion; and low pH levels in caries active areas.

Periodontal Disease

Periodontal disease continues to be one of the most common causes of oral infection and tooth loss in the adult. Seeking to further evaluate preliminary clinical impressions regarding the role of nutrition and heredity, Dr. P. N. Baer conducted a laboratory study which showed a statistically significant reduction in alveolar bone loss in an experimental group of mice maintained on a high protein diet (62 percent casein). On the other hand, a control group of animals, not on a supplemented diet, showed no protective effect. Furthermore, of the two strains of mice studied (STR/N and DBA/2JN), the former was highly susceptible to the disease and the other relatively resistant, thereby indicating a genetic influence.

Calculus Formation

Since it is generally recognized that calculus bears an important etiologic or contributing relationship to periodontal disease, a major effort has been directed toward the application of newer methods of study to understand better its mechanisms of formation. Although enzyme systems associated with the normal calcification process have been studied, there has been little or no information published on the relationship of known enzyme systems (other than phosphatases) to pathologic calcification. In a recently completed report by Doctors Baer and M. S. Burstone, technics of enzyme histochemistry were applied, presumably for the first time, to the study of calculus formation. By fastening mylar strips to the lingual surfaces of the lower incisor teeth of a series of patients with a diagnosis of periodontitis, the collected calculus material could be removed and incubated in substrate solutions for the demonstration of esterase activity. Chosen as substrates were acetates of AS-naphthols because of the potential chromogenicity and insolubility of the enzymatically released naphthol. Following this treatment, it was noted that the bulk of the deposits on the strips (which consisted of clumps of coccoid and filamentous forms of bacteria, leukocytes, macrophages and epithelial cells) had an occasionally variable but intense esterase activity. In considering the significance of this finding, it was suggested that enzyme activity in calculus deposits is correlated with a saponification process ultimately leading to calcifications.

Stomatitis

A viral etiology of recurrent aphthous ulcerations has been suggested by many authors, and herpes simplex virus has been mentioned most frequently. Because of the similarity of factors that can initiate recurrences of so-called "fever blisters" and "canker sores," and because of the proximity of the two lesions, there has been considerable controversy on this point. However, evidence has now been accumulated by various investigators, including several at the Dental Institute, to show that recurrent aphthous lesions are not caused by herpes simplex virus, or another virus. For example, tissue culture technics, using monkey kidney, human skin, rabbit kidney and HeLa cells have failed to isolate herpes simplex virus, or other viruses, from 100 aphthous ulcerations in 31 patients whereas herpes virus only was isolated from 13 of 25 recurrent labial vesicles in 11 patients.

In a current clinical study (conducted by Dr. I. I. Ship in collaboration with investigators at

the University of Pennsylvania) of approximately 2,000 students at the University of Pennsylvania, several additional findings of interest may be noted: (1) 52 percent of the males and 57 percent of the females had a history of recurrent aphthous ulcerations; (2) 38 percent of all the students had a history of recurrent herpes labialis; (3) age, racial background, and various social and environmental differences were not noted in the two categories of disease; and (4) a statistically significant relationship existed between emotional factors and recurrent aphthous ulcerations.

Sjogren's Syndrome

An increasing interest in this syndrome was expressed by Dental Institute investigators during the past year. Characterized, in varying degrees, by keratoconjunctivitis, xerostomia, salivary gland enlargement and rheumatoid arthritis, an effort was made to elucidate the processes responsible for the dry mouth component. In collaboration with Dr. K. J. Bloch and others in the NIAMD, over 20 patients with Sjogren's syndrome have been studied to date. Measured by use of a Lashley cup, a marked reduction or complete absence of salivary flow from the parotid gland was observed in a number of cases. When studied by secretory sialography, a pronounced sialectasis was noted in all patients. Continuing studies are being conducted in two groups; patients with Sjogren's syndrome (with rheumatoid arthritis) and patients with rheumatoid arthritis without Sjogren's syndrome. Findings to date suggest that the syndrome may be due to an autoimmune process which interferes with the function of mucous glands; i.e., in attempting to demonstrate antibodies to saline extracts of human salivary gland, there is evidence that sera of patients with Sjogren's syndrome reacted with the extract and fixed complement.

Dental Pulp

In addition to the common route of pulpal infection, via deep penetration of caries, a condition of noninfective injury may frequently result from the heat, pressure and chemical agents related to operative dentistry procedures. Based on observations by Dr. H. R. Stanley, it is apparent that the major factors determining pulp response to operative technics are the composition of the restorative material (silicate, self-curing resin, zinc oxide and eugenol, oxyphosphate zinc cement, etc.), the rotary speed and applied pressure of instrumentation, and the thickness of dentin. Of particular significance is the finding that the new range of high rotary speeds, in addition to being surgically efficient, provides a considerable degree of protection to the pulp. Thus, 50,000 rpm, with proper water-spray coolant and carbide or diamond cutting instrument, is quite harmless to the pulp, whereas the more conventional speeds of 6,000 to 20,000 rpm are damaging in spite of cooling agents.

Anesthetic Agents

Since general anesthesia for ambulatory dental patients differs in certain major respects from the usual procedures of hospital anesthesia, there is need to obtain baseline physiological data if we are to assure its judicious use and provide a basis for comparison between the variety of existing anesthetic agents. During the past year, Dr. E. J. Driscoll, in collaboration with Doctors G. R. Christenson and C. L. Hebert of the Anesthesiology Department, Clinical Center, assembled data to determine objectively from brain wave activity the precise anesthetic levels at which various oral surgical procedures are carried out with the barbiturates, Pentothal, Neraval and Methahexitol. Of particular interest was that supplementation with nitrous oxide and oxygen does not significantly alter the E. G. patterns, nor does short periods of apnea as previously suggested in the literature. Also, Methahexitol causes a slightly higher blood and pulse pressure than does either Pentothal or Neraval. However, despite this 10 percent elevation, the pattern of rise is quite comparable with all three drugs. Other pertinent findings were related to the evaluation of atropine, scopolamine, and hexocyclium. When given prior to barbiturate administration it was noted that: (1) Atropine alone accelerated the pulse within one minute whereas hexocyclium had no such effect until the barbiturate is added; and (2) hexocyclium is a far superior drying agent to both atropine and scopolamine, as well as having a much shorter duration of action.

The type of information being assembled in these clinical studies is particularly important since without such baseline data, the constant search for safer and more effective anesthetic agents applied to oral surgery has no foundation.

Cephalometric Studies

In a serial cephalometric study of postural changes in maxillary and mandibular full dentures, Doctors P. J. Coccaro and R. S. Lloyd reported a pronounced backward and downward change in mandibular position, and a progressive upward change in maxillary position following insertion. The related changes in morphologic facial height were greater during the first 6-month period than in the 6-to-12 month period, and were primarily noted in the middle facial region rather than in the upper or lower face. Such information, while relatively simple to obtain, demonstrates the important role that cephalometric roentgenography may play in achieving more functional full denture construction. To this end, a continuation program is planned for the collection of pre-extraction records of skeletal and soft tissue facial profiles which will provide a clinical record of morphologic facial height in balance with facial musculature and the natural dentition.

Genetic Studies

In the years 1958 and 1959, a Child Health Survey was conducted in Hiroshima and Nagasaki, Japan under the sponsorship of the U.S. Atomic Energy Commission, the Rockefeller Foundation, the Association for Aid to Crippled Children, and the National Institute of Dental Research. Using facilities of the Atomic Bomb Casualty Commission, and with the cooperation of the Japanese National Institute of Health, a team of examiners, including geneticists, pediatricians, dentists, and anthropologists, undertook to examine a large number of offspring of consanguineous marriages. Included in the total group of 5,033 children were records of many who were deceased. While too early to report any major findings, an example of the type of important information to be gained from this study is a reported death rate that is twice that occurring in a Japanese control group (preliminary study by Dr. W. J. Schull of the University of Michigan). With specific reference to oral aspects of the study, under the direction of Dr. J. D. Niswander, examinations have included the status of soft tissues, an index of malocclusions, recording of developmental failures, estimate of DMF and DEF rates, and anthropometric determinations. Although analysis for specific inbreeding effects has only just begun, a certain number of other preliminary findings are of interest. These include: (1) A higher frequency of supernumerary teeth (3 percent) than is reported for Caucasians, with a significant sex difference (4 percent in males and 2 percent in females); and (2) an accelerated eruption of permanent teeth.

In further studies of a triracial isolate group in Southern Maryland, Doctors C. J. Witkop, A. D. Merritt, R. O. Wolf, B. L. Hanna, L. J. Schneiderman, and Mrs. H. R. Dyson have continued to assemble data to determine the prevalence, mode of inheritance, clinical manifestations, variations in expressivity, and other factors peculiar to the hereditary illnesses found. Examples of more recent findings are: (1) A frequency of dentinogenesis imperfecta and albinism which is the highest reported for any population (3 percent and 1 percent respectively); (2) a frequency of sickle cell trait (20.5 percent) which is the highest reported outside of Africa; (3) evidence that certain speech defects are genetically determined, as in ankyloglossia which is inherited as a dominant trait; (4) the suggestion that an hereditary block in thyroglobulin synthesis accounts for the 8 percent prevalence of goiter in the female members of the isolate population; and (5) the histochemical demonstration of a presumably abnormal protein in the matrix of teeth with opalescent dentin. Other studies have been concerned with certain hematological and serological abnormalities associated with abnormal hemoglobins, blood group and secretor factor distributions in various populations; and with laboratory methods for detecting genetic differences in saliva.

In the study of another selected inbred population group located in North Carolina, findings of interest reported by Dr. Witkop and C. L. Beale of the U. S. Department of Agriculture include: (1) An estimate of fertility that is the

highest for any known racial or ethnic group in the United States (ratio of children under 5 years of age per 1,000 women is 825 as compared with a United States average of 417); and (2) the first description of an hereditary form of benign intraepithelial dyskeratosis involving the oral mucosa and conjunctiva. The latter condition was shown to be transmitted as an autosomal dominant trait and to have a characteristic oral cytological pattern quite similar to that seen in Darier's disease. Following the observation of identical intracytoplasmic bodies in oral epithelial smears from patients who had received certain cancer chemotherapeutic agents (methotrexate and possibly fluorouracil), as well as those who had been on therapeutic doses of X-radiation, it was postulated that the common denominator in all the conditions was a disturbance in nucleic acid metabolism.

In this review of 1960, perhaps the one ingredient that best characterizes the total program effort is the flexibility with which the professional staff utilized its sense of anticipation for unexpected leads and exploited implied opportunities. While a true measure of accomplishment must necessarily await the test of time, the existing framework within which this freedom of scientific expression and pursuit of knowledge operates, is the best assurance for a future of productivity.

DIVISION OF BIOLOGICS STANDARDS

INTRODUCTION

The accelerated pace of research during the past 10 years in the area of infectious diseases has resulted in a significant increase in the number, complexity, and diversity of biological products. At the close of the calendar year, 279 such products were licensed under the provisions of the Public Health Service Act for commercial use in this country and abroad.

These products, which include the vaccines, antitoxins, therapeutic serums, and human blood and its derivatives, are developed for the most part from pathogenic or potentially pathogenic microorganisms. Their preparation requires careful control in order to minimize safety hazards which might occur in the course of processing, and to ensure final products of satisfactory potency. Accuracy is essential in every step, for the end product is the preservation of human life.

Responsibility for the administration of these control measures resides with the Division of Biologics Standards. Effective administration of these responsibilities requires the design and development, within a research context, of adequate and practical standards for the production and testing of biologics, careful surveillance of production methods, and the continuous improvement of testing procedures. Thus the control program of the DBS is necessarily supported by an active and flexible research program, enabling the Division to keep pace with the rapid development of new and improved immunizing agents and to prepare physical references as well as to develop testing procedures for these products once they are ready for commercial production. The essential features and philosophy of this program were discussed in last year's report and need not be repeated.

In addition to providing information as it is needed to meet the problems encountered in this increasingly complex field, the DBS research program also provides a reservoir of trained scientists whose services are available when such problems arise.

Currently staffed with 62 principal investigators and approximately 106 supporting personnel, excluding administrative staff, the Division's research programs include a diversity of projects —many of immediate practical urgency, and having direct relation to the prime responsibilities of the Division. Others may be regarded as having a stake in the future, since demands in this area are likely to arise suddenly and move with unpredictable speed. Although the basic objectives of the Division are clearly known and the areas of research are self-evident from the nature of the mission of the Division, most of our scientists—except those with continuing and full-time administrative responsibilities—are encouraged to devote up to one-half of their time to research projects of their own devising. Many worthwhile leads have developed from such investigations, and many more can be expected to emerge in the future; the basis for this policy, however, is the need for a reservoir of trained and program-oriented scientists.

While the main activities of the Division are covered in some detail elsewhere in this report, a few items of special interest should be mentioned here.

I. The year has been dominated by the problems presented by poliomyelitis vaccine—both the inactivated and live attenuated. A great deal of effort was concerned with matters leading to the eventual licensing of live poliomyelitis vaccine. Numerous conferences were attended by staff members and a number of meetings of the Public Health Service Committee on Live Poliovirus Vaccine were held. The entire situation is perhaps best summarized in the series of papers, presented at the American Medical Association Clinical Session in Washington November 30, 1960, and published in the March 11, 1961 issue of the *Journal of the American Medical Association.*

The main concern of the Division has been the development of standards for the production of live poliovirus vaccine—both physical standards (standard preparations to be used by lab-

oratories engaged in standardization work) and written standards (regulations setting forth the requirements which must be met with respect to safety, purity and potency). Development of the written standards has been a complex and difficult task, conducted, perforce, in an atmosphere of intense public interest and in the face of a variety of freely expressed and sometimes irreconcilable points of view. The preliminary step was the issuance of a report by the PHS Committee on Live Poliovirus Vaccine on August 24th, which outlined the principles. This was followed on August 25th by a set of specifications applicable to the testing of live poliovirus vaccine. After frequent discussions and consultations with those concerned, including the scientists involved as well as prospective manufacturers, a Notice of Proposed Rule Making—the necessary preliminary to the formal adoption of Regulations—was published in the *Federal Register* on November 23, 1960. It had originally been visualized that the manufacturing development would proceed simultaneously with the development of Regulations, so that the live poliovirus vaccine might become available as a licensed product at about the time that Regulations were formally adopted. It does not now appear that this will be so, nor does it appear that any appreciable quantity of vaccine will become available until late in 1961.

II. A problem of another type was presented by the publication of an article by the Massachusetts Department of Public Health stating that, in their experience, the pertussis component of the diphtheria, tetanus, pertussis, and poliomyelitis multiple antigen was unstable. This has given rise to a lengthy and difficult program of examining the stability of the pertussis component of a variety of such preparations. This work was under way at the end of the year and a report will be made early in 1961.

III. The favorable situation with respect to the rise in the potency of inactivated poliomyelitis vaccine (Salk vaccine) reported last year continues and the average potency of the vaccine in 1960 reached unpredicted levels.

IV. In November, investigators from university, pharmaceutical, and NIH laboratories were invited by DBS to present their findings in the development of a satisfactory immunizing agent against measles in order to assess and correlate the progress made since their last meeting in 1958.

Dr. John Enders, whose work with measles virus established the principles for an attenuated measles vaccine, summarized his recent experience with the Edmonston strain of virus grown in chick embryo cells. Clinical data presented by other groups, using live vaccine made from this strain, gave evidence of its capacity to elicit satisfactory protection against natural measles as well as specific antibodies among the vaccinees.

Measles vaccine was shown by three groups of investigators to protect children exposed to natural virulent measles virus against the illness, while the unvaccinated controls showed the expected high incidence of the disease. Evidence of satisfactory antibody titer at the end of 18 months was also presented. Close to 1,000 children have been given this vaccine.

Live measles vaccine, in most cases, caused some clinical reactions—principally fever. However, Dr. Frederick McCrumb, of the University of Maryland, presented information indicating that concurrent administration of live vaccine and gamma globulin markedly suppressed the expected febrile reaction without interfering with the development of antibodies. In addition, Dr. F. A. Gibbs, of the University of Illinois School of Medicine, presented electroencephalographic findings showing that children given live measles vaccine showed little or none of the EEG abnormalities seen in children with naturally acquired measles.

Although various methods of preparing the vaccine have been used, propagation in chick embryo tissue culture is preferred since adventitious agents which might be pathogenic for man are less likely to be encountered.

There has been some question in the past whether a live vaccine of sufficiently high titer could be produced which would maintain its potency during storage. It is now generally agreed that this problem has been solved and that vaccine of high titer can be consistently produced and maintained in stable form in the dry state.

The DBS has developed serologic tests for the detection of measles antibodies, and data pertaining to standardization of the complement fixation and neutralization tests was presented. A dried reference serum has been prepared and its place

as a control in development studies and vaccine production was discussed.

As a result of the conference, an increasing volume of work can be expected from the various investigative groups in acquiring larger clinical experience. The DBS plans to continue to coordinate and expedite these studies since, for the first time, there is a general feeling that vaccination of man against measles is an attainable, practical goal within the immediate future.

V. The exchange of ideas and technical knowledge with other laboratories working in related areas has proved of great value to the Division's research programs. Dr. Gerald L. Van Hoosier, of the Laboratory of Virology and Immunology, returned to DBS in August after a year's study under Dr. Edwin H. Lennette, chief of the Viral and Rickettsial Disease Laboratory, California Department of Public Health. While there, Dr. Van Hoosier worked with a variety of disease agents. His principal projects were the fluorescence microscopy of the enteroviruses, and the chemistry of influenza.

Dr. Samuel Baron, also of LVI, left DBS in June for a year's study in London at the National Institute for Medical Research in the laboratory of Dr. C. H. Andrews, well known authority on respiratory diseases. While there, he will continue his work in the immunology of viral diseases.

Dr. Frank Perkins, of the British Biological Standards Control Laboratory, returned to Hampstead, England, in July after a year's work with the Division. A member of the British Medical Research Council staff since 1955, Dr. Perkins has conducted laboratory studies connected with clinical trials of poliomyelitis vaccine in England, and his year in the DBS viral research laboratories was mutually profitable.

Dr. Mitsuo Yokoyama, chief consultant of the Blood Typing Laboratory and the Rh Center of the Tokyo Medical and Dental University, has been with the DBS Laboratory of Blood and Blood Products for more than a year. An authority on blood grouping and its application to various populations, Dr. Yokoyama is currently studying antibodies to rare human blood group factors in animals by immuno-electrophoresis.

VI. As in past years, there has been a constant flow of visitors through DBS laboratories from foreign countries during the past 12 months. Among these have been scientists from England, Australia, Germany, Russia, Japan, France, and Canada. Others came from Thailand, Finland, South Africa, India, Pakistan, Czechoslovakia, Hungary, Yugoslavia, Venezuela, Chile, and Colombia. Many were interested in the DBS studies relating to poliomyelitis vaccines; some in biologics control procedures in general, some in blood banking. Arrangements to meet the needs for the exchange of scientific information with these visitors is a time-consuming but necessary part of the activities of the Division since many of these visitors are concerned with biological control activities in their own countries.

VII. The Board of Scientific Counselors, which is primarily concerned with the research program of the Division, met with the Director's staff and laboratory chiefs in December. The meeting was essentially one of orientation for the Board's new members, Dr. Benjamin Alexander of Harvard University, and Dr. Scott Neil Swisher of the University of Rochester. They replace Drs. David Bodian, Johns Hopkins University School of Medicine, and Robert Pennell, Protein Foundation Laboratories. Both of the new members appointed for 4-year terms, are well known in the field of hematology. Dr. Alexander is associate professor at Harvard Medical School and associate director of the Beth Israel Hospital in Boston. His special areas of research are blood coagulation, and hemorrhagic diseases, with special reference to the biochemistry of prothrombin. Dr. Swisher is associate professor and chief of the Division of Hematology at the University of Rochester School of Medicine. His special interests are human hemolytic disorders and he is also active in blood banking.

The Board expressed satisfaction with the scope of the DBS research programs and the general capabilities of the research staff, as well as the marked improvement in laboratory facilities. The suggestion was made that the addition of a biometrician to the staff, to promote and extend the use of mathematical and statistical methods and to assist in the design and evaluation of biological research, would be of value to the Division.

VIII. A major event for DBS this year was the long-anticipated completion of the Division's new quarters, which have been under construc-

tion since the spring of 1958. Formal dedication ceremonies took place on June 30, 1960.

His Majesty Bhumibol Adulyadej, King of Thailand, officially dedicated the new DBS building with the unveiling of an inscribed bronze plaque, to be placed in the main entrance.[1] The invitation to participate in the ceremonies was extended to King Bhumibol because of his active role in health measures in his own country and because of the long association of his family with medical science. Additional interest developed following the return of the U. S. Cholera Research Advisory group, headed by Dr. Joseph Smadel, which visited the SEATO and other Asian countries in 1959 and was granted an audience by King Bhumibol in Bangkok. The dedication ceremony itself demanded extraordinary organizing effort on the part of those directly concerned, particularly Dr. Tomlinson, assistant director, Mrs. Butterly, information officer, and Mrs. Lynch of my immediate staff.

The move into the new building was initiated in September 1960, and completed in January 1961. Although beset with all the inherent difficulties and the consequent dislocation of resettling laboratories which had been housed in four separate buildings, the advantage of having all operations under one roof should have a favorable influence on our programs.

For example, it has been possible to reorganize the functions of the Division in the field of virology—a step which had been visualized for some time. The Division's program in virology was strengthened by the addition of Dr. Joseph E. Smadel who joined the DBS staff on August 7, 1960.

The reorganization involved the establishment of three new laboratories, replacing the former Laboratory of Viral Products: the Laboratory of Viral Immunology, headed by Dr. George Hottle; the Laboratory of Virology and Rickettsiology, with Dr. Smadel as chief; and the Laboratory of Biophysics and Biochemistry, headed by Dr. Caspar W. Hiatt.

Good progress has also been made in improving coordination of various projects previously scattered, although some programs are still delayed due to the lag in completion of specific building alterations. Because of increased program demands, space—particularly in the animal areas—is more limited than had been anticipated when the building was originally designed; in fact, as we reevaluate our needs we have come to the conclusion that additional space will be necessary to meet the continuing and expanding responsibilities of the Division.

The Division now consists of six laboratories. The more important activities of these laboratories are summarized as follows.

LABORATORY OF CONTROL ACTIVITIES

The Laboratory of Control Activities, supported by sections on control tests, pyrogens, and reference standards, is responsible for activities dealing directly with licensed establishments in relation to the licensing and control of biological products.

Its activities include:

(1) 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, scientific and professional qualifications of personnel, and the evidence of continued safety, purity, and potency of products for which an application for license is being evaluated. License applications are usually reviewed by committees or groups consisting of appropriate staff members.

(2) Supervision of annual and special inspections of licensed establishments and of those for which an application for license has been made.

(3) Release of individual lots of biological products for distribution by manufacturers on the basis of manufacturer's review, DBS tests, and other information relating to the safety, purity and potency of that particular lot of the product.

(4) The establishment and distribution of physical biological standards, reference preparations, and control materials. A small culture collection is also maintained, mainly for use by the Division and licensed manufacturers.

(5) Review of requirements and regulations now in effect for such constructive revision as is

[1] "Science and Peace will triumph over ignorance and war, nations will unite, not to destroy, but to build, and the future will belong to those who will have done most for suffering humanity."

. . . Louis Pasteur

needed, and for the development of requirements and regulations for new products.

(6) 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 and release of individual lots of licensed products.

During the 12-month period, December 1, 1959–November 30, 1960, a total of 7,206 control tests were carried out to insure the sterility, safety, potency, and purity of licensed biological products. These included: routine products for release, 5,984; inspection samples, 1,193; and complaint investigations, 29. The results of these tests served as a basis for the release or rejection of individual lots or products. In addition, 1,386 cooperative service tests were done on biological products not licensed.

During the same period, 4,894 lots of biological products were submitted for release by licensed manufacturers. Of these, 4,848 lots were released, 12 lots rejected, and 34 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, 12; serums, 2; vaccines, 5; toxoids, 6. A total of 112 tests were required to complete a satisfactory standardization of these lots. These tests include flocculation reactions, animal protection tests, animal potency tests, neutralization 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 January 1, 1960, and December 9, 1960.

	Ampules
Cultures	2,200
Serums	675
Vaccines	1,015
Viruses	717
Toxins	274

Official standards, reference, and control preparations currently maintained include 64 items.

Standards, reference preparations, and cultures were distributed to research or control laboratories of licensed manufacturers, health departments, or universities in this country and abroad as follows:

	Ampules
Antitoxins	337
Serums	1,635
Vaccines	1,655
Toxins	126
Cultures	462
Viruses	268

LABORATORY OF VIRAL IMMUNOLOGY

Some of the responsibilities of the former Laboratory of Viral Products were assumed by the Laboratory of Viral Immunology at the time of its formation in July 1960. These responsibilities include both research and control activities.

Work with vaccines to prevent poliomyelitis constituted the main activity of the laboratory. The properties of the polioviruses which have been used in the preparation of the oral, attenuated poliovirus vaccine have been investigated, and steps have been taken to acquire a supply of the viruses for use as reference preparations. The revelation that vaccines prepared in Macaca kidney cell cultures contained a contaminating agent (the vacuolating virus) has stimulated an examination of vaccine preparations in cell cultures prepared from African green monkeys, in which the virus can be readily detected. Studies on neurovirulence of the vaccine viruses for monkeys have continued; the results were reported at the Second International Conference on Live Poliovirus Vaccines held in Washington in June, 1960.

The control of the killed virus poliomyelitis vaccine continues to be an important part of the program. The decline in incidence of poliomyelitis in 1960 has been accompanied by a decrease in demand for the vaccine. With the stocks of vaccine remaining in the manufacturers' cold storage, production of the vaccine has been greatly curtailed. One manufacturer, Merck Sharp & Dohme, has developed a purified vaccine which was marketed for the first time during the summer.

During the year, the following tests were carried out on killed poliomyelitis vaccine:

Tissue culture safety tests	54
Monkey safety tests	32
Potency tests in monkeys	45
Potency tests in chicks	101

The Laboratory participated in a joint study of potency of poliomyelitis vaccine sponsored by the World Health Organization. The purpose of the study was to correlate the results of potency tests in monkeys and chicks and in tissue culture by the neutralizing antibody combining test. Among the vaccines included in the study was the current DBS reference vaccine.

The relative merits of potency tests in monkeys and chicks continue to be studied. It is planned that an evaluation of the chick test in relation to the monkey test will be possible when the results of the WHO study are made available.

Although the potency of poliomyelitis vaccine has continued to increase during the year, occasional problems with individual lots continue to appear. A proposal to raise the minimum acceptable level by 50 percent was made late this year in order to eliminate vaccines which show low potency.

During the year, the following tests were carried out on adenovirus vaccine:

Tissue culture safety tests	14
Monkey safety tests	8

A strengthened interest in adenovirus vaccine developed during the year. One new license was issued to Pitman-Moore Laboratories, bringing the total number of licensed manufacturers to three. Recently, other establishments have expressed an interest in this vaccine. An emerging problem concerns the continued potency and immunogenicity of the products available.

Comparison of the neurovirulence for monkeys of the strains of attenuated polioviruses after intramuscular inoculation has been made. It was found that the Lederle Type I strain has the ability to cause paralysis in monkeys after inoculation of at least $10^{7.7}$ PFU of virus. While the other strains all have the capacity to produce histologic lesions of poliomyelitis they do not cause paralysis. These results were also reported at the Second Conference on Live Poliovirus Vaccines, and have been stressed in the reports of the PHS Committee on Live Poliovirus Vaccines.

Studies on reversion of attenuated polioviruses to increased neurovirulence for monkeys after passage through the human alimentary tract indicate that this can occur. Furthermore, it has been found that passage of virus in either a liquid tissue culture system or in a plaque system causes changes in the virus. It was concluded that all studies characterizing the excreted virus must be done directly on fecal material.

By means of the immuno-inactivation method for detecting virus antibody in serum, it was possible to find extremely low levels of antibody to enteroviruses in the sera of persons with hypogammaglobulinemia.

Delayed hypersensitivity in guinea pigs has been established to vaccinia virus following inoculation of live or dead virus; the normal cycle of infection and recovery was observed despite the inhibition of antibody mechanisms by use of radiation and antimetabolites.

LABORATORY OF VIROLOGY AND RICKETTSIOLOGY

The Laboratory of Virology and Rickettsiology was established in the summer of 1960, bringing together a number of groups of DBS workers and one group formerly in NIAID.

The objectives of the laboratory are to improve the materials and procedures now available for immunizing man against a number of viral and rickettsial diseases, e.g., influenza and typhus, and to develop the necessary immunologic tools for use against certain of the diseases which are not as yet adequately controlled by immunoprophylaxis, e.g., measles. In order to attain such objectives, the efforts of the staff range widely from basic biological and virological research to classical immunological work on the preparation and assay of potent antigens and on their evaluation in man.

One of the most active fields of interest encompasses two new live attenuated viral vaccines—poliomyelitis vaccine and measles vaccine. The principal problem encountered with live attenuated polio vaccine—one which has engaged the attention of members of this Laboratory as well as others in DBS and in industry—centers around methods for producing the live vaccine free of extraneous agents, particularly the ubiquitous foamy virus and vacuolating virus which

contaminate many of the monkey kidney tissue cultures used for growing polio viruses. Part of the effort, which is in the hands of Dr. Harry Meyer and his group, is concerned with improving and developing serological methods for determining whether vacuolating virus infection is or is not present in monkeys prior to the time their kidneys are used for the preparation of tissue cultures.

Dr. Anthony Morris and his group, in collaboration with investigators of NIAID, have studied the pathogenicity of vacuolating virus in man. In brief, when given by the respiratory routes in appreciable amounts, vacuolating virus causes no obvious disease but elicits specific antibody production and can be recovered from the upper respiratory tract a week or so after inoculation. The work of others had already indicated that similar amounts of virus when fed by mouth failed to multiply in man.

Another approach made by Mrs. Hope Hopps has concentrated on the development of continuous cell lines from several simian hosts, with the objective of obtaining a tissue culture procedure for detecting and maintaining vacuolating virus unhampered by the presence of this agent as a contaminant in primary monkey cell cultures. This approach has not yet been successful, but the results have been sufficiently encouraging to continue the venture.

Many of the pressing problems associated with the development of measles vaccine are similar to those mentioned above which plague the live poliovirus vaccine program. One of the most important of these is the frequent infection of monkeys with measles virus by the time they reach the Laboratory. Although monkey cell cultures are not used for the growth of attenuated measles virus (chick embryo tissue cultures are employed), susceptible monkeys are essential for the experimental work on the immunogenicity and pathogenicity of attenuated strains and will be necessary in the future for certain types of assay procedures which will be applied to each commercial lot of vaccine.

Serological methods for selecting susceptible monkeys and procedures for bringing the nonimmune monkeys from their natural habitat to the investigator without intercurrent infection are being examined. During the year, a collaborative effort was initiated between this Laboratory and the Department of Pediatrics at Walter Reed Army Medical Center which should provide DBS with a clinical testing outlet for its investigations on live measles vaccine.

Dr. Morris's section has as its primary mission the development and improvement of methods for immunizing man against the rickettsial diseases, particularly typhus and Q fever. Although the subject is not new, and reasonably good vaccines are available, there are cogent reasons for continuing to press on.

In typhus immunization, there is great need for (1) stable preparations which can be stockpiled by highly developed countries for use in a limited emergency, and (2) a live attenuated vaccine which can be produced in the event of catastrophe, even in underdeveloped areas. Progress along both of these lines is satisfactory. A killed purified dried *Rickettsia prowazeki* preparation is being studied on a continuing but limited basis in the Laboratory and on a collaborative basis at the University of Maryland School of Medicine in volunteers. Methods for producing, assaying, and stabilizing the E strain of *R. prowazeki* (originally employed by Gallardo in the early 1940's and extensively studied subsequently by Fox) are being developed.

The disease, Q fever, is spreading rapidly in the bovine population of the United States, and increased numbers of human cases are to be anticipated. Although a potent experimental Q vaccine is available, there has been little general demand for its use outside the military services, and no licensed commercial Q vaccine is being produced. Efforts are being made to develop a set of recommendations which would provide an essential step in the eventual licensing and commercial production of Q vaccine. In addition, experiments are under way to study the feasibility of a combined Q and typhus vaccine.

Viral hepatitis is a matter of interest to several groups in DBS. The contamination of blood products by the virus associated with homologous serum jaundice constitutes one of the serious problems in medicine. Moreover, continuing efforts to transmit the agent of viral hepatitis in systems other than volunteers have given discouraging results in this Division and in many laboratories throughout the world. Despite these negative results, occasional flurries of enthusiasm have swept the field, lasting until it became ap-

parent that each newly discovered "hepatitis virus" was unrelated to the disease in man. Despite this background of frustration and occasional wishful thinking, the recent work of Dr. Joseph O'Malley on an agent which may have some relation to viral hepatitis in man again raises a hope. This virus, recovered in tissue culture from the NIH–6 icterogenic human plasma pool, which had been used some years ago in volunteer studies, is associated with what appears to be a specific serological response in human beings. Serum specimens from some of the former volunteers in viral hepatitis studies are without antibodies prior to infection, but certain antibodies against the new agent during convalescence. Because of the potential importance of the work, investigation of this new agent is being pursued.

At the present time, the only continuing responsibility for the routine assay of commercially produced vaccines in this laboratory is connected with the influenza and adenovirus vaccines. The currently available assay procedures in both instances leave much to be desired, but productive experimental investigations which might solve some of the problems have been limited. Efforts along these lines will be intensified during the coming year.

A number of the current investigations of the Laboratory bear little direct relationship to the primary mission. Nevertheless, some of these indirectly support the responsibility of the DBS, and others are interesting and productive. Among these are the studies of Dr. Lawrence Kilham on rat virus, and those of Dr. Bernice Eddy on polyoma virus and what appears to be a hitherto undescribed tumor in hamsters. The work of Dr. C. P. Li's group on the antibacterial and antiviral properties of abalone juice calls attention to an entirely new source of antibiotic substances.

LABORATORY OF BACTERIAL PRODUCTS

The work of the Laboratory of Bacterial Products is devoted to research and regulatory activities. During the year, several changes in organization and staff were made. The Section on Cancer Products was transferred to the Laboratory of Virology and Rickettsiology, and the name of the Section on Haemophilus Studies was changed to Section on Bacterial Vaccines. The Section on Bacterial Toxins continued to operate with a deficiency of staff members. Two new staff members—Dr. Harold Baer and Dr. Sotiros D. Chaparas—commenced work in the Section on Allergenic Products. Dr. Lajos Csizmas, visiting scientist for 3.75 years, resigned on October 1.

Studies relating to pertussis products, cholera vaccine, Schick test toxin, and PPLO contamination of tissue cultures were continued. New studies on typhoid vaccine, tuberculin, and blood group substances were initiated. Studies on immunological responses to multiple antigen vaccines and sterility test media for fungi were discontinued due to staff resignations; it is hoped that they can be activated in the near future.

Due to the day-to-day problems which have arisen in regard to toxicity and potency of pertussis vaccine, the Laboratory has continued to carry out the control testing of this product. Constant surveillance is maintained and investigations are carried out to find the cause of undue mouse toxicity and other undesirable changes. A special investigation was undertaken in connection with a complaint of instability of potency of pertussis vaccine in quadruple antigen vaccines.

The following tests relating to regulatory actions were performed:

Potency Tests	
Pertussis vaccine (lots for release inspection, complaint)	219
Anti-*haemophilus influenza* Type b *serum*	5
Haemophilus influenza typing serum	7
Freedom-from-toxicity tests	
Pertussis vaccine	244

In the cholera vaccine study, the main problem remaining before the use of the newly developed quantitative mouse protection test for potency evaluation can be recommended is the preparation of dried reference vaccines. Unlike the results obtained in the drying of other bacterial vaccines, a marked drop—as much as tenfold—was obtained with cholera vaccine. Study of this problem will be pursued. In coordinating this study with the SEATO Cholera Research Program, bacteriological equipment was selected for shipment from NIH. Dr. John Feeley of this Laboratory, organized the equipment in the

newly established Pakistan-SEATO Cholera Research Laboratory in Dacca; he also participated, while in Dacca, in the first Conference on Cholera under the auspices of the SEATO Program. A large number of cholera cultures from Thailand have been received, examined, dried, and distributed to participating cholera research scientists.

The diphtheria toxin to be designated as the U. S. reference for the Schick test toxin was dried commercially. Studies are now in process (1) to determine stability during the process of drying and during storage at high temperatures, and (2) to relate its activity to that of the international reference preparation. Based on the results of the total study, a proposed revision of the requirements for this product will be prepared.

In the studies on pleuropneumonia-like organisms in tissue cultures, it was demonstrated that fluorescent antibodies against a tissue culture PPLO strain reacted specifically with PPLO-contaminated tissue cultures but not with PPLO strains from other sources or with 24 different bacterial species.

Participation in the laboratory phase of a World Health Organization project on the evaluation of typhoid vaccine has been initiated. This laboratory is one of a number of international laboratories participating in the laboratory evaluations of two specially prepared lots for use in clinical field trials in three countries. It is hoped that out of the various tests employed, one will provide results which will show a correlation with protective activity in man and can be recommended eventually as the standard laboratory test for potency evaluation of typhoid vaccine.

In the newly activated Section on Allergenic Products, a study was initiated on an analysis of the antigen complexes of various mycobacteria in order to obtain basic information applicable to improvement in the specificity of tuberculins. In cooperation with LBBP, specific blood groups were found to be present in urines.

The laboratory is serving as a depot and distribution center of ragweed pollen for the Committee on Standardization of Allergens, which is functioning within the extramural activities of NIAID. This and other liaison activities provide helpful connections relating to the development of regulatory standards for allergenic products.

Regulatory activities, in addition to the control testing of pertussis vaccine and *Haemophilus influenza* products, have consisted of reviews of license applications for bacterial products in single and multiple antigen vaccines and of revisions in manufacturing procedures, participation in yearly inspection of establishments, and in the formulation of additional standards or revision of standards for bacterial and allergenic products for inclusion in U. S. PHS Regulations for Biological Products.

LABORATORY OF BLOOD AND BLOOD PRODUCTS

The research program of the Laboratory of Blood and Blood Products has as its goal the improvement of testing procedures used for the control of biologic products derived from blood. The investigations cover all phases of blood collection, processing, shipment, and storage. The research interest encompasses not only improvements for existing control procedures but also is directed toward new tests for control of new products.

Many of the products under development have immunological or biological activities for which there are no established test criteria. This uniqueness often requires that research on fundamental properties be carried out before definitive control tests can be devised. A good example is the investigation of components of the coagulation system that was started 2 years ago in anticipation of introduction of such products on the market. Information gained from these studies has been applied in standards for fibrinolysin (human).

Other research interests of importance to the control program include:

Studies of blood containers, particularly those of plastic, to determine possible effects of such materials on the blood. Investigations of several aspects of immunological reactions of blood group antigens and antibodies to enable more significant standards to be established.

Determination of changes that occur during processing and storage of blood and blood products. This is a continuing fundamental study

designed to yield information leading to better standards for purity, potency, and expiration date.

Studies of the use of platelets in leukemia. As part of this work, the effects on donors of repeated large platelet donations by plasmapheresis are being investigated to establish dating period and needs for specific control procedures.

An "extramural" program of the Laboratory derives principally from its obligations to other organizations. Many of these obligations stem from the legal requirements of the control functions of the Laboratory, others from a need to utilize all available sources of information in establishing standards for blood and blood products. As the number of establishments engaged in collecting and processing blood—now more than 400—increases, the need for a centralized organization to help coordinate such matters as technician training, reference serums, and consistent technical information becomes more apparent. Within the limitations of time and staff, the laboratory has assisted in each of these areas. Arrangements have been formalized for the expeditious handling of requests to store or to supply blood or rare and unusual types. Using a technique developed in this laboratory, such blood is stored at extremely low temperatures, allowing preservation for long periods of time. The laboratory is now engaged in the preliminary aspects of a study of the interchangeability of American equipment for taking, storing, and giving blood. This work is being done on a cooperative basis with the American Standards Association and the International Standards Organization.

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