NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT
INTRAMURAL RESEARCH PROGRAM

ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR
1985

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health

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The Intramural Research Program is broadly concerned with the biological and neurobiological, medical and behavioral aspects of normal and abnormal human development. In addition to four major clinical research and training programs in the areas of genetics and endocrinology, a diversity of developmental models are under study in twelve fundamental research Laboratories, drawing upon observations in bacteria, Drosophila, yeasts, viruses, molluscs, frogs, rodents, and subhuman primates. Disciplines employed in these studies include biochemistry, virology, molecular biology, immunology, pharmacology, genetics, cell and neuronal biology, biophysics, mathematical and theoretical biology, reproductive physiology, and comparative ethology.

In November 1984, a new Laboratory was formed within the Intramural Research Program, the Cell Biology and Metabolism Branch. Dr. Richard Klausner, previously associated with the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, was recruited to lead the new Laboratory, and he was joined by two senior colleagues, Drs. Gilbert Ashwell (NIADDK Institute Scholar) and Joseph Harford. The Cell Biology and Metabolism Branch focuses on the developmental aspects of various cellular organelles and receptors, and in particular, the structure and function of these important elements in cell biology. The Branch's laboratory activities take advantage of the extraordinary opportunities in cell biology occasioned by the advent of recombinant DNA and monoclonal antibody techniques. The Branch's clinical research activities reflect the Institute's interest in developmental aspects of cell physiology and metabolism, especially the metabolism of iron. Building 18 was extensively renovated to accommodate these new research interests, and a large addition to Building 18 was constructed as well.

The Laboratory of Comparative Ethology, established late in 1983, continues to undergo a major building program. An extensive outdoor facility for free-ranging primates, employed in observational studies on the genetics of behavioral development, is now in place, and construction of a three-story indoor facility, as well as breeding quarters and a newborn nursery, has begun. These facilities are located at the NIH's Animal Center in Poolesville, Maryland.

The Institute continues to acquire additional laboratory space. Construction is well underway for a three-floor addition to Building 6. This will provide twenty-five new laboratory modules, as well as an extensive animal facility, and will be ready for occupancy in 1986. This new space will accommodate the Laboratory of Developmental Pharmacology, several sections of the Laboratory of Developmental and Molecular Immunity, and the DNA/protein sequencing and synthesis activity of the Endocrinology and Reproduction Research Branch. During the past year also, the Human Genetics Branch, the Developmental Endocrinology Branch, and the Endocrinology and Reproduction Research Branch, moved to newly renovated laboratory and office space in the Clinical Center. The Laboratory of Theoretical and Physical Biology will move to expanded and renovated space

in the Clinical Center in the summer of 1986. Finally, plans are being made to add sixteen additional modules in Building 36 for our two neuroscience Laboratories.

In major personnel actions during the past year, Drs. James Russell, Greti Aguilera, Joseph Harford, and George Chrousos were granted tenure. Dr. Jacob Maizel transferred to the NCI to direct a new Branch concerned with computer applications in molecular biology, and Drs. Wilbert Nixon and Bela Gulyas vacated laboratory positions to take up new responsibilities in the extramural program.

The Institute's staff fellowships for physicians in adult, pediatric, and reproductive-endocrinology, as well as the clinical research fellowship in human genetics, continue to thrive, and in the past year, we have initiated a program which offers three years of full-time training in laboratory research for physicians whose previous laboratory experience has been minimal. These physicians are offered stipends through the new NIH Intramural National Research Scholarship Award (NRSA) mechanism.

The NICHD has developed still other mechanisms for the provision of post-doctoral stipends that do not utilize regular government positions, as a response to the decreasing number of such positions in virtually all Federal agencies. These new mechanisms include, in addition to the Intramural NRSA, a contractual program for the support of intramural training administered by the National Research Council of the National Science Foundation, primarily intended for Ph.D.s who are within five years of receipt of the doctoral degree. We have also been extremely successful in identifying new donors of stipends, including endowments by private industry and foundations. Contractual funds administered by the NIH's Fogarty International Center have been employed for the support of sabbatical visits by junior and senior scientists from abroad. Moreover, a number of post-doctoral fellows from abroad have been awarded stipends for training in our Laboratories under the terms of formal bilateral agreements generated between the NIH and many countries in Europe, Asia, and the Middle East, and several medical students supported by the new Howard Hughes Foundation program at the NIH are working in our Laboratories during an elective year. Finally, our Summer Student Program was very successful this year, with more than fifty undergraduate and graduate students working in our Laboratories; most were here on a voluntary basis.

Peer review of intramural research, conducted by the Institute's Board of Scientific Counselors and ad hoc experts, continues to receive great emphasis, with rigorous site visits to each Laboratory at three to four year intervals. During the past year, visits were made to the Laboratory of Developmental Pharmacology and the Laboratory of Comparative Ethology, with detailed critiques prepared as a consequence of these visits. The membership of the Board of Scientific Counselors reflects the increasing diversity of research interests within this Intramural Program. The current Board membership includes:

Aron Moscona, Ph.D., Louis Block Professor of Biological Sciences, University of Chicago (Chairman)
Roger Guillemin, M.D., Chairman, Laboratory for Neuroendocrinology, Salk Institute
John C. Marshall, M.D., Ph.D., Professor of Medicine, University of Michigan

Lewis P. Lipsitt, Ph.D., Professor of Psychology, Brown University
Allen H. Neims, M.D., Ph.D., Professor and Chairman, Department of Pharmacology and Therapeutics, University of Florida
Story C. Landis, Ph.D., Associate Professor of Neurobiology, Harvard Medical School
Harold Amos, Ph.D., Professor of Bacteriology and Immunology, Harvard Medical School
John Phillips, Jr., M.D., Professor of Human Genetics, Vanderbilt University School of Medicine.
Merry R. Sherman, Ph.D., Head, Biochemistry Laboratory, Sloak-Kettering Institute

Other developments in the past year include the strengthening of all aspects of the management of animals employed in our research, under the supervision of the Institute's full-time veterinarian, Dr. John Donovan. The Institute's Animal Research Committee, chaired by Dr. Charles Strott, works closely with Dr. Donovan in this regard.

We have continued to develop new computer-based administrative procedures in the Office of the Scientific Director so as to maximize the efficiency with which our resources are shepherded. These administrative approaches are ensuring the maximum yield with respect to scientific productivity while the current climate of constrained resources persists.

Seminars sponsored by the various Laboratories and Branches in this Program were numerous and well attended throughout the year, such that this Institute organized a relatively large fraction of the NIH's overall roster of intramural seminars and workshops. During the past year also, three major conferences with participants from throughout the world were hosted by Laboratories of the Intramural Research Program, including:

Cytochrome P-450 Genes and Their Regulation (Laboratory of Developmental Pharmacology)
Pineal and Retinal Relationships (Laboratory of Developmental Neurobiology)
Molecular and Developmental Neurobiology (Laboratory of Neurochemistry and Neuroimmunology and Laboratory of Developmental Neurobiology)

During the year, we were especially honored by the award to Dr. Igor Dawid (Chief, Laboratory of Molecular Genetics) of the Department of Health and Human Service's highest honor, the Distinguished Service Award, for his landmark contributions to developmental biology. Dr. John Robbins was awarded the Public Health Service Meritorious Service Medal for his work on the development of new bacterial vaccines, and Dr. Lynn Loriaux was the first recipient of the NIH's new Annual Award for "Clinical Teacher of the Year." Dr. Andreas Chrambach received the NIH Director's Award for his numerous innovations in molecular separation techniques, and Drs. Bruce Nisula and Gordon Cutler received the PHS Commendation Medal for their clinical research on thyroid and pituitary disorders. Additionally, many of the Institute's senior investigators held honorary lectureships and visiting professorships during the year; major prizes for their research accomplishment were awarded to a number of our scientists by various universities and societies.

Finally, during the coming year, we shall be sponsoring two distinguished senior scientists in the Fogarty Scholars-in-Residence Program, Professor Donald Brown of the Carnegie Institute (Baltimore) and Professor Itzhak Parnas of the Hebrew University (Jerusalem), who will work in our Laboratories during their sabbaticals.

In December of 1982, this Institute embarked upon an ambitious program designed to improve its intramural scientific productivity, even as the rate of growth in biomedical research support declined. As one reflection of our success in this regard during the past three years, NICHD intramural scientists this year published twice the number of peer-reviewed original scientific reports, appearing in journals of stature, than had been the case prior to the beginning of this new direction. Moreover, one out of every two of our post-doctoral scientists is now fully supported by a stipend awarded from a non-NIH source--reflecting, we believe, the current quality and productivity of this Intramural Program.

With regard to the major research interests and scientific results during the past year, particularly notable are the following:

Laboratory of Molecular Genetics--
Igor Dawid, Ph.D., Chief

Investigators in this Laboratory use the tools of molecular and cellular biology to answer questions about gene transmission and recombination, and the regulation of genetic functions during development. The range of model systems under investigation includes bacterial and animal viruses, transformed animal cells, yeasts, mouse and Xenopus embryos, and the fruit fly Drosophila melanogaster. Although this diversity of model systems is broad, the Laboratory relies on a commonality of basic interests, conceptual approaches and technologies. Recombinant DNA and gene transfer methods are emphasized. The development of novel vectors is being pursued for the introduction and expression of isolated genes in animal cells.

Dawid and a senior colleague, Tom Sargent, have continued to study gene expression during embryonic development of the frog Xenopus laevis, and in particular have focused on a group of genes that are activated for the first time during the transition from blastula to gastrula stages of Xenopus. Several of these genes, which have been named DG ("differentially expressed in gastrula"), have been analyzed by sequencing, localization of their products in the embryo, and determination of their developmental expression. Analyses of thirty DG RNAs during development has demonstrated that all are restricted in their expression to embryonic or tadpole stages, and that most DG RNAs persist in the embryo for just one to a few days. No DG gene studied to date is expressed in the adult; thus, the DG set of genes is distinctive in its developmental specificity. However, genes that are activated at the neurula stage, several hours subsequent to the activation of DG genes, are frequently active in the adult. Three of the most abundant DG RNAs encode cytokeratins, which are abundant structural components of epidermal and epithelial cells. The proteins encoded by these three cytokeratin genes are structurally very similar to the cytokeratins of mammals and birds. Dawid and his colleagues have localized the cytokeratin RNAs to the epidermis. Further, one of these genes has been isolated and sequenced, revealing an exon/intron arrangement very similar to that of a human

cytokeratin gene. Another DG gene (DG42), which is expressed for less than one day during the blastula to early neurula stages, encodes an RNA which has been localized to the endoderm, providing for the first time a very early endodermal marker. Current studies are directed toward the identification and isolation of DG42's predicted protein product.

In other experiments, using keratin gene expression as a marker for ectodermal differentiation, DG42 as the endodermal marker, and actin gene expression as a mesodermal marker, Dawid's group has sought to determine the factors and structural properties which influence differentiation of the three germ layers in Xenopus. By preventing cell-cell interactions mechanically, these investigators were able to show that the initial differentiation of the ectoderm and endoderm is independent of these cell interactions and must therefore depend on factors inherited in the egg. On the other hand, the establishment of the mesoderm does require cell-cell interactions, i.e., embryonic induction. In the coming year, keratin genes will be analyzed further to delineate the overall pattern of the expression of this important gene family during frog embryogenesis. Isolated genes will be injected into the embryo to reconstruct their normal regulation of expression and if this reconstruction is successful, the responsible DNA sequences will be determined as will the factors required for regulated expression. An attempt will be made to identify further DG gene products and to identify the spatial and temporal aspects of expression of these products. Using tissue-specific RNAs as molecular probes, work will continue on the analysis of embryonic induction. For the first time, considerable information is becoming available, through these studies, on the regulation of gene expression during early embryonic events which set the stage for the entire developmental series that leads from the newly fertilized egg to the adult. Xenopus laevis is a particularly attractive model for such studies because it is a vertebrate, its embryonic tissues are easily accessible, and a large amount of embryological and molecular information is already at hand.

A second group, directed by Dawid, employs the fruit fly, Drosophila melanogaster, which offers unique advantages among animals for studies on the mechanisms through which genes regulate, and are in turn regulated by, cell and tissue differentiation. This group has had a particular interest in homeotic genes, which are required for the normal determination of segment identity during fly development. Recent work has focused on the fs(1)h locus of Drosophila: Female flies mutant in this locus are either sterile or may produce offspring that show various malformations. Among the most interesting homeotic transformations are those seen in flies born of mothers mutant in fs(1)h and who themselves also carry mutations in chromosome loci such as trithorax and bithorax. The fs(1)h locus has now been cloned by chromosomal walking and the limits of the locus have been established. The RNA molecules (7.6 and 5.9 kb) transcribed from the fs(1)h locus persist for the first few hours of postfertilization development but rapidly decay thereafter. These recent observations have permitted Dawid and his colleagues to propose the following model of the maternal function of the fs(1)h gene: The fs(1)h product accumulates in the egg and is transmitted to the embryo where it is required for the proper expression of the embryo's trithorax gene. The product of this gene in turn is required for the normal expression of the bithorax complex. This model explains why combined hypodosage of the fs(1)h locus and the trithorax locus leads to homeotic transformations, whereas hypodosage of either of these loci alone does not result in such a phenotype. During the next year, the functions of the fs(1)h gene will be studied with the aid of the cloned probes now available and sequence information will be obtained in order

to predict the protein products of the locus. With sufficient sequence information from appropriate cDNA libraries, this group intends to synthesize peptides corresponding to the predicted protein, generate antibodies against these peptides, and then to employ the antibodies in identifying and localizing the fs(1)h product within the organism and within the cell.

In the course of analysis of the fs(1)h locus, a new family of repeated gene sequences has been found in Drosophila. These short sequences (pen) are interspersed in the genome and to some extent, are expressed as parts of the fs(1)h transcript. Studies in the next year will involve the isolation and analysis of other genes which contain copies of the pen repeat; these studies should allow a test of the possibility that the pen repeat is associated with a specific functional class of genes as with the homeobox repeat model. The pen transcripts appear to be developmentally regulated. Finally, Dawid's group has studied the expression of interrupted rRNA genes in Drosophila. These genes do not contribute to the production of rRNA, but some transcripts are nevertheless formed under the control of insertion sequences. When Drosophila cells were cultured in the presence of chloroquine or ethidium bromide, drugs which intercalate into DNA, it was found that the level of transcription from interrupted rRNA genes increased at least 10-fold, suggesting that the relative inactivity of interrupted rDNA is due to the packaging of these interrupted genes in chromatin and that a change in torsional stress of the DNA brought about by intercalating drugs relieves transcriptional repression.

The group headed by Hiroto Okayama has been interested in the identification and isolation of new oncogenes beyond the approximately 30 presently known. Oncogenes comprise a limited set of cellular genes, which when expressed, normally appear to regulate the cell cycle and cell growth--probably via the elaboration of growth factors and/or growth factor receptors and other elements of the cell's "signal transduction" pathway. When abnormally expressed, the oncogenes appear to induce cellular transformation to a malignant phenotype. Undoubtedly, oncogenesis is extremely complex and may require the interaction of many oncogenes beyond those presently identified with the available test systems. Appropriate test systems, which usually employ mutant cells, have proven extremely difficult to generate, and therefore Okayama has attempted to develop a system for the detection of unknown DNAs (that may or may not contain hitherto unrecognized oncogenes) in which expression is guaranteed in virtually any desired cell independent of that cell's normal spectrum of gene expression. With this new expression system it should be possible for Okayama to clone molecularly and to characterize any cellular gene, the activation of which leads to induction of full or partial transformed phenotypes. In the past year, Okayama has modified a cDNA expression system that he had previously developed together with Paul Berg. The new approach comprises the following steps: A cDNA clone expression library is constructed using mRNA from SV40-transformed human fibroblasts; the library is transduced into NIH 3T3 mouse cells; colonies that have lost the capacity for contact inhibition are identified; and integrated cDNA is recovered from such colonies for amplification in E. coli and subsequent characterization. Using this approach, DNA complementary to the RNA from the SV40-transformed human cells was cloned in a vector that supplies the necessary controls that eventuate in the expression of the information content of these cDNAs when they enter a mammalian cell. The vector also contains a separate selectable marker (the neo gene) which allows preliminary selection of transfected cells. Okayama has now obtained 62 NIH 3T3 colonies that do not exhibit the contact inhibition of growth characteristic of the original 3T3 cells. DNA from these cells has transmitted this

growth property to fresh cells. While a few of these transformed cells carried the SV40 T-antigen, the majority did not and may thus be carrying new human oncogenes. At least one cDNA clone so far recovered that repeatedly induces the same transformed phenotype in NIH 3T3 cells has no detectable homology to the known oncogenes. Further characterization and DNA sequencing of this clone is in progress. In related experiments, Okayama is developing a host-vector system for more efficient gene transfer into mammalian cells. In E. coli, a λ receptor protein (Lam B) plays a key role in infection by bacteriophage λ by providing a binding site on the outer bacterial membrane and subsequently inducing conformational changes in the phage tail protein which lead to injection of DNA into the cells. Using a pcD expression vector, the Lam B gene was inserted into transfected mouse cells in order to establish a stable cell line expressing the Lam B protein. When these cells were transfected with a λ phage expression vector containing the transforming DNA described above, the frequency of transformation was 10 to 20-fold higher than that obtained previously, and should permit more efficient transduction of cDNA libraries into NIH 3T3 cells. Efforts will now continue to further increase the efficiency of λ phage and its receptor-mediated gene transfer into mammalian cells.

The group headed by Alan Hinnebusch is concerned with the regulatory pathway in yeast known as "general amino acid control." Yeast is a eukaryotic organism which offers a level of genetic and molecular analysis that is difficult to achieve in higher eukaryotes. A multitude of yeast structural and regulatory mutations have been identified and located on the yeast genetic map, and the cognate wild-type DNA sequences encoding many of these genes have been isolated. Mutations can be introduced into cloned yeast DNA sequences in vitro and reinserted back into the yeast genome in place of the wild-type sequences. In this way, novel mutations have been constructed and analyzed in vivo in their correct chromosomal locations. It is becoming clear that yeast cells share with cells of higher eukaryotes a variety of important structures and processes. As a result, it is possible to study these features of eukaryotic cell biology in yeast using a powerful arsenal of molecular and genetic techniques. Moreover, results obtained in yeast should provide useful paradigms to guide studies of analogous processes in higher cells. As an example, the mechanism of coordinate regulation of the transcription of unlinked genes is central to the control of cellular differentiation in higher eukaryotes. The "general amino acid control" found in yeast is a useful model of this type of gene regulation. In this system, the rate of transcription of at least 30 unlinked genes is coupled to amino acid availability. Hinnebusch's group has now established a hierarchy of control genes by genetic and molecular studies, suggesting that three genes, GCN1, GCN2, and GCN3, encode negative regulators for GCD1 which is in turn a negative regulator of GCN4. The GCN4 product is a positive regulator of the genes which are ultimately affected, such as the HIS4 gene which regulates the synthesis of histidine. The regulation of expression of GCN4 has now been shown to occur at the translational level. This was accomplished by generating fusion genes in which the 5' untranslated part of the coding region of the GCN4 gene has been fused to β-galactosidase to provide an easily assayed product. Culture under conditions of amino acid starvation and in various genetic backgrounds has demonstrated that the primary level of regulation of the fusion product is translational. This regulation is mediated by four short open-reading-frames in the 5' untranslated region of the GCN4 mRNA; it does not depend on the GCN4 promoter, but does depend on wild-type GCN2, GCN3, and GCD1 products. These conclusions are based on the fact that a GCN4 gene fusion from which the four short open-reading-frames have been removed is constitutively derepressed, but a construct which contains the normal

5' untranslated region and a foreign promoter is regulated normally. Of these four short open-reading-frames, the largest effect is exerted by the first (5'-proximal) reading frame.

The effects of the deletion in the GCN4 leader suggest certain possibilities for the mechanism of translational regulation of GCN4. Hinnebusch proposes a model consistent with the "scanning" mechanism of translation initiation in eukaryotes in which ribosomes must first associate with the 5' end of a mRNA and then translocate downstream until an AUG codon suitable for initiation is encountered. Insertion of a short open-reading-frame into the 5' prime leader of the mRNA would inhibit the efficiency of reinitiation. If reinitiation is inefficient, the fraction of ribosomes that remain attached to GCN4 mRNA after translation of all four upstream open-reading-frames will be very small. If so, the GCN1, GCN2, and GCN3 factors might act in response to amino acid starvation to increase the probability of reinitiation. The GCD1 product may promote the dissociation of ribosomes from the mRNA at the upstream termination codons by antagonizing this function; the GCN products could increase the fraction of ribosomes that will remain attached to the mRNA and continue scanning downstream to reinitiate. Hinnebusch is also pursuing the identification of GCN gene products. For example, if GCN4 is a transcriptional activator of amino acid biosynthetic genes, it should be located in the nucleus. If the other transacting factors in the general control system work as translational effectors of GCN4, they should be located in the cytoplasm--probably in association with ribosomes. These possibilities will be tested by immunofluoresence after preparing antisera against the products of GCN and GCD genes. Moreover, the antisera will be used for purification of the GCN4 protein product as well as the positive regulator encoded by GCN3. In any event, further analysis of the molecular details of this translational control system in yeast should provide important insights into the mechanism of protein synthesis and the ways in which it can be regulated in higher eukaryotic cells.

In other experiments, Hinnebusch's group has cloned two different yeast genes on the basis of their sequence homology to the ras genes found in certain oncogenic animal retroviruses. Molecular and genetic analysis of these yeast genes has shown that the encoded proteins carry out an essential function in yeast related to the activation of cAMP-dependent protein kinases. The activation of these kinases is believed to trigger initiation of the cell division cycle. A homologous ras protein in human cells can complement a yeast ras mutant, suggesting that the ras proteins may carry out similar functions in all eukaryotic cells. A mutation in only one of the two yeast ras genes is not lethal, suggesting that the two ras proteins can substitute for one another. However, the two ras genes are differentially regulated by a carbon source such that a ras 2 mutation changes the cell phenotype, whereas the ras 1 mutation does not. Further results support the notion that the ras 2 phenotype arises from insufficient ras gene expression and not from the absence of a function restricted to ras 2. Other correlations suggest the possibility that the level of ras function is an important determinant of the rate of cell division, and that modulation of the division rate is achieved, at least in part, by modulation of the level of ras 1 transcription. Such a role for ras function in yeast is entirely consistent with the role which ras proteins appear to play in the control of growth of higher eukaryotic cells. Elucidation of the molecular details of the important function of ras genes in regulating cell division in yeasts should provide great insight into the effects of mutant ras proteins on abnormal cell proliferation in animal cells.

The section headed by **Michael Cashel** is also attempting to understand how diverse cellular activities are coordinately regulated. Here the model is the bacterium, wherein large sets of genes are turned on or off in response to nutritional impoverishment and to other environmental signals. All cells probably possess such "global control" systems that govern large domains of gene expression in addition to the regulatory circuits known to apply to specific genes, pathways, and operons. Cashel's major interest is in the domain of gene expression regulated by the ppGpp (guanosine 3', 5' bis-pyrophosphate) global control system, factors affecting ppGpp regulation, and other global control systems which might interlock with the ppGpp system. The role of ppGpp in the regulation of ribosomal RNA synthesis has long been a focus of this laboratory's research; this work has now been expanded to include studies on the metabolism of ppGpp and on ppGpp effects on histidine operon expression. The domain of gene expression regulated by the ppGpp global control system includes genes encoding the enzymes responsible for synthesis of RNAs and proteins; these are generally inhibited by ppGpp. The synthesis apparatus is strictly regulated as a function of growth rate, with the primary site of regulation occurring at the level of rRNA transcription. However, ppGpp also stimulates certain genes and of such positively regulated operons, the histidine operon has a particularly strong dependence on ppGpp.

With respect to the ribosomal RNA operon, Cashel's group has focused on anti-termination, a mechanism which permits ribosomal transcripts to read through normally efficient terminator signals. This feature is shared with bacteriophage λ transcripts, and recent experiments have shown that the E. coli nusA, nusB, and nusE, genes known to be involved in λ anti-termination, are also involved in the case of ribosomal transcripts. Anti-terminated transcripts are terminated by super-terminators. A search for other genes required for super-termination was carried out and yielded mutations in three locations. One of these has been identified as the rpoB subunit gene of RNA polymerase. A series of overlapping deletions in the super-terminators has now been prepared which will be used in dissecting the functional determinants of this region.

A second series of experiments carried out by Cashel's group was directed towards an analysis of the spoT gene in E. coli. This gene is responsible for the degradation of ppGpp. Different mutants in spoT have been obtained and combined with various relA mutants. In this way, different levels of intracellular ppGpp have been generated in cells cultured in the same medium. This experiment allowed a test of the effect of changing ppGpp levels on growth rate without changing nutritional conditions. An inverse correlation between ppGpp level and growth rate was found. The spoT gene has been cloned and sequenced, which will allow the generation of null mutations and a further study of its properties.

The regulation of histidine biosynthesis in Salmonella is affected by ppGpp. Two histidine regulatory mutations have been studied and shown to be alleles of gyrA and gyrB, the genes encoding the two subunits of DNA gyrase. This observation suggests that superhelix density affects transcription of the histidine operon.

The group directed by **Robert Crouch** is concerned with RNA processing and enzymes involved in DNA replication. Since all growing cells actively synthesize DNA and ribosomal RNA, these studies are quite basic to our understanding of cell growth and division. Crouch's work is concerned with two types of RNA

processing, generation of RNA primers for DNA replication, and ribosomal RNA processing in higher eukaryotes. There is a particular interest in RNaseH, an enzyme that degrates the RNA strand of RNA/DNA hybrids. In E. coli, rnh mutants which also carry a recB temperature sensitive mutation are temperature sensitive for growth. Using this double mutant in a complementation test, it has been possible to isolate several mutant RNaseH genes from E. coli, the RNaseH from Salmonella, and a complementing DNA sequence from yeast which probably is the yeast RNAseH gene. These clones will permit molecular and genetic studies on important aspects of the enzymology of DNA replication.

Crouch is also studying the mechanism of chicken rRNA processing. This year, he has shown that U3 RNA (a small nuclear RNA) can form stable complexes with rRNA precursors, supporting the notion that U3 RNA is involved in rRNA processing. Further studies will be concerned with the exact nature of the interaction between these two RNA species.

The section directed by **Heiner Westphal** is concerned with eukaryotic gene regulation and control, and has a particular interest in the results obtained when cloned genes or specific gene products are microinjected into test cells. One system under study in this laboratory employs human adenovirus (a cause of human respiratory disease as well as a tumor virus in rodents). In collaboration with Martin Rosenberg, Westphal has studied the functions of the adenovirus E1A gene product. The E1A gene is of special importance because its product acts as a transcriptional modulator and is also directly involved in malignant transformation. Wild-type and modified E1A proteins were produced in E. coli and microinjected into cultured cells. The major properties assayed were complementation of growth of a mutant virus lacking the E1A gene, and translocation of the E1A product to the nucleus. Deletion analysis has demonstrated that the translocation function is encoded by a region close to the C terminus of the E1A protein, whereas complementation of the growth of mutant virus required a contiguous region in the interior of the protein. Thus, information for nuclear localization and for viral gene activation is encoded by distinct domains of the adenovirus E1A gene.

The second research direction in Westphal's group involves the generation of transgenic mice. In these experiments, cloned gene constructs of interest are microinjected into fertilized mouse eggs and the genetically modified embryos are then carried to term by surrogate mothers. The resulting transgenic progeny are examined for marker gene expression. This approach allows the study of the function of genes and gene regions in the normal life cycle, and should provide us with an extremely detailed understanding of the mechanisms of developmental regulation of gene activity. Moreover, these studies are a prelude to gene therapy in humans. Westphal's group has now succeeded in creating and analyzing three new transgenic mouse strains carrying different chimeric gene constructs; analysis is directed toward spatial and temporal control of the expression of the inserted genes. Each of these gene constructs contains the gene for bacterial chloramphenicol acetyl transferase (CAT) under the control of a promoter/enhancer region derived from either the mouse α A-crystallin or the α 2 (I)-collagen gene, or the promoter/enhancer region (LTR) derived from Rous sarcoma virus (RSV). Constructs containing these relatively short segments from the 5' region of the crystallin and collagen genes were expressed in precisely the spatially and developmentally correct fashion; the crystallin construct was expressed only in the fiber and epithelial cells of the lens, while the collagen construct was expressed most intensely in the tail. Constructs carrying the RSV-LTR were expressed in muscle and connective

tissues, reflecting the known tissue preference (and disease specificity) of this avian retrovirus. Finally, Westphal's group has generated one RSV-CAT transgenic strain which is characterized by a dominant trait of embryonic lethality, with the mutation apparently induced by the integration of the foreign DNA. This strain demonstrates a chromosomal translocation which is probably the result of the insertion of the foreign gene. During the coming year, the sequences flanking the inserted gene will be characterized in an attempt to identify the cause of embryonic lethality. Other gene constructs under study in the transgenic model include various oncogenes/promoter fusions.

Westphal's group is also exploring the possibility of generating loss-of-function mutations in predetermined genes of the mouse using "antisense" RNA as a negative regulator. In E. coli, antisense RNA is able to interfere with the expression of a specific mRNA, and this negative control is a physiological regulatory mechanism. Experimentally, expression of specific genes in frog oocytes or in fruit fly embryos has been neutralized by the injection of antisense RNA. In the coming year, Westphal's group will attempt to extend this principal to transgenic mice. In transgenic cells and embryos containing the CAT gene, an antisense CAT DNA construct will be introduced. Neutralization of CAT expression in vivo by an antisense DNA construct would open the way for generating loss-of-function mutations. For example, the expression of any proto-oncogene whose physiological function is unknown could be neutralized by an antisense construct directed by a suitable promoter to a specific target tissue in a transgenic mouse. One could also use this system to create mouse analogs of important human genetic diseases caused by loss-of-function mutations. To produce a favorable ratio of antisense:sense RNA in a target tissue, Westphal and his colleagues will introduce the bacteriophage T7 polymerase gene which should be extremely efficient in inducing the synthesis of large amounts of antisense RNA. Finally, this group also intends to study gene constructs containing homeobox sequences homologous to those of the homeotic genes of the fruit fly, and in particular, they will attempt to identify those tissues of the developing mouse embryo which express homeotic RNA--presumably involved in encoding the body plan.

Judith Levin's group is concerned with the mechanism of replication of enveloped RNA viruses, specifically murine leukemia viruses (MuLV). Studies on the RNA tumor viruses are of particular interest since these viruses establish a chronic infection in the host cell by integrating viral genetic information into the host chromosome, often disrupting normal gene function. A detailed understanding of the steps involved in viral replication may permit the design of agents which would limit the oncogenic potential of these viruses. Moreover, evidence is accumulating that a number of eukaryotic genetic elements have been created by transfer of information from RNA to DNA during the development of the eukaryotic genome. Current interest is focused on the process of reverse transcription in an effort to correlate genetic structure with enzymatic function. Portions of the MuLV pol gene have been cloned in E. coli. By the use of suitable expression vectors, fusion proteins between parts of the pol gene and β-galactosidase have been produced, and injected into rabbits to elicit antibody production. These sera react with reverse transcriptase and with the pol gene-encoded endonuclease derived from virus-infected cells. In other studies, revertants of the frameshift pol mutant clone 23 have been isolated, and efforts are underway to make molecular clones of these revertants to determine the mechanism by which the mutation is repaired. Experiments involving in vitro mutagenesis of selected regions of the MuLV genome have also been initiated.

Robert Weisberg's section is concerned with the mechanisms of genetic recombination, especially as exemplified by the insertion of the bacteriophage λ genome into the E. coli chromosome. These studies are very significant because the insertion and excision of viral and other DNAs is known to change the physiology of the host organism. Moreover, viruses provide an important mechanism of genetic transfer and exchange in the microbial world, and recombinational mechanisms are also at play in the symbiotic relationship demonstrable between certain eukaryotic viruses and their hosts.

Recombination between bacteriophage λ and its host E. coli (site-specific recombination) is effected by a pair of reciprocal strand exchanges that occur within special sequences called attachment sites. Weisberg has previously shown that base substitution mutations in the central segment of an attachment site interfere with recombination by preventing homologous pairing between the mutated site and its wild-type partner. This group's recent results suggest that the same mutation also prevents conversion of a Holliday structure--an X-shaped DNA molecule that is a postulated intermediate in recombination--to recombinant products. It therefore appears possible that a direct interaction between homologous regions is also necessary for this step in the reaction. Weisberg and his colleagues had previously shown that endonuclease I of bacteriophage T7 cleaves Holliday structures. They have now extended their original observation, made with a short cruciform DNA substrate, to several other substrates, including Holliday structures formed from phage λ attachment sites. They have made the surprising observation that introduction of a base substitution mutation into the central region of the λ Holliday structure affects the symmetry of cleavage: Instead of obtaining all four products in equal yield, only two products are obtained. It appears likely that the effect of the mutation is to limit the extent of branch migration of the Holliday structure.

Weisberg and his group have also discovered that HK022, a temperate coliphage related to λ, produces a protein that causes RNA polymerase to terminate transcription when it encounters specific sequences in the early region of the phage λ chromosome. Termination occurs at or near sequences called nut sites, and requires host-encoded proteins called nus factors. Nut sites and nus factors are also required for a contrasting process: Antitermination of transcription by the λ N protein. It is of considerable interest that termination and antitermination of transcription appear to have mechanistic steps in common.

Laboratory of Developmental Pharmacology--
Daniel W. Nebert, M.D., Chief

This Laboratory studies genetic mechanisms of drug metabolism, with particular reference to the induction of drug metabolizing-enzymes by drugs as well as environmental chemicals. There is particular interest in relationships between the genetics of these enzyme systems and fundamental biological events such as teratogenesis, toxicity, carcinogenesis and mutagenesis. Recent studies in this Laboratory have concentrated on attempts to understand drug-induced gene expression at the molecular level. Other projects employ the classical methods

of clinical pharmacology and pharmacogenetics. One long-range goal is to design molecular biology-based assays to predict the individual genetic risk in humans of drug-induced birth defects and chemically induced malignancies.

Daniel Nebert's section is interested in the structure and expression of genes encoding "Phase I" drug-metabolizing enzymes (e.g., aryl hydrocarbon hydroxylase), which generally are cytochrome P450 proteins. The P450 gene superfamily is composed of at least five gene families, and a protein from any of these five families has diverged more than 60% from a protein in any of the other four families. These families include genes which are: (1) 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; "dioxin")-inducible; (2) phenobarbital-inducible; (3) steroid-inducible; (4) gene(s)that encode enzymes responsible for cholesterol side-chain cleavage; and (5) genes responsible for steroid C21-hydroxylase polymorphism. Nebert's group has studied the TCDD-inducible P450 gene family in greatest detail, partly because of its relationship to aryl hydrocarbon-induced carcinogenesis, and partly because the inducer-receptor complex controlling the two genes in this family has been well characterized. During the past year, the P_1450 and P_3450 genes of the mouse were isolated, sequenced and mapped. The two genes appear to reside adjacent to one another in the middle portion of mouse chromosome 9 and to be controlled by the same cytosolic receptor, yet exhibit striking differences in developmental and tissue specificity, differential sensitivity to inducer concentrations, and transcriptional versus post-transcriptional regulation. Cotransfection of pSV2-neo plus pSV0-cat-derived constructs containing upstream P_1450 sequences has led to the isolation of the promoter region, a negative control element (which interacts with a negatively autoregulated loop), a positive control element (presumably the inducer-receptor-associating region), and an upstream activation element (most likely an endogenous enhancer).

During the past year also, the P450Coh protein, responsible for an inbred mouse polymorphism involving coumarin metabolism, was purified and a specific antibody was developed. This antibody will be used to study the mouse phenobarbital-inducible P450 gene family. The P450PCN protein, inducible by steroids such as pregnenolone-16 α - carbonitrile, has been purified and a specific antibody was developed. A full-length cDNA clone has been isolated and sequenced. The protein sequences, deduced from the nucleotide sequences, allow Nebert and his colleagues to conclude that the TCDD-inducible, phenobarbital-inducible, and steroid-inducible P450 gene families have each diverged from a common ancestral gene more than 200 million years ago, and that the homologous P_1450 and P_3450 genes separated from each other at least 65 million years ago. The human P_1450 gene and its flanking regions was also been sequenced and mapped. Nebert's group now hopes to develop an assay, based on recombinant DNA technology, to assess the human aryl hydrocarbon hydroxylase phenotype. Such an assay may predict which individuals are at increased risk for certain types of environmentally-caused birth defects, cancers, and toxicity.

Howard Eisen's section compares the mechanism of action of the glucocorticoid receptor and the Ah (aryl hydrocarbon) receptor. Emphasis is placed on purification of the Ah receptor, development of anti-receptor antibodies, and use of somatic cell genetics to isolate variants defective in the induction of cytochrome P_1450. It is hoped that these studies will lead directly to the use of recombinant DNA methods to clone the gene(s) for the Ah receptor. During the past year, significant progress has been made in purification of the Ah receptor with the use of high performance liquid chromatography and affinity

chromatography (polycyclic aromatic dyes linked covalently to agarose). Eisen's group has continued to study mutant clones of mouse hepatoma cells (Hepa-1) which do not express the Ah receptor ("r^-" phenotype); these clones and revertant clones are being used for "rescue" of the Ah receptor gene by DNA-transfection. Eisen's group has identified a human cell line (hepatoblastoma, $HepG_2$) that contains an Ah receptor with high affinity for TCDD; aryl hydrocarbon hydroxylase activity and human P_1450 mRNA are highly induced in this cell line by TCDD. This line should provide a good model for studying the relationship between the Ah receptor and the induction of human P_1450 mRNA. In other studies, antibodies produced in Eisen's laboratory to the glucocorticoid receptor have been used to investigate the subcellular localization of glucocorticoid receptors in intact cells, and to study glucocorticoid sites of action in the mammalian brain.

The Section on Drug Biotransformation, under the direction of **Ida Owens**, studies the regulation of UDP glucuronosyltransferase(s), one of the major classes of "Phase II" drug-metabolizing enzymes. These transferases catalyze the conjugation of many potentially toxic exogenous, as well as endogenous (e.g., bilirubin), compounds to glucuronic acid. Many highly fat-soluble substrates, converted to oxygenated products by the phase I cytochrome P450-dependent monooxygenases, are then detoxified by transferase through conjugation and, thus, transformation to highly water-soluble and excretable metabolites. Owens' group is particularly interested in understanding the genetic linkage in the regulation of certain monooxygenases and certain transferases by the Ah receptor in mice. This section is also interested in induction by phenobarbital-like compounds. The regulation of the transferase enzymes is being studied by means of DNA, RNA and protein chemistry. Five mouse transferase clones have been isolated and shown to be 85% similar by nucleotide sequence analysis and cross-hybridization studies. These clones represent members of a subfamily, with their unique sequences contained in the 3' region of the cDNAs. $pUDPGT_m$-1 (1800-bp insert) and $pUDPGT_m$-2 (1600-bp insert) differ by one nucleotide (converting an isoleucine to methionine). A third clone, $pUDPGT_m$-3, contains an unusually long insert of 4300 bp compared to other transferase cDNAs which encode mRNAs ranging from 1900 to 3000 nucleotides. The detection of 35 genomic clones (inserted into λEMBL-3) with sequence homology to $pUDPGT_m$-1 suggests that multiple and closely related genes exist and that the gene for $pUDPGT_m$-1 is most likely duplicated. At least two of these clones encode proteins with 50-kDa molecular weights, have one or two potential glycosylation sites, and contain hydrophobic transmembrane amino-acid sequences near the carboxy terminus.

In order to study aromatic hydrocarbon-inducible transferase forms, a cDNA library was recently constructed in λgt11 using 3-methylcholanthrene-induced mRNA. This library is being screened with previously developed mouse transferase antibody. Furthermore, human mRNA has been isolated and translated *in vitro* to produce a 48-kDa protein immunoprecipitable by mouse transferase antibody. A human cDNA library has been constructed and successfully probed with the insert from $pUDPGT_m$-1 to isolate and partially purify two human transferase cDNA clones. A human transferase form that is being purified appears to have similar properties to a mouse 3-methylcholanthrene-inducible form. Transferase studies at the molecular level will enable Owens and her colleagues to determine sites of regulation, the heterogeneity of the system, and substrate specificity of the different transferase forms.

Peter Mackenzie is studying the regulation and expression of several subfamilies of the rat UDP glucuronosyltransferase gene family. Three cDNA clones encoding different forms have been isolated and sequenced. The mRNAs complementary to two of these clones ($pUDPGT_r$-1 and $pUDPGT_r$-3) are 85% similar by sequence analysis and are not elevated by 3-methylcholanthrene or phenobarbital treatment. They are transcribed from genes belonging to the same subfamily. The mRNA complementary to the third clone, $pUDPGT_r$-2, is only 65% similar in sequence to the other cDNAs. It is elevated 5-fold by phenobarbital and is transcribed from a gene belonging to a second subfamily. Sequence studies have also shown that the transferase forms of both subfamilies encoded by the three cDNAs contain signal peptide and membrane anchoring regions, and potential asparagine-linked glycosylation sites. In vitro translation studies indicate that the signal sequence is most likely cleaved during insertion into the endoplasmic reticulum. Experiments are in progress to express those transferase cDNAs which contain complete coding regions in transferase-deficient cells, in order to characterize each form by its catalytic activity and substrate specificity. The regulation of each member of these two subfamilies as a function of age, tissue distribution and administration of prototypic inducers will be investigated with the use of both cDNA and genomic clones. Studies are also progressing on the human transferase gene family, associated with a number of serious heritable disorders of liver function as well as neonatal jaundice.

Laboratory of Neurochemistry and Neuroimmunology--
Harold Gainer, Ph.D., Chief

This Laboratory utilizes the tools of molecular biology and immunology in a cell biological context to study the biosynthesis, packaging, transport, secretion, and functions of biologically active neural peptides and proteins. Various model neuronal systems in organisms ranging from molluscs (squid) to vertebrates (e.g., frog, chick, and rat) are used to examine the development and function of defined neural pathways in vivo. Organotypic cultures of these systems are also studied. Techniques which are used include peptide chemistry, monoclonal antibodies and immunochemistry, light and electron microscopy, immunohistochemistry and cytochemistry, subcellular fractionation, and tissue culture.

This Laboratory is concerned with the development, functional organization, and interactions between three major integrative systems in the body--the central nervous system, endocrine system, and the immunological system. In particular, the Laboratory is studying various secretory peptides, intracellular membrane systems, and cytoskeletal proteins which are found in these organ systems and which are essential to their function.

The section directed by **Harold Gainer** has focused on the large number of neural peptides which have been identified as chemical transmitters of information in the nervous system. These known peptides (and presumably those still to be discovered) act as conventional neurotransmitters in a synaptic, paracrine, or autocrine fashion, as well as in neuroendocrine systems. In addition to the well-known hypophysiotrophic regulatory peptide hormones (e.g., CRF, LHRH, and TRH), there are neuropeptides involved in a wide variety of other CNS functions (e.g., pain, blood pressure control, and possibly memory). This section's goal is to study the cell biology of peptidergic neurons in the context of their

regulatory functions in the nervous system. In particular, Gainer and his colleagues study the expression of neuropeptides during CNS development and their impact on the development of organismic functions.

These studies are mainly concerned with two specific peptidergic systems in the hypothalamus, the oxytocinergic and vasopressinergic neuronal systems, and the LHRH neuronal system. The hypothalamo-neurohypophysial system is studied because the neurons that constitute it (i.e., the oxytocin and vasopressin magnocellular neurons) are prototypic of peptidergic neurons in the central nervous system. These neurons populate two defined topographic sites in the brain (the paraventricular and supraoptic nuclei), and have a specific axonal pathway and termination site, all of which are accessible to experimental manipulation in vivo by stereotaxic, morphological, and biochemical-pharmacological techniques. Access to all three of the critical neuronal structures, i.e., the cell body, the axon, and the terminal, in the hypothalamus, median eminence, and posterior pituitary, respectively, permits a cell biological analysis of this system. The cell bodies in the hypothalamus are responsible for the biosynthesis of the vasopressin (AVP) and oxytocin (OT) prohormones. This Laboratory first identified the prohormones, and suggested a molecular structure for the prohormones based on peptide mapping analysis of these molecules synthesized in vivo. Since that time the complete amino acid sequences and the genes for these peptide hormones have been elucidated. In addition, the magnocellular vasopressin neurons synthesize another neuropeptide, dynorphin, whereas the oxytocin neurons also synthesize enkephalin. Some vasopressin neurons in the paraventricular nucleus also synthesize CRF (corticotropin releasing factor). The phenomenon of neuropeptide co-existence in these neurons is now well documented, and this lab is involved in elucidating the biological significance of, and mechanisms involved in generating, this state of coexistence. The LHRH (leutinizing hormone-releasing hormone) neurons found in the hypothalamus project principally to the median eminence. The secretion of LHRH into the portal blood system regulates the development and function of the reproductive system. In addition to being the subjects of cell biological studies, the embryonic development of these two neuronal systems is currently also under intensive study. This Laboratory has also recently introduced the developing Xenopus laevis embryo as a model system of neuronal development. Two projects are currently underway in this area: 1) Investigation of the cell lineage and fates of distinct peptidergic neurons in the Xenopus hypothalamus (e.g., vasotocin, mesotocin, and LHRH neurons), and 2) the analysis of certain cytoskeletal proteins (e.g., neurofilaments and microtubule-associated proteins) and their role in generating neuronal morphology and function in the developing brain.

Much of the past year's activity has been devoted to laying the groundwork for these developmental studies. This includes the development of relevant monoclonal and polyclonal antibody reagents, establishing a sophisticated immunochemical and immunocytochemical (at the electron microscope level) technology, and generating a data-base for the biological model systems. These scientists have succeeded thus far in generating 7 monoclonal antibodies against neurophysins in the AVP and OT cells, 18 polyclonal antibodies against the AVP, OT, and LHRH peptides and their intermediate and prohormone forms, 5 monoclonal antibodies against neurofilaments in the squid giant axon, and over 50 monoclonal antibodies against Xenopus brain cytoskeletal proteins. These antibodies are currently being utilized in many studies ranging from the molecular to the morphological levels, and are being evaluated for their phylogenetic cross-reactivities. Immunocytochemical studies by M. H. Whitnall using the neuro-

physin antibodies have shown that oxytocin and vasopressin cells in the fetal hypothalamus express their prohormones simultaneously and migrate to their brain nuclei at similar rates. However, they express the post-translation modification mechanisms which generate the peptide products, and their neurite outgrowth, at dramatically different rates, the vasopressin neurons being considerably more precocious. Preliminary radioimmunoassay studies by M. Altstein have confirmed these observations. Current studies are directed at understanding the mechanisms and biological significance of this differential behavior. S. Wray has examined LHRH neuron development in the rat from the neonate (2 days pn) to adulthood. Her observations are that smooth LHRH cells are transformed to irregular LHRH cells during puberty, and that this occurs independently of gonadal steroid feedback. Current efforts are being directed at determining whether specific synaptic inputs to the LHRH cells are modified during this dramatic morphological change. This involves the use of double-label electron microscopic immunocytochemistry (EM-ICC).

Gainer's group continues to develop capability in ultrastructural localization of antigens by EM-ICC. The point of this work is to demonstrate unequivocally, by these techniques, the intravesicular location of various enzymes and peptides (precursors), to study the routing of these antigens through the membrane systems of the cell, and to identify pre- and post-synaptic cells. This has involved new technological developments by M. Whitnall, S. Wray, and S. Key in EM-ICC which allow for good ultra-structure combined with good antigenicity. New fixation procedures, embedding media, and most recently the application of immunogold techniques, has allowed this group to: 1) Show the co-localization of an opioid peptide, dynorphin A (1-8) and vasopressin, in common secretory vesicles, 2) locate the dynorphin 1-8 in smaller secretory vesicles in the Brattleboro rat, a mutant which does not contain vasopressin, 3) disprove a hypothesis that axonal endoplasmic reticulum is used instead of vesicles for hormone transport during dehydration stress, 4) show that the oxytocin precursor is located in secretory vesicles during fetal development, and 5) show that CRF and AVP are co-localized in the same nerve endings and vesicles in the median eminence, and that this coexistence is enhanced greatly by adrenalectomy.

The Xenopus development program (B. Szaro and K. Conway) has succeeded in establishing breeding conditions in this Laboratory which allow for the study of wild-type and various mutant strains. Computer programs have been established for 3-dimensional analysis of neurons in brain, and immunological identification by Conway has revealed over 4000 AVT and MT neurons in the adult Xenopus brain. A 3-D map of their distribution is in progress. B. Szaro is analyzing the 2-D gel maps of Xenopus brain cytoskeletal proteins, and has succeeded in making potent antibodies against some of these proteins. These antibodies will be used in analysis of protein expression in development, and will be the basis of a molecular cloning strategy in the near future. M. Lang has studied the AVT receptor's development in the Xenopus kidney A6 cell line. He has demonstrated that the development of functional AVT (V-2) receptors in the cultured A6 epithelial cell line is dependent primarily on the development of an epithelial morphology in culture. Only when the morphology of the cell is appropriate, does the AVT receptor become coupled to the adenylcyclase. This correlation is intriguing and suggests a common mechanism regulating both phenomena. Studies of the ligand selectivity of the A6 line AVT receptor showed that it is a novel V-2 receptor.

Recent studies on the squid axon model have continued to focus on the Ca^{2+} activated protease's pattern of cleavage of its endogenous substrate, the neurofilament protein. This group has found that the three molecular components, the neurofilament proteins, the protease, and the protein kinase--are present in a complex in axoplasm. All are synthesized in the cell body, but the kinase acts only when the complex enters the axon, and the protease activates only at the nerve terminal. Thus, the genesis of neurofilament structure is determined topographically in the neuron, i.e., biosynthesis and assembly in the cell body, phosphorylation in the axon, and proteolysis in the terminal. This topographic organization of regulation of post-translational modification is currently under study with regard to mechanism, and studies are also devoted to the possibility that it is an error in this mechanism which underlies Alzheimer's disease.

Peng Loh's section studies brain and pituitary peptides which are involved in intercellular neurocommunication and fetal development. The emphasis has been on the ACTH/endorphin/α-MSH family of peptides. Endorphin is an opiate peptide that is found in brain, pituitary and placenta. α-MSH is present in brain and pituitary, but in humans it is present in the pituitary only during pregnancy and in the fetus. This peptide has been proposed to have an effect on fetal growth and development. ACTH is a pituitary peptide which is traditionally known to stimulate steroidogenesis. However, it is also found in brain. All of these peptides have been shown to have central nervous system effects and are thought to act as neurotransmitters and neuromodulators. The major focus has been to continue to study the enzymology and regulation of biosynthesis, packaging and secretion of this family of peptides. Within the past year two interrelated projects have been pursued.

The ACTH, α-MSH and endorphin peptides are synthesized in the intermediate lobe of the pituitary from a common, glycoprotein prohormone (pro-opiocortin) of about 32,000 daltons in size. Recently these investigators have assayed for several enzymes involved in the processing of this prohormone. A converting activity which cleaves at the paired basic residues which flank these peptides in the pro-hormone has been detected in bovine intermediate lobe secretory vesicles. This prohormone converting enzyme (PCE) has now been purified to homogeneity and characterized as a 70,000 molecular weight glycoprotein. It has a pH optimum of between 4.0-5.0 and is functional at the acid intravesicular pH. Purified PCE is inhibited by pepstatin A, but not by PMSF, DFP (serine protease inhibitors), TLCK or EDTA. The thiol protease inhibitor, dithiodipyridine, had a partial inhibitory effect. PCE specifically cleaved mouse pro-opiomelanocortin (POMC) between the basic residues and on the carboxy side of the Arg at Lys-Arg pairs to yield ACTH, β-endorphin and a 16K N-terminal glycopeptide. It also cleaved human β-lipotropin to yield β-MSH and β-endorphin, and proinsulin to yield the A and B chains of insulin, suggesting that PCE can also cleave Lys-Lys and Arg-Arg pairs of basic residues in addition to Lys-Arg. PCE does not cleave single basic residues. An enzyme with characteristics indistinguishable from the intermediate lobe PCE has also been purified from bovine neural lobe secretory vesicles that cleaved the endogenous prohormones of this lobe to yield their respective hormones. Generation of antibodies to PCE is now in progress. An Okayama-Berg bovine intermediate lobe cDNA library has been prepared which will be ultimately used for screening for PCE clones, using an antibody to the enzyme as a probe. Other processing enzymes, e.g., a carboxypeptidase B-like enzyme that removes the C-terminal basic residues from the cleaved peptides, has been detected in rat neural lobe, anterior lobe and intermediate lobe secretory vesicles. In addition, an aminopeptidase B-like

enzyme which removes N-terminal basic residues has been partially purified from bovine neural lobe and intermediate lobe secretory vesicles. These enzymes appear to be acid metalloproteases which are highly stimulated by Co^{++}.

The regulation of synthesis of pro-opiomelanocortin (POMC) has been studied using the toad intermediate lobe as a model system. Organ cultures of the toad neurointermediate lobe show that dopamine effectively down regulates the biosynthesis of POMC. The dopamine receptor in the toad intermediate lobe has been pharmacologically characterized as being in the D2 category and is negatively coupled to adenylate cyclase. Presence of dopamine in the culture medium resulted in a decrease in intracellular cAMP and POMC synthesis in the intermediate lobe. This decrease in POMC synthesis was prevented by the addition of 8-Bromo-cAMP to the medium. Work is now in progress to determine the mechanism by which the cAMP activates and/or maintains POMC mRNA transcription and translation.

James Russell's group studies the nerve terminal, a highly specialized region of the neuron, separated from the neuronal soma by an axon whose function is to release neurotransmitter substances when stimulated by an electrical signal carried by the axon. Thus the nerve terminal plays the central role in the nervous system function that operates by signal transmission between cells (by means of secretion of neurotransmitters). Modulation of the quantity of the transmitter released at the terminal may form the basis for all central nervous system functions, including integration of information, long term information storage, and retrieval. Because of this complexity (cellular heterogeneity and complex organization), a basic understanding of the molecular mechanisms of nerve terminal function in the central nervous system is still lacking.

The program of Russell's unit, established this year, is focused on studying the biochemistry and physiology of the nerve terminal using neurohypophysial neuroendocrine cells as the model system. The hypothalamo-neurohypophysial system represents a model of central nervous system neurons because of their homogeneity and discrete localization, which are eminently accessible for experimentation. The nerve terminals of these neurons are discretely localized in the neurohypophysis, where they are accessible to experimental manipulations both in vivo and in vitro. These nerve terminals can be isolated from the neurohypophysis without contamination by the post-synaptic membrane, unlike nerve terminals from other regions in the central nervous system.

Over the past several years, this laboratory has been involved in the elucidation of the biochemical processes underlying the biosynthesis and the post-translational modification of the prohormones for vasopressin and oxytocin. This work led to the description of the secretory vesicle hypothesis, which states that the primary site of post-translational modification of secretory proteins is the secretory vesicle, and that the vesicle provides the highly organized microenvironment for these biochemical reactions. Neurosecretory vesicles from posterior pituitaries were prepared in a highly purified and stable form. These vesicles were shown to maintain an intravesicular pH of 5.5, by means of an electrogenic proton-translocating Mg^{++}-ATPase on the membrane. The vesicle membranes were also shown to possess a b-type cytochrome (cytochrome b561), which functions as an electron transporter to intravesicular semidehydro ascorbic acid, which is generated by the activity of the peptide α-amidating enzyme. The intravesicular ascorbic acid content was found to be in the order of 20 mM. Recently, a preparation of highly purified nerve endings (neurosecretosomes) has been obtained in order to study the kinetics of

neuropeptide secretion from these terminals in vitro. Because of the homogeneity, this preparation for the first time allows the study of intra-terminal calcium ion homeostasis, and membrane calcium channels and their physiological regulation. Work is underway to isolate different membrane systems from these nerve terminals in order to study the biochemical mechanisms of calcium ion transport and its regulation by intracellular and extracellular signals.

Optical studies of nerve terminal activity, begun in collaboration with B. Salzberg, continue. Russell has extended the voltage-sensitive dye work to mouse and rat pituitaries. These new studies revealed a light scattering signal correlated with the secretory process at the nerve terminals. In addition, two different secretion models were developed: 1) An intact mouse posterior pituitary system stimulated electrically in vitro allowing radioimmunoassay of secreted neuropeptides following varied stimulation frequencies and pharmacological paradigms (using this system, Russell found that the dependence of secretion on extracellular calcium ions exhibits a relationship of one Ca^{++} per secretory event); 2) a neurosecretosome model (equivalent to synaptosomes from brain but from the neurohypophysis), which will be used for basic studies on peptide secretion.

Laboratory of Comparative Ethology--
Stephen J. Suomi, Ph.D., Chief

Research in this Laboratory is focused on the development of behavior in humans, as well as primates and other animal models. The interactions of genetic and environmental factors are explored, using a comparative mammalian approach, so as to determine the origins and evolution of various behavioral phenotypes. Experimental results in animals are correlated with the results of longitudinal studies in human infants and families. At the same time, hypotheses concerning behavior in the animal models are correlated with results obtained by physiological and biochemical neuroscience techniques.

The section directed by **Stephen Suomi** studies genetic and environmental interactions through comparative ontogenic study of rhesus monkeys living under semi-natural conditions. The neuroanatomical, electrophysiological, and biochemical concomitants of various behavioral phenotypes are determined and these phenotypes are tracked developmentally from birth through senescence. Monkeys bred to yield specific behaviors under stress (e.g., "laid-back" or "up-tight") are used extensively in these longitudinal investigations. Suomi's approach involves long-term developmental study of individual rhesus monkeys of known genealogy which are reared in physical and social settings that are experimentally determined and periodically manipulated in a systematic fashion.

Behavioral and physiological data encompassing several levels of analysis are routinely gathered from each subject under controlled environmental conditions at predetermined age points from birth to maturity. Several long-term studies initiated or continued during the past year have focused on individual differences in the monkeys' reactions to mild environmental challenges at various points during development. These differences in "stress reactivity" appear to have an important genetic component, can be detected very early in life, and are relatively stable over major periods of development--although both the behavioral and physiological differences between subjects are largely masked when environmental challenges are absent.

The interaction between genetic and environmental factors affecting reactivity is reflected in one major study in which rhesus monkey neonates, selected as potential "high reactors" ("up-tight") on the basis of their genetic pedigrees, were cross-fostered to multiparous adult females of predetermined reactivity who also differed in their maternal style, as assessed by their care of previous offspring. These high-reactive infants were compared to control infants ("laid-back") who were likewise foster-reared. Results of nephew reflex tests and temperament ratings confirmed the reactivity status of the infants, with highly reactive individuals having more extreme scores on measures of orienting, muscle tone, behavior inhibition, and predominant state within the first month after birth. Nevertheless, the highly reactive infants were effectively buffered from environmental challenges by their foster mothers, even those who were high reactors themselves. However, when these infants were briefly separated from their foster mothers at 6 months of age, they reverted to previous response styles, i.e., highly reactive infants displayed more extreme behavioral, adrenocortical, and catecholamine responses to separation than did control infants. When reunited with their foster mothers, the highly reactive infants once again displayed normal activity and social interaction patterns. Several months later, the infants were placed in a small social group containing age-mates and an elderly (>25 years) male-female pair ("foster grandparents"). The highly reactive infants were slow to adapt to peer group introduction, but the highly reactive infant who had been raised by the foster mother judged most nurturant was the first individual to establish a close social relationship with the older monkeys, and as a result it became the highest ranking member of its peer group. Follow-up study of these cross-fostered monkeys will be continued at least through adolescence, and a replication of the study is currently in progress.

Other long-term longitudinal studies in Suomi's section examined juvenile and adolescent rhesus monkeys who were either mother-reared for their first 6 months of life or hand-reared in a neonatal nursery and placed in peer groups at one month of age, but whose social rearing environments had been otherwise identical after 6 months of life. When these monkeys were 8, 20, and 32 months of age, they were each briefly separated from their social group. Analyses performed to date indicate that (a) there were no major differences in behavioral profiles during group housing as a function of early rearing conditions, but (b) there were major differences in behavioral responses to separation as a function both of age and early rearing conditions, with qualitatively different patterns displayed at the different ages, and quantitatively more extreme reactions shown by early peer-reared juveniles. In contrast, there were no major differences in physiological response to separation as a function of rearing conditions: Both mother and early peer-reared monkeys displayed marked increases (over preseparation levels) of plasma cortisol, heart rate, and CSF MHPG (a norepinephrine metabolite), moderate decreases in CSF norepinephrine (NE), small increases in CSF 5-HIAA (a serotonin metabolite), and no systematic change in the dopamine CSF metabolites HVA and DOPAC. Moreover, age differences in physiological reactions to separation were modest at best, with older subjects generally exhibiting more moderate changes from preseparation values. Thus, although brief social separation produced major changes in both behavior and physiological activity in juveniles of different ages and rearing backgrounds, the behavioral measures seemed more sensitive to differences in these variables, while the physiological measures were more robust across age and rearing background differences.

Detailed analyses of behavioral and physiological effects of the antidepressant drug imipramine administered to adolescent monkeys, both during brief separation and during periods of group housing, were also completed this year. Substantial individual differences in behavioral reactions to imipramine treatment during separation were found: Most high-reactive subjects showed decreases in self-directed behavior and became more passive, whereas low-reactive adolescents (who had displayed little self-directed behavior prior to imipramine treatment) showed activity increases. Imipramine administered during periods of group living was not associated with pronounced behavioral change in either high or low reactive monkeys. The physiological data presented a more complicated picture: While imipramine clearly reduced separation-elevated levels of plasma ACTH, it had no apparent effect on plasma cortisol levels. Imipramine given during periods of group housing significantly elevated levels of NE and lowered levels of the NE metabolite MHPG, but had no consistent effects on either 5-HIAA or dopamine metabolite levels. Separation significantly elevated MHPG and 5-HIAA, and lowered NE, during placebo periods, while imipramine treatment during separation elevated NE above preseparation baseline levels (and reduced MHPG below those levels) without affecting separation-induced levels of 5-HIAA. No consistent drug nor separation effects were found for HVA and DOPAC. Thus, the locus of physiological change produced by imipramine treatment during separation was apparently limited to NE among the monoamines and ACTH within the HPA axis.

Another project initiated this past year involved direct comparison of members of the LCE rhesus monkey colony with rhesus monkeys originating in other laboratories or feral colonies. The immediate purpose of such comparisons is to determine the generality of the various reactivity differences that have already been observed within the LCE colony. As a first step, a group of six juveniles was obtained from Dr. Seymour Levine's colony at Stanford. These subjects had been reared in social groups and had experienced short-term separations while still at Stanford; they were shipped to the LCE this past year. Of particular interest is the fact that all of the rhesus monkeys in the Stanford colony seem to be similar to the LCE's low reactive monkeys, at least in terms of their behavioral reactions to short-term separation. Suomi's group is interested not only in comparisons of physiological reactions to challenge in these monkeys but also in any long-term advantages or disadvantages they might exhibit when integrated with LCE monkeys in mixed social groups.

Finally, Suomi and his colleagues continued their longitudinal study of a group of 12-year-old rhesus monkeys and two generations of their progeny, all of whom were moved from Wisconsin and now live year-round in a 5-acre enclosure on the Poolesville grounds. The 12-year-old adults were all laboratory-born, hand-reared in a nursery, and subsequently put together as a mixed-sex peer group. Despite the fact that none of these now middle-aged monkeys (nor any of their progeny) have had any physical exposure to any other monkeys since they were first moved outdoors as juveniles, they have consistently exhibited the full complement of species-normative social behavior and group organization reported for rhesus monkeys born and living in feral environments. Not only did all group members successfully adapt to the climatic extremes of the Maryland winter and summer, but they also demonstrated seasonally based changes in foraging patterns, diet, and social behavioral activities, including the emergence of a distinct breeding season that once again was consistent with existing data from rhesus monkey groups living in feral environments. Futhermore, the observed similarity of the present group's patterns of social organization and demographic dynamics to those of wild groups was extended this past year to

include adolescent male peripheralization, i.e., the group's expulsion of the first male offspring to approach adulthood (this young adult male subsequently was successfully introduced to the previously mentioned group of monkeys from Stanford). These results serve to reinforce the impression that major elements of rhesus monkey behavioral repertoires have deep-rooted genetic foundations.

The section headed by **David Symmes** utilizes behavioral observations and detailed sound spectrographic analyses to characterize the conditions under which specific patterns of vocalization are emitted by squirrel monkeys and to identify the information content of each distinct vocal signal. In addition, neuroanatomic and pharmacological techniques are utilized experimentally to modify the production of particular types of vocalization, while subtle species and subspecies differences in sound spectrographic patterns of generically similar vocalizations are investigated in genetic studies that involve hybrid and back-crossing selective breeding techniques. The construction of outdoor group housing cages on the Poolesville grounds for the LCE squirrel monkey colony this past year has greatly facilitated the study of vocal exchanges in naturalistic settings.

One study completed this past year focused on a type of vocalization, termed the "chuck," used in affiliative contexts by adult female Gothic arch squirrel monkeys. The relatively high incidence of chuck exchanges between affiliated females was confirmed, and the temporal parameters of such exchanges clarified by higher resolution analysis. Socially popular monkeys uttered chucks which resulted in prompt chuck "answers" from others; less popular monkeys were answered more slowly, and they often repeated themselves. The latency-of-response distributions correlated significantly with ranked affiliative popularity. A significant new finding was that chucks used as answers were higher in peak frequency of several acoustic features than chucks preceded by above average intervals of silence (Symmes has called them "question" chucks). Answers which terminated an exchange (i.e., which were the last in a sequence of 2 or more) were higher in frequency than those which continued an exchange, providing evidence that the original speaker detected and responded to the acoustic difference. Both findings are based on large samples of data and rigorous analyses of variance.

N. Masataka successfully carried out another study on the influence of separation distance on the characteristics of a different type of squirrel monkey vocalization, the isolation call or peep (IP). This work has been done utilizing the new outdoor habitats constructed near the laboratory at Poolesville. The results showed that duration of the IP (alone among standardized descriptors of the structural details of this call) varied positively with separation distance. Both juveniles serving as separated subjects (placed singly in a small restraining cage at varying distances from the habitat) and adults serving as home troop members used longer IPs at longer distances. The differences were significant for all monkeys involved. These two sets of findings are of considerable interest in that they provide the first evidence that important information can be conveyed via systematic variation within the same call type emitted by a particular individual, rather than by variation in the sequence of different call types. Such vocal flexibility has not previously been reported in the New World primate species.

Other studies conducted by **J. Newman** in collaboration with NIMH researchers focused on the production of vocalizations emitted by squirrel monkeys under more tightly controlled laboratory conditions. Two experiments employed behav-

ioral pharmacological techniques to examine the role of specific neurochemical substrates in mediating vocal behavior. The first study utilized drugs that have affinity for binding alpha-adrenergic receptors. One such drug, clonidine, in a dose of 0.1 mg/kg i.m., consistently decreased or eliminated IPs in subjects separated from conspecifics. Yohimbine in a dose of 0.2 mg/kg reversed the clonidine-related suppression of vocalization in this context, and, in addition, enhanced production of another vocalization, the twitter. In a second pharmacological study, benactyzine, an anticholinergic, was found to potentiate the induction of alarm calls given in response to a model predator. This same drug failed to alter significantly production of IPs. These findings indicate that benactyzine can increase alarm call rate in squirrel monkeys under defined laboratory conditions and may serve as a useful pharmacological probe to study chemical mechanisms mediating production of alarm calls. In a third study, performed in collaboration with Dr. Paul MacLean of NIMH, the role of the medial frontal cortex in vocal production was studied following anterior limbic and neocortical ablations. Postoperative testing completed on four animals provides evidence for a concerted involvement of pregenual and preseptal limbic cortex, together with an as yet undefined area of adjacent neocortex, in the normal expression of the IP when the subject is visually and acoustically separated from his conspecifics.

Previous research in this section had demonstrated consistent differences in the sonographic structure of IPs between Roman arch and Gothic arch (sub)species of squirrel monkeys, and a hybrid breeding program was initiated. This past year, J. Newman analyzed IPs from Roman and Gothic hybrid infants through their first year of life. One cross-mating involved parents from sympatric populations (Loreto Province, Peru), while the other cross involved individuals from geographically separate populations (Roman-arch male from Bolivia, Gothic-arch female from Guyana). As reported last year in a larger group of infant hybrids, the "sympatric" infant produced IPs classified as Roman (like the father, in this case), while the "allopatric" infant's IPs were more Gothic in character. The first viable F2 hybrid from the project's breeding program was born during 1985. It had the Roman-arch type of facial phenotype. Tape recordings of its IPs were made at one day, one week and four weeks of age. This infant consistently produced IPs typical of F1 hybrids from sympatric matings, i.e., intermediate in acoustic character to IPs from the parents. This trait was particularly apparent in this individual, since the father (F1 offspring of Bolivian x Guyanan cross) consistently produces IPs with a Gothic character, while the mother (F1 offspring of a sympatric Roman x Gothic cross) consistently produces IPs of the Roman phenotype. Addtional F2 hybrid births are expected this year. The importance of genetic mechanisms for the development of normal communication and their involvement in communicative disorders is widely recognized but poorly understood. The availability of primate models for studying the genetic transmission of species-specific communication patterns provides a unique opportunity for analyzing the genetic mechanisms underlying the expression of communicative behavior in primates.

Finally, this past year M. Biben carried out a detailed analysis of play behavior in squirrel monkey juveniles. In this study of social play in 10 yearling squirrel monkeys of the Roman-arch variety, two types of play wrestling were identified which differed in the degree of roughness and were used with different frequencies by males and females. Partner choice and type of play were shown to be strategies used by animals to maximize their chances of winning in play interactions. By using some behaviors selectively to initiate play, animals were able to manipulate the type of wrestling that occurred. Both sexes

initiated play more often with same-sex partners, and males responded more often than did females to initiation attempts by either sex. While dominance relationships outside of play remained stable, role reversal characterized both male-male and female-female play, but not male-female play. Males dominated females both during and outside of play. These results suggest that social play, and particularly the strategies of role reversal and partner choice, may serve important functions in the socialization of young animals. Animals living in complex social groups should benefit from knowing when and how to assume different social roles later in life. This hypothesis will be tested directly in studies currently underway.

The section headed by **F. Pedersen** investigates the influence of early experience in the family on the cognitive, emotional, and psychosocial development of human infants and children, as well as how certain neuroendocrine functions affect behavioral development. These researchers utilize detailed behavioral observations of children interacting with family members in their natural home environment, structured play and test sessions in the LCE laboratory playroom, detailed interviews, and various standardized questionnaires to generate a longitudinal data base. This past year, additional indices, i.e., salivary cortisol and telemetry-based heart rate and vagal tone, were developed for inclusion in future longitudinal studies.

Several studies completed this past year under Pedersen's direction examined phenomena associated with maternal separations typically experienced by infants in our contemporary society. One series of analyses highlighted distinguishing features of different types of separation experiences. Maternal separations associated with employment occurred more frequently, were of longer duration, began earlier in the baby's first year, and were more likely to occur in a patterned or predictable way than recurrent separations associated with non-reimbursed activities such as health and sports groups, classes, and volunteer work. In addition, caretakers for infants during employment-related maternal separation were less likely to be the infants' fathers, and care was more likely to take place in another home and with other children present than in nonemployment-related maternal separations. Of special interest was the finding that infants whose substitute care arrangements involved experience with peers were more likely to have secure attachment relationships with their mothers, employed or not, when they began their second year of life. This finding is noteworthy in view of other evidence linking early peer experience with greater autonomy and more advanced levels of play later during childhood, and nonhuman primate data indicating that early peer experience can compensate in part for loss of mother via separation (in laboratory settings) or death (in feral environments).

The effects of recurrent maternal separation on fathers' relationships with their infants were examined in another study. Here, the interactions of fathers and their one-year-old infants were compared between men who had relatively extensive experience with their infants in the first year on a one-to-one basis (in situations when the mother was out of the house) and men whose experiences with the infant rarely were independent of the mother's availability. On virtually every measure, men who had more extensive experience with infants on a one-to-one basis in the first year showed numerically higher rates of interaction when observed in a family setting. Significant differences were found in the men's vocalization rates (playful sounds) and in a rating of reciprocal positive affect between father and infant. The infants also showed significantly higher rates of offering toys to their fathers,

further supporting the reciprocal nature of these effects. Further analyses are being pursued to test whether the one-to-one experience had a truly causal impact on fathers or whether the results can best be explained as dispositional differences in fathers, a preference to interact with infants that manifests itself in both electing more one-to-one experiences and in higher rates of interaction as observed in the family setting.

J. Demetre this past year completed a major study investigating the cognitive processes utilized by infants in their processing of adults' manual actions and the nature of developmental changes that might occur in the manner of information processing utilized by the infants as they grow older. Three kinds of procedures were employed: First, an experimental procedure in which the experimenter performed various actions "live" for the infant under different conditions of prior exposure to the object in the static state was used to assess comparative processing. Second, a series of procedures involving presentation of visual stimuli via a video recorder was used to assess infants' sensitivity to perceptual and productive components of action. Third, joint play sessions of mother and infant with toys were recorded on videotape for subsequent assessment of infants' productive capacities and the specific details of maternal stimulation with objects. The measure of interest in the first two procedures was duration of visual fixation. Categorical and frequency data of infant and maternal manipulative behavior were coded for the third procedure. These included the frequency with which mothers and infants performed object-specific (demonstrative) acts, the variety of actions produced by the mother with any given object, and the extent to which the infant's manipulations of objects matched those of the mother in terms of content. Results analyzed to date from 9-month-old and 16-month-old subjects indicate that an adult's object-specific actions are attended to longer by 9-month-olds, but not 16-month-olds, following prior exposure to the object in a static state. This indicates that 9-month-olds process actions in terms of stimulus features of the object encoded in memory (comparative processing). Further experimental manipulation of the actions presented to infants indicates that the mere juxtaposition of a hand and an object previously experienced in isolation, and a hand moving an object previously experienced as static, are not sufficient in themselves to elicit comparative processing in infants of this age. These findings suggest that changes accompanying specific actions with objects alone are responsible for the eliciting characteristics of manipulative actions. Such actions serve to highlight differentially or transform certain features of the object which are not readily decoded by infants when presented with static objects.

R. Klein's major research project encompasses a series of studies examining the behavioral correlates of endocrine disorders, including children with precocious puberty, Turner's syndrome, and growth hormone deficiency. A first objective was to determine whether these children are at risk for problems in psychosocial adjustment. In a sample of children with precocious puberty, it was found that these children do, in fact, show an above-normal incidence of a variety of adjustment problems. A current objective is to ascertain the factor(s) responsible for this finding. Analyses during the past year have focused on three issues: (1) Closer examination of the previously reported relation between age and behavior problems revealed that children who did not receive treatment until 8 years old were at very high risk (85%) for clinical levels of behavior problems. Klein is currently determining what characteristics of this group could be responsible for this finding. (2) There was no immediate effect of treatment (an LHRH analogue) on reported behavior problems even though the treatment was effective in reducing sex steroid and gonado-

tropin levels. This suggests that the adjustment difficulties seen in this sample are not the direct result of hormonal action. (3) Comparison between the idiopathic cases and cases matched on the basis of sexual development-stage data from an ongoing NIMH study of normal puberty showed several differences in behavioral adjustment. In several areas the probands showed evidence of greater adjustment difficulties than did the control cases, particularly with regard to hyperactivity. (4) In contrast to previous research, Klein found no evidence of verbal IQ superiority in a sample of girls aged 5 to 8 with idiopathic precocious puberty.

Finally, data collection for a long-term project investigating the development of mastery motivation was completed. Three of the studies employed the same sample of 75 children to study the interrelationships between cognition, motivation, and the environment at 6, 12, and 30 months, and 6 1/2 years of age, respectively. At each age point, methods were introduced that were developmentally appropriate yet conceptually similar to the methods used to collect data at the earliest age. A separate study was conducted to investigate the expression of mastery motivation in Down syndrome infants. The current focus of analysis of data collected in the 6, 12, and 30 month studies is directed toward explicating the relationship between mastery motivation, competence, and social abilities. The results indicate that in 6- and 12-month-old infants, manipulative activities directed toward success with objects represent an important dimension of their behavioral repertoire. Moreover, these early manifestations of the motivation to be competent are an important aspect of later cognitive abilities. While these findings extend through to the early school-age years, it appears that the quality of mother-infant attachment serves to mediate these relationships. With regard to the development of mastery motivation and social competence in Down syndrome (DS) infants, it appears that in comparison to a cognitively normative sample, DS infants are not as adept at integrating the object-related and socially-directed domains into ongoing streams of behavior.

Laboratory of Developmental Neurobiology--
Phillip G. Nelson, M.D., Ph.D., Chief

This Laboratory investigates the neurobiologic mechanisms relevant to development of the nervous system, with emphasis on studies at the cellular membrane and molecular levels. The basis for short- and long-term interaction between nerve cells is studied electrophysiologically and biochemically. Combined molecular and morphological methods are used in the analysis of experiential modifications of brain function and gene expression.

Information transfer in the nervous system is effected by the mechanisms responsible for synaptic transmission and this has been of focus for LDN activities. Results important for an understanding of the long-term modulation of synaptic efficacy have been obtained this year, with regard both to presynaptic transmitter release mechanisms and to postsynaptic receptor properties. Significant progress has been made on understanding neuron-glial relationships related to neuronal survival and development. A number of cell-specific immunocytochemical probes were produced which have augmented substantially the ability of the Lab to analyze differential development of neuronal sub-populations in culture systems. Successful cloning of the choline acetyltransferase gene has opened up the possibility of rigorous analysis of the regulation of

the expression of this important gene. A new concept of pineal-brain relationships has been introduced by the striking finding that processes from pineal cells extend into the nervous system. Progress has been made in the immunologic and molecular genetic analysis of the regulation of pineal gland metabolism.

In the section headed by **P. Nelson**, D. Brenneman has now made a compelling case for a peptide-mediated interaction between neurons and glia that has a major impact on neuronal survival and maturation during a critical period of nervous system development. Vaso-active intestinal peptide (VIP) is released, as a consequence of electrical activity, by the VIP-containing neurons in Nelson's spinal cord cell culture system. This VIP acts upon glial cells and causes them to release a macromolecular factor which is required by neurons for survival and development. The evidence for this novel and important schema is as follows: Previous studies showed that a population of spinal cord neurons grown in culture died during a critical developmental period, and that electrical blockade further decreased neuronal survival and expression of the cholinergic marker, choline acetyl-transferase (CAT). Addition of VIP (at 10^{-12} to 10^{-8} M concentration) prevented both the naturally occurring cell death and that which was associated with electrical blockade. This group has now shown that addition of VIP antiserum or a carboxy fragment of VIP (VIP_{10-28}) produces decreases in neuronal survival and CAT activity that are indistinguishable from those produced by electrical blockade with tetrodotoxin (TTX). VIP_{10-28} acts at concentrations of 10^{-12} to 10^{-8} M. We have shown that high affinity VIP binding sites are present on non-neuronal glial cultures, and conditioned medium from glial cultures stimulated with VIP (10^{-10} M) prevented TTX-mediated neuronal death, whereas conditioned medium from similar cultures not treated with VIP had no such effect.

This work is particularly exciting for two reasons: 1) It represents a concrete example of neuron-glial interaction of major significance for neuronal survival and development that lends itself to analysis at the membrane and molecular levels. Elucidation of the molecular mechanisms that govern these multiple neuron-glial interactions is among the foremost challenges to clinical and cell biological investigation. 2) The activity-dependent neuronal survival that is mediated by glial-derived trophic material involves cholinergic neurons. Cholinergic systems in the mammalian central nervous system are becoming increasingly identified as playing a critical role in the expression of plasticity in the nervous system. Learning and memory deficits are associated with pharmacological or neuropathological interruption of these cholinergic systems, and understanding the molecular factors involved in their maintenance is of primary significance to neuroscience.

It is a high priority, for the effective analysis of the culture systems used here, to be able to identify clearly the different cell types in the cultures. **E. Neale** has achieved substantial progress in this regard with a number of cell specific immunocytochemical probes. Neurons can be distinguished from non-neuronal cells; cholinergic, GABAergic and various peptidergic neurons can be identified, as well as oligodendrocytes, astrocytes, and fibroblasts. Receptors (e.g., opiate and muscarinic cholinergic binding sites) can be quantitated both biochemically and radioautographically. Cholinergic neurons consist of both large motoneuron-like cells and smaller cells of various shapes; the number of these cholinergic neurons in a culture can be affected

markedly by culture conditions. The number of astrocytes is reduced substantially in serum-free medium. Further exploitation of these powerful techniques is anticipated.

The physiology group has continued studies aimed at understanding nervous system function in terms of discrete physiologically identifiable membrane molecules, in particular ion channels and receptors, as well as cell biologic mechanisms such as internal Ca^{++} sequestering systems. These investigators had previously shown that major portions of the synaptic bouton apparatus mediating synaptic transmission may be inactive under physiologic conditions and would represent a synaptic reserve available for activation under appropriate circumstances. They have now shown that inadequate intracellular regulation of calcium ion concentration and the blocking effect of high $[Ca^{++}]_i$ on the voltage-sensitive calcium conductance mechanism may account for some of this synaptic inactivation. This group uses depolarizing voltage clamping pulses to induce calcium currents (I_{Ca}) which carry calcium ions into the neuron. Repetitive activation of this sort produces an inactivation of I_{Ca} due, at least in part, to an increase in $[Ca^{++}]_i$. This inactivation is much more prominent in dorsal root ganglion (DRG) than in spinal cord (SC) neurons. These investigators also show that repetitive activation of DRG neurons is accompanied by a much greater reduction in transmitter output than is the case for SC neurons. DRG synaptic boutons contain a lower concentration of mitochondria than do SC boutons; mitochondria are related either directly or indirectly to calcium sequestration. These workers suggest that the ability to maintain an appropriate $[Ca^{++}]_i$ varies between different cells and for individual boutons, and represents a mechanism for regulating the long term functional status of synaptic structures.

The excitatory synaptic response evoked by interaction of presynaptically released transmitter with its postsynaptic receptor has been elucidated. Previous work had shown that two classes of excitatory receptors could be identified, those activated selectively by aspartate or n-methyl-D aspartate (NMDA), and those activated by kainate or quisqualate. The use of antagonists selective for these different receptors indicates that an excitatory amino acid receptor, but not the NMDA type, mediates fast excitatory neurotransmission between spinal cord neurons. The time course, voltage dependence and reversal potential of the excitatory synaptic currents have been examined. The synaptic current decays as a single exponential with a time constant of 0.5 - 1 msec; this sets an upper limit on the channel lifetime of synaptically activated channels. No voltage sensitivity of the decay time constant or channel conductance was detected between -80 and 0 mV. Calculations based on recent estimates of the conductances of single kainate or quisqualate activated channels suggest that about 300-750 channels are activated per synaptic site.

The ubiquitous distribution of NMDA activated channels and their powerful effect on neuronal excitability indicate the importance of understanding the properties of these receptors and their relationship to kainate activated channels. Previous work had shown that Mg^{++} ions enter and block the NMDA channel but have no such effect on kainate channels. Experiments with other divalent cations suggested that Ca^{++} ions might penetrate the NMDA channel but not the kainate channel. Direct experiments indicate that this is the case. This selective Ca^{++} ion permeability has important implications for the physiological role of amino acids, beyond that traditionally ascribed to fast excitatory transmitters, because of the broad metabolic regulatory role of intracellular

Ca^{++}. It is of interest that recent evidence suggests that intracellular calcium activity is elevated during long-term potentiation (LTP, an important form of neural plasticity) and that NMDA receptor antagonists block LTP.

Nelson and his colleagues have begun the study of the receptors and channels related to excitability and synaptic functions during early developmental stages in culture. This promising area has been opened up by technical advances using various types of patch recording electrodes. They have shown that the density of voltage-sensitive Na^+ channels increases markedly during the first week in culture, but the kinetics of Na^+ activation are not altered. A differential developmental pattern for excitatory amino acid receptors has been demonstrated, with kainate activated channels showing a much more dramatic increase during the period of active synapse formation than do NMDA or quisquilate activated channels.

Infection of sensory neurons in culture with herpes viruses has revealed highly specific effects of the viral infection on functional membrane constituents. Na^+ channels, but not Ca^{++} channels, and specific K^+ channels were blocked by one virus strain. Cell fusion and electrical coupling were induced by other strains. Acyclovir, which blocks replication but not absorption or penetration of the viruses, prevented the electrophysiological effects of the viruses.

A membrane conductance mechanism important for regulation of neuronal excitability has been demonstrated in a subpopulation of sensory ganglion neurons. In those cells, entry of calcium ions during the action potential produces a large increase in chloride permeability, resulting in a prolonged post-spike depolarization.

This group has also begun physiological experiments directed at understanding central cholinergic function. As mentioned above, they can now visualize presynaptic cholinergic structures with choline acetyltransferase immunocytochemistry, and postsynaptic muscarinic receptors using radioautographic techniques. They have developed a system incorporating cholinoceptive (and potentially cholinergic) superior cervical ganglion neurons into the spinal cord cultures. They have demonstrated post- and probably pre-synaptic muscarinic effects on the components of this system, but substantial further work is required.

The group under **B. K. Schrier** has produced cDNA libraries, presumed to be differentiation-specific, by cascade hybridizations, and subsequent cloning characterization of sequences specific for the differentiated and undifferentiated state of NS20Y neuroblastoma cells is currently in progress.

A cDNA clone corresponding to the mRNA for human choline acetyltransferase has been identified by screening a λgt11 library prepared from mRNA of human basal ganglia with a monospecific polyclonal antibody to human CAT. The identified clone (λChat7) contains a 1100 bp insert. A 160 Kd protein synthesized by the λChat7 lysogen was recognized by the anti-CAT antibody and by an anti-β galactosidase antibody. This fusion protein substantially inhibited staining of the neurons in spinal cord cell cultures by the antibody. The insert hybridized to mRNA in Northern blots of an RNA from CAT-producing neuroblastoma clones, but not from neuroblastoma clones that did not express CAT activity. Availability of this probe for the choline acetyltransferase gene is particularly exciting in that it will enable study of regulation of the enzyme at the primary transcriptional level. This is a highly regulated gene and the in-

volvement of central cholinergic systems in a number of extremely significant disease states makes understanding of its genetic organization especially important.

In the section led by **David Klein**, an important finding this year evolved from interest in the molecular similarities between the pineal gland and the retina. Klein previously had initiated a collaboration with investigators in the National Eye Institute with the intention of accelerating research on the pineal gland. The basis of this interest in pineal-retinal relationships is that the pineal organ in cold blooded vertebrates is a photoreceptor--a third eye--with morphological and biochemical similarities to the retina. Klein has worked with Igal Gery, an immunologist interested in a highly antigenic protein in the retina, the S-antigen. This protein, which is involved in regulating the cyclic nucleotide response to photostimulation in the retina, is found in the pineal gland but in no other tissues. With antiserum against S-protein, Klein initiated a series of collaborations with comparative pineal morphologists, Theo van Veen of the University of Lund, and Horst Korf of Julius Liebig University, who have used the antibody to study the pineal gland and retinae of vertebrates and the photosensitive organs of several invertebrate classes. The antiserum has proven to be the best available reagent to identify pineal cells specifically, primarily because the antigen is not present in brain. In the course of examining the distribution of the S-antigen stain in the mammalian pineal gland, Korf and Klein discovered that pineal processes were also identifiable with this reagent. These enigmatic processes had been thought to be short and terminate within the pineal gland. However, with the S-antigen antiserum, it was possible to trace these processes out of the pineal gland itself. Careful investigation revealed that these processes travelled through the habenula and they were traced as far as the posterior commissure. The processes appeared to be neuronal in character: They had varicosities and bifurcations. Many questions remain to be answered regarding these processes. However, it is clear from this discovery that a new area of pineal research has opened. The pinealocyte had been thought of and studied as a neuroendocrine cell, communicating via chemical messages in the blood. Now the pinealocyte must be considered also as a neuron, capable of delivering high concentrations of chemicals directly to discrete brain areas.

Klein's section has made other advances in understanding neural regulation in the pineal gland. David Sugden, a Visiting Associate, and Jiri Vanecek, a Visiting Fellow, have investigated the atypical neural mechanism which regulates cyclic AMP and cyclic GMP in the pineal gland. Several years ago, Klein published preliminary evidence that in this tissue, norepinephrine acts through two receptors to control these cyclic nucleotides, alpha-1 and beta-1 adrenoceptors. Using dispersed pinealocytes, it was possible to dissect this mechanism and unequivocally establish that in the case of both cyclic nucleotides, beta-1 adrenergic activation is a requisite, and that alpha-1 adrenergic activation potentiates this event. Further studies done with Wayne Anderson and Thomas P. Thomas of the National Cancer Institute established that the alpha-1 affect on cyclic AMP was mediated by a phospholipid product, diacylglycerol, and that it acted by promoting the association of a calcium-, phospholipid-dependent protein kinase with the membrane, with the resulting phosphorylation of a membrane protein. The mechanism through which cyclic GMP is regulated has not been elucidated. This development has important implications in neurobiology, because it provides a model to explain how neuromodulators might act.

A goal of this section is to obtain the molecular tools required to determine how specific genes in the pineal gland are regulated. Klein's group has come closer to this goal through several efforts. First, it has been possible for J. Weller to establish a bovine pineal cDNA library, through the cooperation of William Strauss, LDN. This library has been screened with anti S-antigen antiserum and several clones were identified. Independently, Cheryl Craft, a postdoctoral fellow, has been working with Toshi Shinohara of the National Eye Institute to isolate S-antigen cDNA clones from retinal libraries. They have been successful, and it is expected that Craft will now pursue problems of pineal molecular biology independently. It is hoped that it will be possible to compare the pineal and retinal S-antigen cDNA to determine if they reflect the same or different genes. The goal of studying pineal gene products of special interest has been furthered through the efforts of Sugden, P. Voisin and Klein. Together, they have purified hydroxyindole-O-methyltransferase, the enzyme which forms melatonin from N-acetylserotonin, and have prepared antisera. Weller is now using the antisera to screen the pineal cDNA library. Sugden, Klein and Voisin have also been able to obtain a partial sequence for the enzyme. Currently, Valentine Cena, a Guest Researcher in the section, is continuing the isolation of this molecule using high performance liquid chromatography, and preparing sufficient amounts of fragments to allow the complete sequence of the enzyme to be determined. He is also examining the question of whether this enzyme is phosphorylated. Finally, the pineal enzyme of greatest interest to neurobiologists, N-acetyltransferase, is now being obtained routinely in highly enriched preparations. These preparations, from sheep and rat pineal glands, will be used to raise an antiserum and to obtain the full sequence of the molecule. This enzyme is of special interest because its activity is increased 100-fold through an adrenergic-cyclic AMP mechanism involving both new gene expression and protein synthesis.

An intriguing study involving the pineal-retina question is being conducted by Tony Ho, a Visiting Fellow in the Section. This involves rhodopsin kinase in the pineal gland. Last year, Klein and Robert Somers of the National Eye Institute discovered that the pineal gland had about as much of this enzyme as the retina. However, the pineal has no detectable rhodopsin, and the question of the function of this enzyme was raised. To answer this, Ho, Somers and Klein are now in the process of determining whether there is a pineal substrate of purified retinal rhodopsin kinase, and the identity of this substrate. It is suspected that rhodopsin kinase in the pineal gland, as in the retina, phosphorylates a membrane-associated receptor. In the retina the receptor is rhodopsin; perhaps it is an adrenergic receptor in the pineal gland.

Laboratory of Theoretical and Physical Biology--
David Rodbard, M.D., Chief

This Laboratory conducts a wide range of computational and theoretical studies, applying mathematical, statistical, and computer-based techniques to the analysis of complex clinical, biological, and pharmacological problems. Experimental work in the Laboratory involves the study of receptor-ligand interactions; the mass spectroscopic analysis of metals, steroids, and amino acids in man; and the physical-chemical characterization of peptides using polyacrylamide gel electrophoresis and related approaches.

The section directed by **D. Rodbard** analyzes the interaction of hormones and neurotransmitters with their receptors using a combined theoretical and experimental approach. With this approach, the group has previously demonstrated the existence of the mu-1 subtype of opioid receptor, which is responsible for analgesia and the hypothalamic control of pituitary function. During the past year these investigators have characterized other multiple subtypes of the opioid receptors in adrenal medulla, designated K1, K2, and K3. These findings have been made possible by the development of new mathematical and statistical methods for studying dose-response curves in the presence of multiple ligands (the "multi-ligand" design); the combination of modelling and biochemical approaches; and computerized methods for optimization of experimental design, analysis and validation of results.

Analysis techniques utilizing non-linear least-squares have been developed which allow simpler, more informative experiments to be performed. With regard to opioid receptor systems, a Monte-Carlo study of the validity of these statistical techniques was completed showing them to be valid in the situation of mu-1 and mu-2 opioid subtypes. A parametric means of characterization of asymmetric dose-response curves was developed and tested extensively on practical problems. An alternative, "nonparametric," splined-based method of characterizing such curves, testing parallelism and calculating potency, is currently under development. A new statistical method for detecting non-randomness was developed and tested. This method is an alternative to the commonly used mean-square successive differences test. Ligand-binding studies continue to be an important source of information regarding the functioning of receptors for drugs, hormones, and neurotransmitters, yet the assumptions surrounding these studies have been poorly appreciated, resulting in sometimes significant misinterpretation of results and poorly founded theories. Proper understanding of these studies is aided by use of the computerized analyses developed by this group, wherein an explicit statement of assumptions must be made.

The existence of multiple opioid peptides and diverse functions of opioids suggests the existence of multiple receptor subtypes. As noted above, the previous studies of Rodbard and his colleagues on the mu-1 opioid receptor have been extended and validated by Monte-Carlo studies to demonstrate that the mu-1 site is not a statistical artifact of the modelling process. Moreover the existence of at least three subtypes of the kappa-receptor of bovine adrenal medulla membranes has been demonstrated: K_1 is selective for ethylketocyclazocine; K_3 is selective for etorphine; and the major subtype is K_2 which has high affinity for both ethylketo-cyclazocine and etorphine (and for D-ala^2, D-Leu^5 enkephalin as well). Studies in other laboratories suggest that dynorphin $^{1-13}$, Met-enkephalin-arg^6-gly^7-leu^8, and Met-enkephalin-arg^6-phe^7 are the endogenous ligands for these three receptor subtypes. The methodology of "blocking" and "modelling" has been considerably advanced in the course of these studies. The demonstration of these opioid receptor subtypes now mandates a thorough reevaluation of the pharmacology, biochemistry and function of opioid receptors. These studies will provide a basis for the development of new selective agonists and antagonists which in turn will be necessary for further dissection of these intricate systems which appear to control neurotransmitter and endocrine systems.

A major accomplishment in the past year has been the development of several innovative computer programs to assist physicians, paramedical personnel and patients with home monitoring of blood glucose and self-adjustment of insulin dosage. These programs, for the IBM-PC and Apple II classes of microcomputers,

provide data storage and retrieval, graphical and statistical analyses, and "expert" consultation regarding adjustment of insulin dosage, and timing of glucose measurements. The programs also alert the patient to the need for conversation with a physician. Human engineering factors have been optimized to facilitate acceptance, and the programs have multiple fail-safe features. Rodbard's group has worked out a general flexible approach for the development of customized treatment plans ("algorithms") for individual patients. The computer permits examination of the discrete and continuous "glucose profile" to evaluate median circadian patterns, or changes by day of week with appropriate weighting and smoothing. The program provides a detailed analysis and interpretation of the glucose profile. The ancillary documentation for the programs has developed into a book, explicitly describing a versatile new approach to management of patients with type-I diabetes. These programs are now being evaluated in controlled clinical trials at medical centers throughout the country, with special reference to their use in children, adolescents, and during pregnancy.

A major new activity has been the development of several approaches to the analysis of sequential hormonal measurements, for the detection of "pulses" of episodic secretion. These methods are objective, statistically valid, rational, simple, sensitive and reliable, and have been incorporated into interactive computer programs for the DEC-10 and IBM-PC. They permit the evaluation of the "half-life" of the hormone, and estimation of the instantaneous rate of hormone secretion. These programs are now being applied to several areas of clinical investigation. In addition to this definitive approach to analysis of episodic hormone secretion, Rodbard, P. Munson, and other colleagues have developed improved computer programs for the analysis of radioimmune assay data and for the analysis of enzyme and membrane transport systems. These programs have been applied during the past year to the analysis of thymidylate synthetase (to define the role of polyglutamate derivatives of methotrexate) and for the analysis of cocaine sensitive and insensitive transport of catecholamine by chromaffin cells in vitro.

N. Gershon has employed an image analysis method to examine protein-cytoplasmic matrix interactions wherein studies demonstrate that the cytoplasmic matrix, which is composed of a number of filamented systems, slows down the diffusion of proteins to a great extent by transient binding. Although the association-dissociation process can occur very quickly, most proteins are bound to the cytoplasmic matrix. In these studies also, Gershon calculated the binding rate of a ligand diffusing to membrane receptors as a function of the internalization (endocytosis) rate. It was shown that when the internalization rate is relatively slow, the effect of the lateral distribution of the receptors on the ligand intake is not large compared with the situation wherein the internalization occurs instantly. These image analysis studies that measure the volume fraction of the cytoplasmic matrix have shed light on molecular transport to the cytoplasm. Additionally, the amount of surface area associated with the cytoplasmic-matrix is important in understanding the role of hydrated water in the cell physiology. Finally, the rate of intake of ligands by membrane receptors with different internalization conditions sheds light on a number of aspects of endocytosis.

In another area of interest, Gershon employed three-dimensional reconstruction using the color capability of the computer graphic system to enhance electron microscopic visualization of parts of the cell center. This center is implicated in forming and maintaining the cell shape and its filamentous systems.

The organization of the endoplasmic reticulum "whorl body" and the Golgi-apparatus of hypothalamic neurons were also studied, again using three-dimensional reconstruction and computer-based image analysis. These studies should permit a description of the mechanism of endoplasmic reticulum shape changes and their relation to the Golgi-apparatus. Gershon and his colleagues also digitized and processed many sections of rat brain in order to develop a single, full-stereotaxic representation of the brain in its entirety. This approach should also allow these investigators to represent a projection of neurotransmitters, peptides, and receptors in color in their respective locations. A similar reconstruction will be used to evaluate the effects of teratogens on mouse embryos (many sections of mouse embryos at different stages of development have been digitized). The same approach can be used to follow the migration of germ cells within embryos. All of these studies should further our understanding of how the cell acquires its shape, and organizes its organelles. Moreover, these tissue studies should reveal new functional relationships in the brain and in embryos.

The section directed by **A. Chrambach** has continued its studies on the methodology of polyacrylamide gel electrophoresis, isoelectric focusing and related techniques. Pilot studies are examining the feasibility of new approaches to "two dimensional macromolecular mapping". A major effort has been made to obtain stable pH gradients for isoelectric focusing, avoiding problems of low conductance. The combination of soluble and immobilized ampholytes appears to offer significant improvement over previous methodology. Analysis of available data for the migration of virus particles in very dilute agarose gels has revealed "non-linear Ferguson plots", inconsistent with most previous data from gel electrophoresis. This has led to theoretical studies, with modification and extension of the application of the "Ogston model". It appears that the effective gel-fiber radius and coating or density vary systematically as a function of gel concentration. New methods have been developed for electrophoretic fractionation of cell organelles and vesicles, and for multimillion molecular weight immunogens using agarose electrophoresis. An improved apparatus for rapid staining and destaining of gels has been developed. The theoretical analysis of isoelectric focusing using multiple mono- and bivalent buffer constituents has been pursued.

Several of the practical applications of Chrambach's approach to macromolecular analysis achieved during the past year follow: Using agarose gel-electrophoresis, this group determined the particle size of covalently linked protein-polysaccharide conjugates (e.g., meningococcus) to be used as experimental vaccines (see description of work in the LDMI). These physical measurements will be important if they correlate with the degree of immunogenicity of these preparations, to be employed in the prevention of invasive bacterial infections in children. In another study, Chrambach and his colleagues demonstrated the applicability of quantitative agarose gel-electrophoresis to the analaysis of sub-cellular particles--here, clathrin-coated vesicles from brain and liver. The method appears promising for analytical and preparative studies on vesicles involved in metabolite traffic across cell membranes in general. During the past year also, Chrambach and his colleagues employed quantitative polyacrylamide gel-electrophoresis and isoelectric focussing to demonstrate that in hypertensive disease, wherein inactive renin accumulates in the circulation, the block responsible is at the level of the enzymatic cleavage of prorenin to renin. It may be possible to target this block for pharmacologic intervention. Further, Chrambach's group, using PAGE with glutaraldehyde crosslinking of the peptides prior to staining, was able to separate angiotensins I, II, and III.

The separability of these three peptides demonstrates applicability of the method to peptide separation and isolation in general. Chrambach's work on the theoretical analysis of isoelectric focusing, using multiple buffer constituents, has led to a substantial advance for all studies which rely on isoelectric focusing, and in particular, the widely applied practice of two-dimensional macromolecular mapping which aims at the detection of minor component changes (characteristic of developmental changes) against the background of a constant multi-component pattern. Finally, Chrambach and his colleagues have been pursuing the development of a rapid, continuous electrophoresis apparatus that would allow physical characterization of each species in a complex mixture, thus permitting the multi-component analysis of biological fluids and clinical samples, as well as industrial quality control. During the past year, Chrambach's group found that non-crosslinked liquid polyacrylamide possesses a sieving capacity similar to that of crosslinked gels; with this finding, the capillary apparatus becomes applicable to molecular and particle sieving, with optical analysis and on-line computer evaluation of migration rates. Redesign of the capillary apparatus has permitted very rapid size and net charge estimates for each component.

The group led by **A. Yergey** has made several important advances in the application of liquid-chromatography-thermospray mass spectrometry (LC-MS) to compounds of biochemical and clinical interest. New methods have been developed for measurement of deuterated glucose and cortisol in human plasma, and are now being applied to clinical investigation of the role of cortisol metabolism in normal puberty, as well as to biochemical analysis of the pathophysiology of various inborn errors of metabolism (e.g., glycogen storage diseases). Studies of metabolism of stable isotopes of calcium in neonates (to examine the role of vitamin D supplements), in pregnancy, lactation and in disorders with ectopic calcification are continuing with collaborators at NIH and throughout the country. LC-MS has now been applied to a host of compounds, including the carnitine derivatives important in fatty acid and drug metabolism, studies of glucuronide derivatives of drugs, and a novel amino acid (hypusine). Pilot studies have indicated the feasibility of new approaches to characterization of oligo- and small polypeptides, and these methods are now being applied to growth factors derived from neoplasms.

As noted above, one principle objective of Yergey's work is to employ mass spectrometry to elucidate the kinetics of calcium metabolism in normal children and adults as well as to evaluate disease-related changes in this metabolism. Stable isotopes make such studies possible in children and in women of childbearing age, studies that are not possible with radioactive calcium tracers; stable tracers also permit repeated measurements. Yergey and his colleagues employ thermal ionization-isotope ratio-mass spectrometry together with a quadrupole mass filter in order to measure tracer enrichments in serum, urine, etc. The isotope ratio measurements are analyzed with a multi-compartmental mathematical model. Two stable isotopic tracers, one given i.v. and the other orally, are used which permit studies on absorption as well as excretion. During the past year, Yergey has refined the instrumentation such that he can now detect a 2% change in the natural ratio of $^{43}Ca/^{48}Ca$. Population statistics have been obtained in a group of normal children for the mean residence time of calcium in plasma (11.1 ± 2.5 hrs) and the fraction of calcium absorbed from the diet. These studies have now been extended to pregnancy and lactation.

Another objective of Yergey's group is to develop and apply new methods for analysis of biological materials that require mass spectrometric analysis, but which have not previously been amenable to such analysis by reason of volatility, thermal lability, or charge state. The basic approach involves a direct interface of high performance liquid chromatography effluent with the mass spectrometer source. Ions are desorbed directly from vapor droplets that are heated rapidly during passage from the HPLC capillary through the ion source by a mechanism that resembles other desorption techniques. Thermospray LC/MS has the important advantage over other methods of a) a chromatographic inlet, b) applicability to analysis of mixtures, and c) simplicity of sample preparation. Recent applications include the identification of novel fatty acid conjugates of carnitine in patients with Reye's syndrome, organic acidurias and valproic acid toxicity; the separation and quantitation of cortisol in human plasma; and the separation and quantitation of glucose as an aspect of turnover studies in glycogen storage disease.

In summary, the LTPB has had an extremely productive year, with its wide ranging multidisciplinary program for the development of new strategies, biochemical methods, and computer programs to permit improved analysis of ligand-receptor systems; for purification and characterization of protein and polypeptide hormones; for studies on the metabolism of metals and organic compounds in man and in vitro; and for more effective utilization and interpretation of laboratory and clinical data.

Human Genetics Branch--
Michael A. Zasloff, M.D., Ph.D., Chief

This Branch's interests range from studies on the etiology, diagnosis, and treatment of genetic and developmental disorders of young people to very basic studies on eukaryotic gene expression utilizing recombinant DNA methodology. Current clinical research projects involve studies of the natural history, diagnosis and therapy of genetic disorders of lipid and carbohydrate metabolism, the mucopolysaccharidoses, heritable disorders of bone and connective tissue, and lysosomal storage diseases. Other work concerns in vitro models of cellular differentiation, and the genetics of alcohol-related syndromes.

During the past year, work in the section directed by **M. Zasloff** has concentrated on the mechanism of processing of eukaryotic RNAs; the basic cellular mechanisms involved in transport of biological information from the cell nucleus to the cytoplasm; and the expression of"ALU" sequences and other natural sense RNAs. Clinical research in the section has focused on the treatment and pathophysiology of hereditary heterotopic ossification disorders in humans; the molecular basis of osteogenesis imperfecta; and the pathophysiology of the mucopolysaccharidoses. Over the past several years, this group has been studying the expression of a human $tRNA_i^{met}$ gene, a species cloned by this laboratory from the human genome. One of the several genes cloned contained a mutation within the T loop of the tRNA coding region. Since such a mutation would have generated a tRNA with a pyrimidine in a highly conserved site, occupied only by a purine in bacterial and eukaryotic tRNA species, Zasloff and his colleagues suspected that this variant human tRNA might be defective. In a series of experiments utilizing both in vitro and in vivo systems, they demonstrated that this mutation had profound effects on the processing reactions which mature the primary gene transcript to tRNA, and the transport mechanism which delivers the

tRNA from the nucleus to the cytoplasm. As a result of these findings, the laboratory was directed to an exploration of the processing enzymes actually utilized in eukaryotic tRNA biosynthesis as well as the mechanism of nuclear tRNA transport.

These investigators have now isolated the nuclear enzymes which are responsible for processing the pre-$tRNA_i^{met}$ primary transcript from X. laevis oocytes. The enzyme which processes the 3' terminus is a single polypeptide of about 97,400 in size. It generates a mature end and releases the 3' trailer intact. Its most striking feature is that it will only cut the primary gene transcript after the short 5'leader has first been removed. This substrate restriction of the 3' processing enzyme thus imposes a cutting order on the maturation pathway and demands that the 5' processing enzyme act initially. The 5' processing enzyme is a very complex structure consisting of some 15 polypeptides with an aggregate molecular weight of about 400,000. It appears as a ring-like structure on electron microscopic examination and may undergo transition to more complex physical forms on activation in the oocyte nucleus. The current interest is to define the cell biology of these enzymes, the structural requirements of each, and their role in tRNA transport.

In the area of tRNA transport, this group has determined the portions of the $tRNA_i^{met}$ molecule recognized by the mechanism in X. laevis oocytes. They constructed 30 different point mutants by in vitro methods and determined the transport phenotype by micro-injection and micro-dissection methods developed in this laboratory. The data demonstrate very clearly that the two highly conserved loops of the tRNA, the T and D loops, are critical for transport. In addition, every mutant defective in transport was also inefficiently processed. This result indicates that both the processing and transport mechanisms see the same portions of the tRNA molecule, and opens the possibility that one of the processing enzymes functions as a component in tRNA transport.

Studies also began in the area of mRNA transport this year. The system utilized is the thymidine kinase (TK) gene of Herpes simplex I in the X. laevis oocyte. Injection of the gene into the X. laevis oocyte nucleus results in the appearance of active enzyme several hours after injection. When kinetics of mRNA synthesis were followed, it was found that RNA is generated in two "phases:" Initially, TK RNA accumulates in the oocyte nucleus, and most is degraded. By 2 hours, however, newly made RNA appears to be effectively transported to the cytoplasm. The details of this system are under study.

Zasloff's section has also explored the molecular biology of a particular ALU sequence in the mouse this past year, and has discovered a new pathway by which this sequence generates a small RNA in the cytoplasm of mouse cells. The RNA studied is contained in the antisense orientation to the first intervening sequence in the mouse alpha-fetoprotein gene. They find that the primary transcript of this ALU sequence is processed by an endonuclease, transported from nucleus to cytoplasm, and packaged as ribonucleoprotein. The levels of this processed RNA vary between different mouse tissues, but are present in greatest abundance in fetal liver. These workers believe that this conversion pathway represents one means by which a natural antisense RNA is handled by the cell.

Studies on the pathophysiology of fibrodysplasia ossificans progressiva (FOP) continue, evaluating the therapeutic efficacy of the synthetic retinoid 13-cis retinoic acid in this disease. Current data suggest that the Vitamin A derivative is effective in reducing the appearance of new ossification centers.

Other clinical studies focus on diagnostic modalities of use in this rare condition, and it was shown that both Tc99 bone scanning and CT scan procedures effectively delineate the progress of the disease. The CT scan, furthermore, demonstrated the presence of new bone within fascia surrounding muscle, substantiating the view that the disorder expresses itself in tissues in that location rather than within muscle itself. Because of an early report that certain prostanoids were elevated in media bathing fibroblasts grown from active lesions of patients with FOP, plasma levels of PGE2 were assayed in some 25 patients this past year. From this RIA-based measurement, all patients were found to have markedly elevated levels of this compound. Currently, an attempt is underway to define the structure and identity of this prostanoid by direct methods.

Studies were begun in the area of collagen molecular biology as it relates to human bone disease, principally, osteogenesis imperfecta (OI). In the clinical sphere, many pedigrees were collected and appropriate tissues and blood samples were gathered to provide sources of DNA and RNA. The basic purpose of the effort is to define the molecular lesion in osteogenesis imperfecta so as to learn more about the normal mechanisms involved in bone development and growth in man. Initial efforts are directed toward an analysis of the collagen polypeptides and their genes since several studies from other laboratories have pointed to these loci as fundamental to the defect in some cases. Related to this area, J. Marini in this group has attempted to define whether the same splicing patterns are utilized in production of collagen a-2 Type I polypeptides from bone and skin fibroblasts, an issue that is important in the study of connective tissue diseases in man. Initial studies from this laboratory suggest that the 5' ends of a-2 differ in these two tissues. This group is in the process of determining the mRNA structure by cloning tissue-specific mRNA species from these two sources. Several clinical studies in osteogenesis imperfecta were also initiated this year including an endocrine study, which seeks to learn whether the striking differences in growth velocity exhibited by some but not all patients with O.I. might reflect the activity of the growth hormone-somatomedin axis.

Efforts in the treatment of the mucopolysaccharidoses (MPS) were continued. Initial reports that implantation of human amnion epithelium could effectively correct the biochemical defect in several of the MPS disorders spurred a clinical study in this area. After analysis of almost 20 children over the past year, J. Muenzer, et al. have been unable to detect any increase in circulating lysosomal enzyme or any obvious clinical response. One value of the study, however, was the establishment of clinical criteria which should provide a framework on which to gauge any future attempts at treatment. In addition, several clinical features of the MPS conditions not fully appreciated previously were uncovered. It was discovered that a surprisingly large number of children with MPS I and II develop hydrocephalus prior to or coincident with deterioration of CNS function. This suggests that early shunting may be of some use in these conditions. Other studies using electroretinography to assess the state of activity of the retinal neuronal population demonstrated that patients having the MPS disorders with CNS disease generally have rather severe rod-cone degeneration. These results should lead to improved management of visual problems of children with these conditions and provide objective measures of the neuronal health of the CNS in individuals with MPS. Basic studies on the purification of iduronate sulfatase also continue. Efforts have been underway for some time to purify this enzyme in order to initiate studies on the defect in the Hunter Syndrome. A new procedure utilizing preparative poly-

acrylamide gel electrophoresis under non-denaturing conditions was developed this past year, substantially improving purity and yield, and it is hoped that homogeneity (or something close to it) will be achieved in the coming year. With this protein in hand, attempts at the eventual cloning of the involved gene can be considered.

The section led by **A. Mukherjee** has been studying: (i) The function and genetic regulation of a steroid-dependent small molecular weight (15K) protein, uteroglobin, and (ii) the genetic predisposing factors in Fetal Alcohol Syndrome (FAS). During the past few years, several proteins have been characterized as endogenous anti-inflammatory substances. These proteins are potent phospholipase (PLA) inhibitors and exert their anti-inflammatory effects by reducing the level of tissue prostaglandins. Some of these proteins are: lipomodulin, macrocortin and renocortin. All of these proteins are induced by glucocorticoids, and thus it has been suggested that the anti-inflammatory effects of glucocorticoids are mediated by the above mentioned proteins. Uteroglobin is a progesterone-induced anti-inflammatory protein in the rabbit uterus and corticosteroid-induced protein in the tracheobronchial epithelium of the rabbit. During the past year this section investigated whether uteroglobin is also an inhibitor of phospholipases, thus explaining its similarity to the above mentioned proteins. They found that uteroglobin inhibits PLA2 derived from porcine pancreas as well as from cultured mouse macrophages. A dose dependent inhibition of PLA2 by uteroglobin was observed, with a maximum inhibition at a concentration of 20 μM of this protein. Mukherjee speculates that previously observed effects of uteroglobin, including its immunomodulatory, platelet aggregation inhibitory, and uterine antimotility effects, could be explained by one central effect, i.e., PLA-1 inhibition.

In a related study, this group found that in humans, a protein similar to uteroglobin can be identified by radioimmunoassay, Western blot and HPLC. The human protein has so far been identified in human tracheobroncheal, uterine and prostatic epithelium by immunofluorescence. Further characterization of this protein, including its function, is now being investigated.

Involvement of genetic factors in the pathogenesis of fetal alcohol syndrome (FAS) is strongly implicated from the facts that a) only 33% of alcoholic mothers give birth to FAS babies, and b) there are instances of fraternal twins born of chronic alcoholic mothers wherein only one of the twins is afflicted with FAS. During the past year, Mukherjee and his colleagues have shown that thiamine deficiency in the mother can lead to intrauterine growth retarded (IUGR) babies in the rat. These IUGR pups have been followed-up with regard to postnatal growth and learning ability. It was found that the IUGR babies born of thiamine deficient mothers remained significantly lower in their body weight and size, and they were significantly deficient in their learning ability as judged from their exploratory behavior compared to their control counterparts. These data suggest that postnatal growth retardation and deficient learning ability, characteristic features of FAS, may be related to thiamine deficiency in utero. Since some strains of rats are more susceptible to thiamine deficiency than others, these sensitive strains are now being studied to see if they have a transketolase with a high Km for thiamine pyrophosphate (TPP). If so, these animals will be used as models to study predisposing genetic factors in FAS in more detail. To characterize these animals biochemically, a method was developed for purifying the apotransketolase from various tissues to determine the Km for TPP. Since the effect of thiamine deficiency is most pronounced

in the brain, and since most of the studies on transketolase have been done on skin fibroblasts or RBC's, it will be important to show whether or not the Km for TPP of the fibroblast or RBC enzyme is similar to that of the brain enzyme.

The studies in the section directed by **Janice Chou** have concerned regulation of gene expression during normal and abnormal differentiation processes. Studies on the expression of the human placental alkaline phosphatase (PAP) gene were continued. This heat-stable phosphatase has been considered to be a marker for malignant transformation and is under developmental control. Tunicamycin, an inhibitor of protein glycosylation, was found to suppress PAP biosynthesis in addition to inhibiting protein glycosylation in general. The non-glycosylated PAP monomer, formed in the presence of tunicamycin, had a degradation rate similar to that of the glycosylated monomer. Tunicamycin suppressed PAP mRNA activity leading to the observed decrease in biosynthesis. Studies of the expression of the PAP gene at the molecular level have been extended over the past year. Genes encoding PAP subunits were cloned. Efficient isolation of these genes was accomplished by probing a phage λgt 11 recombinant DNA expression library with polyvalent antibodies directed against highly purified PAP made in this laboratory.

Studies on α- fetoprotein (AFP) gene expression in temperature-sensitive (ts) fetal liver cells were continued. It was demonstrated that AFP synthesized in cells exhibiting a transformed phenotype is a glycoprotein of 65,000 daltons. A non-glycosylated AFP-specific polypeptide of 48,000 daltons was the precursor of a glycosylated AFP-specific polypeptide of 59,000 daltons which existed only intracellularly. The 59,000 dalton polypeptide was processed to the 65,000 dalton AFP found in both cells and medium. This group showed previously that the non-glycosylated form of mature AFP is a polypeptide of 66,000 daltons. They now have found that these two AFP variants arise by translation of two mRNAs of 20S and 16S which are produced from a single AFP gene. Preliminary experiments indicate that the 16S RNA differed from the 20S AFP mRNA at the 5' end.

Chou and her colleagues have established adult hepatocyte lines by transforming adult liver cells with SV40 tsA mutants that are ts in the gene required for maintenance of transformation. The adult hepatocytes should complement the fetal hepatocytes in delineating the mechanisms controlling differentiation, transformation, and maturation. The SV40 tsA mutant-transformed adult hepatocytes are ts for growth and differentiation. They synthesize greatly increased levels of albumin and transferrin at 40°C, the temperature at which the cells exhibit a normal differentiated phenotype. Chou has now found that the levels of mRNAs for albumin and transferrin are greatly increased at 40°C. These data indicate that control of differentiation is regulated at the transcriptional level. In addition to albumin and transferrin, it was found that these adult hepatocyte lines express two other liver-specific functions, tyrosine amino transferase (TAT) and fibrinogen. The expression of genes for TAT and fibrinogen was also temperature-sensitive and dependent upon the presence of glucocorticoid hormone. These studies thus show that differentiation in these hepatocytes is programmed. The simultaneous activation or inactivation of a spectrum of highly specialized functions in these cells might aid in the discovery of factors that regulate differentiation processes.

In studies on the biosynthesis of human transferrin, an iron-binding glycoprotein and a growth factor for most cultured cells, Chou's group found that HepG2 hepatoma cells produced two species of anti-transferrin precipitable

polypeptides with apparent molecular weights of 74,500 daltons, predominantly extracellular, and of 72,000 daltons, predominantly intracellar. Secretion of the polypeptides into the media occurred 30-60 minutes after translation. In vitro translation studies of RNA isolated from HepG2 cells suggests post-translational processing, perhaps at the membrane level. Human transferrin from normal liver or serum has been shown to be a single polypeptide with an apparent molecular weight in the range of 73,200-79,500 daltons. Production of a second transferrin-specific polypeptide by human hepatoma cells may indicate an altered mechanism in transferrin gene regulation. In the presence of the glycosylation inhibitor tunicamycin, a single polypeptide of 71,000 daltons was produced by HepG2 cells. This suggests that the two transferrin-specific polypeptides may have different extents of glycosylation. When cells are grown in the presence of sodium butyrate, the 71,000-72,000 molecular weight species is induced. This may provide a useful system in which transferrin gene regulation in liver cells may be studied following transformation.

The group led by **William Gahl** continues to pursue the biochemical bases for genetic disorders in man. This involves bench research on cultured cells and physiological specimens, and clinical investigation of pathological manifestations and therapeutic interventions. A primary area of interest remains the lysosomal storage disease, cystinosis. After having shown that the primary defect in cystinosis lies in the deficiency of a lysosomal carrier system for cystine, this group further demonstrated how cysteamine (β-mercaptoethylamine) depletes cystinotic lysosomes of cystine. The drug, which passes through both the plasma and lysosomal membranes and participates with intralysosomal cystine in a disulfide interchange reaction, produces cysteine and cysteine-cysteamine mixed disulfide, both of which readily exit the cystinotic lysosomes. This provides the basis for this group's treatment of cystinotic chldren with oral cysteamine. After seven years of a clinical trial, they are finding that long-term cysteamine therapy improves growth if begun before 3 years of age, and slows the inexorable glomerular deterioration that characterizes the disease. Only the future will reveal whether cysteamine therapy initiated very early in life can protect fully against kidney failure. These workers are preparing for the time when that goal becomes reality by extending their knowledge of which other organ systems are clinically involved in cystinosis. They have found cerebral calcifications in a 24-year-old cystinotic man, as well as progressive visual impairment, posterior synecchiae, lens and retinal crystals, severe blepharospasm, and other ophthalmologic complications in several post-transplant cystinotic patients. They now recognize insulin-dependent diabetes mellitus, restrictive lung disease, and decreased salivary gland function as other late manifestations of cystinosis. All of these findings will allow the HGB investigators to chart the course of clinical intervention after the issue of renal deterioration is resolved.

Two other clinically relevant discoveries have been made in the past year. The more important, that individuals with renal tubular Fanconi syndrome have a marked plasma deficiency of free carnitine, derives from the fact that 97% of free carnitine is normally reabsorbed by the renal tubules, which are dysfunctional in Fanconi syndrome. Over 20 children with the reabsorption defect, including 19 cystinotics, had mean (± SD) plasma free carnitine levels of 11.7 ± 4.0 nmol/ml, compared with 42.0 ± 9.0 nmol/ml in normal individuals. The deficiency was shown to result from an increased fractional excretion of free and acyl carnitine, which averaged 33 and 26%, respectively, in Fanconi syndrome subjects compared with 3 and 5%, respectively, in normals. Hepatic oxidation of free fatty acids proceeded normally despite the plasma carnitine

deficiency, as evidenced by normal production of β-hydroxybutyrate and acetoacetate after a 24-hour fast in two cystinotic individuals. However, 10 muscle biopsies revealed a mean 60% depletion of tissue free carnitine and lipid droplet accumulation typical of muscle carnitine deficiency. An ongoing protocol involves supplementation with oral L-carnitine (100 mg/kg/day), and has already revealed that plasma carnitine levels are restored to normal within 24 hours of initiation of therapy. It is hoped that repeat muscle biopsies after 8 months of carnitine replacement will show resolution of the pathological features of muscle carnitine deficiency.

A second finding relates to the administration of cysteamine, chronic (19-59 months) use of which has been found to be associated with a blunting of prolactin release upon TRH stimulation in 5 cystinotic children. No abnormality of TSH release was observed, and there appeared to be no clinical concomitants of the phenomenon.

In one other intriguing disorder, mucolipidosis II ("I-cell disease"), cystine accumulates within lysosomes. The disulfide is the only amino acid stored, and fibroblasts provide the model system for the study of this occurrence. Gahl and his co-workers measured egress of cystine from lysosome-rich granular fractions of normal, cystinotic, and I-cell fibroblasts. The half-times for such cystine egress were 40 min, infinity, and over 100 min, respectively. This suggested that the cystine carrier was impaired in I-cells, perhaps because it required processing by lysosomal enzymes which are deficient in this disease due to the well-known absence of mannose-6-phosphate recognition markers.

Gahl's group is also studying two variants of a storage disorder characterized by the intralysosomal accumulation of free sialic acid, namely, free sialic acid storage disease and its Finnish counterpart, Salla disease. Patients afflicted with these disorders manifest mental retardation and poor development in infancy. Cultured cells have lysosomal inclusions and contain thirty times normal amounts of free sialic acid. These investigators have found that the mutant cell granular fractions display negligible egress of free sialic acid, while normals loaded with sialic acid exhibit a brisk egress of sialic acid. This suggests that the disorders represent lysosomal membrane transport defects.

A 31-year-old man with a 20-fold increase in plasma methionine was also studied. His disorder was diagnosed as methionine adenosyltransferase deficiency by liver biopsy, but he was clinically normal. This carries substantial importance for the five young children (each less than 6 years of age) previously reported with the extremely rare disorder. The subject also represents a unique opportunity to determine sulfur flux from methionine to inorganic sulfate in an individual with a major block in the transsulfuration pathway.

In a clinical protocol, Gahl and his colleagues are treating six pyridoxine-nonresponsive homocystinuric patients with betaine, a source of methyl groups which stimulates the remethylation of homocysteine to methionine. The double-blind, placebo-controlled crossover study is designed to determine if betaine therapy improves generalized osteoporosis, a significant complication of homocystinuria. Computerized tomography is employed as a sensitive indicator of vertebral body bone density, which is low in homocystinuric patients.

Finally, this group has shown that cysteamine can react in vivo with apolipoprotein E. Isoforms of this protein, which have cysteine replacing arginine at one of two substitution sites, are charge-shifted by cysteamine treatment both in vitro and in vivo. The demonstration of protein modification by circulating cysteamine makes feasible the treatment by oral cysteamine of diseases with cysteine-for-arginine substitutions which result in nonfunctional proteins. A prime example would be antithrombin III Toyama, in which a single cysteine for arginine mutation results in an antithrombin III which cannot inhibit the clotting cascade, giving rise to life-threatening thromboemboli. Oral cysteamine therapy may correct the functional defect.

Studies by **J. Butler** revealed that pantethine, a derivative of coenzyme A, and WR-1065, a radiation protective agent, both deplete cystine from cystinotic cells by intracellular formation of cysteamine-cysteine mixed disulfide and release of this compound outside of the cell. This, then, might comprise an alternative therapy for patients with cystinosis. In other studies, Butler found that metallothionine in cystinotic cells does not differ from that found in normal cells, indicating that its two-fold excess in cystinotic cells may reflect an abnormality in gene expression. Finally, Butler, in studies on a cystine-storing mutant mouse, demonstrated a defect in cholesterol esterification. The same defect is not seen in patients with cystinosis, but a related defect in cholesterol or fatty acid metabolism may be present in humans with this disease.

The section headed by **J. Sidbury** continues its studies on the treatment of the glycogen storage diseases, the kinetics of calcium metabolism in man, and the pathophysiology and treatment of various forms of childhood obesity. They have shown that glycogen storage disorders can be effectively treated by oral administration of cornstarch, an easy approach which yields predictable blood glucose levels with little fluctuation, replacing the complex and often fatal treatments previously attempted in these diseases. The group continues to study means of optimizing therapy, including a study of the therapeutic efficacy of several different naturally occurring vegetable starches. Studies of the metabolic pathways utilized for glucose production in individuals with the glycogenoses have been initiated, employing stable isotope methodology in collaboration with A. Yergey to trace metabolic routing. Stable isotope methodology has also been applied to a study of calcium kinetics in man, providing the first picture of available pools during pregnancy and in several human disorders of calcium metabolism.

Caddell and Sidbury have begun a study of the consequences of magnesium deficiency in the human neonate, with parallel studies in an animal model (the weanling rat fed varying levels of dietary magnesium). One hypothesis suggests that magnesium deficiency could be linked to the "Sudden Infant Death" syndrome, and Caddell's investigation includes a retrospective analysis of data from 200 premature neonates with apnea neonatorum to learn whether magnesium supplementation is beneficial. Using the weanling rat as a model, studies have also been concerned with catecholamine release in magnesium deficiency, the interaction of magnesium and calcium in furosemide-treated rats, and light and electron microscopic studies of the heart and lung in acute magnesium deficiency.

Cell Biology and Metabolism Branch--
Richard D. Klausner, M.D., Chief

Research in this Branch is directed toward clarifying developmental aspects of intracellular structure and function. Various methods are being utilized to study receptor biosynthesis, dynamics, regulation, and degradation, using the human transferrin receptor and the T-cell antigen receptor as models. (Receptors are a complex set of molecules that mediate virtually all aspects of interactions between the cell and its environment. They mediate cell-cell communication via hormones and neurotransmitters, invasion by viruses, uptake of nutrients, regulation of growth and differentiation, and recognition of specific molecules.) The Branch also conducts clinical research on the fundamental mechanisms and treatment of patients with genetic disorders of iron metabolism (hemochromatosis) to learn how the intracellular traffic of iron is normally controlled, and at what level iron metabolism is abnormally regulated in such patients. In another area of interest, the Branch examines mechanisms by which intracellular architecture is maintained, providing the basis for the function and dynamics of cellular organelles.

R. Klausner's section is engaged in studying the T cell antigen receptor. A complete description of the components of the murine T cell antigen receptor complex has been developed based upon the ability to immunoprecipitate the complex using a clonotypic anti-receptor monoclonal antibody developed by L. Samelson. The receptor recognized by this antibody is expressed on a continuously growing hybridoma, 2B4, which recognizes the carboxy terminal fragment of pigeon cytochrome C. When 2B4 cells are labeled with the hydrophobic radioiodinated covalent probe trifluoromethyl iodophenyl diazarine (TID), four distinct proteins other than the α , β receptor are specifically immunoprecipitated by the A2B4 antibody which recognizes the α - β heterodimer. A variety of other antibodies against T cell surface proteins fail to precipitate this complex. When another T cell hybridoma was labeled with TID and its receptor immunoprecipitated, the same set of associated proteins was observed. The specific co-immunoprecipitation of these proteins from two different cell lines (only by the appropriate monoclonal antibodies that recognize the receptor on each of these lines) ruled out any interpretation other than that the four proteins are specifically associated with the clonotypic α-β chains of the antigen receptor.

After identifying the four protein chains, studies aimed at characterizing their biochemical properties were undertaken. The gamma (γ) chain has a molecular weight of 25,000 daltons. Asparagine-linked carbohydrate chains can be removed by treatment with endoglycosaminidase F, leaving a peptide of molecular weight 14,000. The delta (δ) chain is also a glycoprotein whose molecular weight drops from 21,000 to 16,000 upon removal of the carbohydrate. The δ chain is a phosphoprotein whose isoelectric point becomes more alkaline after treatment with alkaline phosphatase. Removal of both sialic acid and phosphate reveals a very basic protein with an apparent isoelectric point of greater than 8.5. The epsilon (ε) chain is not a glycoprotein and its molecular weight is 26,000. Interestingly, it contains intrachain disulfide bonds and in the absence of reduction its apparent molecular weight is 22,000. The zeta (ζ) chain is also not glycosylated and its molecular weight is 16,000. In the membrane the ζ chain exists as a disulfide linked homodimer. Thus the complete T cell antigen receptor can be described as a seven chain membrane complex composed of six distinct polypeptides.

The addition of specific antigen to the 2B4 T cells results in the rapid phosphorylation of the δ chain of the receptor. This phosphorylation only occurs if the antigen is presented by an appropriate Ia molecule-bearing cell. Furthermore the phosphorylation is inhibited by anti-Ia antibodies which block the activation of the T cell. These T cells can be activated by the mitogen concanavalin A and this also leads to phosphorylation of the δ chain. However, in contrast to antigen, this does not require presenting cells and is not blocked by either anti-receptor antibodies or anti-Ia antibodies. The δ chain can also be phosphorylated by the addition of calcium ionophores in the presence of calcium. The antigen-induced phosphorylation of the δ chain occurs on serine residues. Furthermore at least two distinct sites are phosphorylated in response to antigen stimulation.

The δ chain can also be phosphorylated in cells by the addition of phorbol esters such as PMA. The level of phosphorylation in a population of cells is higher than can be achieved by antigen and presenting cells are not required. There is no evidence for additional sites on the δ chain being phosphorylated by PMA and in fact only one of the two sites phosphorylated by antigen is affected by phorbol esters. Also in contrast to the antigen induced phosphorylation, PMA leads to phosphorylation of the ε chain to nearly the same extent as the δ chain. The residue phosphorylated in response to PMA is serine.

Because PMA leads to the phosphorylation of the δ chain, the activation of protein kinase C by endogenous diacylglycerol in response to antigen stimulation was examined. Antigen, only in the presence of an appropriate presenting cell, leads to the breakdown of phosphatidyl inositol in the T cells as measured by the production of water soluble inositol phosphates. The production of these products must arise from the action of a phospholipase C which liberates diacylglycerol along with the lipid base. Furthermore the stimulated metabolism of phosphatidyl inositol can be specifically inhibited by dibutyryl cyclic AMP. Consistent with the role of lipid metabolism in the phosphorylation is the inhibition of the antigen induced δ chain phosphorylation by cyclic AMP. Cyclic AMP fails to inhibit the δ chain phosphorylation induced by PMA or calcium ionophore. The coupling of antigen-activated receptor to phospholipid metabolism is being analyzed to determine which enzyme or enzymes are directly activated in this process. Data have been obtained which support the activation of a phospholipid kinase as the proximal step in T cell activation. An *in vitro* cell-free assay for this kinase has been developed. It is a magnesium requiring integral membrane protein that utilizes ATP and phosphatidyl inositol to produce monophosphophosphatidyl inositol.

Another group studying receptors of the immune response is headed by Warren Leonard, who recently joined the Branch. He is establishing his own research group studying the mechanism of action and regulation of T cell growth factor (IL-2) receptors. This group adds to the Branch's program on the molecular basis of receptor function in immune activation. While in the National Cancer Institute, Leonard cloned, sequenced, and expressed cDNAs encoding the human interleukin-2 (IL-2) receptor. More recently, he identified genomic phage clones spanning most of the IL-2 receptor gene, and has determined the map of the gene, the sequence of the exons and exon/intron splice junctions, and the location of two of the three IL-2 receptor promoters. Leonard's initial investigations are directed toward the following goals: (1) Determining the difference between high and low affinity IL-2 receptors. Only the high affinity IL-2 receptor appears to be biologically active. When the IL-2 receptor cDNA is

transfected into mouse L cells, only low affinity receptors are expressed. The leading hypothesis for the difference is that associated subunit(s) must be present to form a high affinity receptor, although the possibility of a second gene cannot be excluded. Studies will focus on (a) expressing the IL-2 receptor cDNA in T cells to determine if high affinity binding is achieved, and (b) performing chemical cross-linking experiments with ^{125}I-IL-2 and precipitations with both anti-IL-2 receptor antibodies to identify other subunits. Preliminary experiments are consistent with either the existence of associated subunits or with the ability of more than one IL-2 molecule to bind to a receptor molecule. (2) Making use of the promoter regions of the gene to search for DNA binding proteins. Pilot studies will be performed using the methods of Wu (exonuclease III) and of Emerson and Felsenfeld (essentially a Western blotting methodology). (3) In a collaborative study, the promoter regions are also being studied by Uli Siebenlist, who is evaluating DNaseI hypersensitivity. (4) Looking for potential enhancer elements. Two different DNA fragments that have their 3' termini near the 5' end of the cDNA and extend different distances 5' have been ligated to the chloramphenicol acetyltransferase (CAT) gene. These constructs differ in their apparent promoter activity in CAT assays, suggesting that the longer construct includes critical DNA regions for optimal promoter function. Attempts will be made to identify the critical regions.

A second area of research in the CBMB focuses on the cell and molecular biology of iron metabolism. Two groups work in this area. One, directed by **Joe Harford**, studies the human transferrin receptor. Treatment of K562 cells, a human erythroleukemia cell line, with the iron chelator desferrioxamine raised the levels of the transferrin receptor approximately 3-fold over that of control cells. In contrast, the levels of the receptor were reduced significantly by provision of exogenous iron to cells in the form of diferric human transferrin, ferric ammonium citrate, or hemin. Using metabolic pulse labeling of cells with [^{35}S]-methionine, receptor-specific immunoprecipitation, and SDS-polyacrylamide gel electrophoresis, the rate of biosynthesis of the transferrin receptor was found to be elevated by desferrioxamine and reduced by iron provision. None of these treatments had any effect on the rate of transferrin receptor degradation. The alteration in receptor biosynthesis was correlated with corresponding changes in the level of receptor-specific mRNA. This was determined utilizing a cDNA probe for the receptor to probe Northern blots of RNA preparations from cells under various states of iron availability. Hemin was found to be a particularly potent modulator of receptor biosynthesis. It had been suggested by others that hemin directly affected receptor expression by a mechanism other than supplying iron to the regulatory iron pool. By performing crossed concentration dependence experiments with hemin and desferrioxamine, this model was shown to be erroneous. The effect of hemin on receptor biosynthesis proved to be fully attributable to the ability of hemin to supply chelatable iron.

To determine whether receptor mRNA levels were altered via changes in the transcription rate, nuclei were isolated and *in vitro* transcription experiments performed. Nuclei from cells treated with hemin were compared with those from desferrioxamine-treated cells. Transcribed RNA was labeled with [α-^{32}P] uridine and the mRNA encoding the transferrin receptor was selected by hybridization to an immobilized receptor cDNA clone. These experiments indicated a significantly higher rate of receptor mRNA synthesis in desferrioxamine-treated

cells than in cells treated with hemin. Manipulations of intracellular iron result in greater than 100-fold differences in receptor mRNA. The half-life of this specific mRNA is quite short, being less than 30 minutes in K562 cells.

Promotor activity is present in an isolated region of the gene containing approximately 120 base pairs upstream of the transcription initiation site. This is demonstrated by expression of the human receptor after transfection of mouse L cells, using genomic DNA for the receptor that contains the 5' upstream region. However, this expressed gene fails to demonstrate regulation by iron. Furthermore, when this promotor is attached to the bacterial enzyme chloramphenicol acetyl transferase, it will support the transcription of the enzyme in short term transfection assays. Again no regulation of this expression by iron is seen.

Treatment of K562 cells with a monoclonal antibody (OKT9) against the transferrin receptor has marked effects on receptor dynamics and the survival of the receptor. The antibody employed recognizes a receptor determinant distinct from the ligand binding site. OKT9 treatment leads to a rapid ($T\tau < 10$ min) redistribution of receptor such that a lower percentage of the receptors involved in iron uptake are displayed on the cell surface. On a somewhat longer time scale the number of receptors in the "cycling pool" is reduced. Radiolabeling of K562 cells with either [^{35}S]-methionine or by lactoperoxidase-catalyzed surface iodination allowed the half-life of the transferrin receptor to be assessed. Treatment with OKT9 resulted in a decrease in this parameter from 8 hr to 3 hr. Although transferrin receptor turnover is unaffected by ligand, the antibody treatment results in a "down-regulation" of the receptor analogous to the ligand-mediated down regulation observed in other receptor systems such as those for insulin or epidermal growth factor. Based on continued iron uptake from [^{59}Fe] transferrin, it appears that receptors with bound OKT9 continue to traverse the transferrin cycle. The profound effect on receptor half-life is apparently accomplished by a relatively subtle decrease in recycling efficiency per cycle from 98% to 95%.

The second group studying iron metabolism has been headed by Jos van Renswoude, a Visiting Scientist in the CBMB. This group has focused on the intracellular aspects of iron metabolism. A substantial fraction of iron, delivered to the cell via the transferrin cycle, ends up in ferritin. The remainder is distributed over a variety of intracellular targets, e.g., heme proteins, iron-sulfur centers, etc. The regulation of the distribution of newly delivered iron between storage/detoxification (ferritin) and utilization (e.g., heme synthesis) compartments has been studied using various perturbants of cellular iron homeostasis. These include: diferric transferrin at saturating concentrations, ferric protoporphyrin IX (hemin), the cell-permeant iron chelator, desferrioxamine, and the monoclonal anti-transferrin receptor antibody, OKT9. It was found that the fractional distribution of ^{59}Fe to ferritin ranges from 0.05 to 0.7 and that it is strictly correlated with the size of the total intracellular ferritin pool. When the cellular ferritin levels are high, a relatively high percentage of the newly delivered iron will go to ferritin. When ferritin levels are low, a relatively small fraction will become ferritin-associated. This correlation between ferritin level and fractional delivery of iron to ferritin is formulated as a nomogram, characteristic of the type of cell studied. The distribution of iron to ferritin proved to be independent of the amount of iron taken up by the cell, per unit time. The distribution function therefore has the characteristics of a partition. The first implication of this finding is that the fractional delivery of iron to ferritin

in K562 cells (growing exponentially in culture) can simply be predicted from the ferritin level at the time of iron entry. Second, this partition drives the distribution of intracellular iron that may serve to maintain cellular iron homeostasis vis-a-vis utilization processes such as the biosynthesis of iron-containing proteins. Ferritin levels reflect the iron status of the cell. Sustained iron supply (diferric transferrin, hemin) leads to an increase in cellular ferritin through enhanced biosynthesis, whereas iron deprivation via chelation of intracellular iron (desferrioxamine) or via reduction of iron input via down-regulation of the transferrin receptor (with OKT9) results in a fall of the intracellular ferritin concentration as a result of decreased biosynthesis. The effects of perturbation of cellular iron homeostasis on ferritin levels are rapid in these cells. The ferritin concentration can vary over a 50-fold range within 36 hours. The degradation of this ferritin is complex and under continuing study. Ferritin degradation is characterized by an obligatory lag period before degradation is started, and there are preliminary data suggesting that the light and the heavy subunit turn over at different rates. The lag period may represent a set of chemical/physical changes within the ferritin molecule, necessary to allow complete proteolytic breakdown of the molecule. Regulation of the degradation of ferritin by different drugs has been observed and these act at the level of the lag phase.

The above mentioned relationship between fractional delivery of iron to ferritin and existing ferritin levels in K562 cells bears a striking similarity to the "rule" which governs intestinal iron absorption: The percentage of an oral iron load that is absorbed, i.e., goes into the circulation, is quantitatively inversely proportional to the total body iron stores and hence to cellular ferritin levels. The implication of this similarity is that intestinal iron absorption defects might be studied using model systems, such as K562.

In the third area of this Branch's research activity, Ignacio Sandoval, a Visiting Scientist, directs a group studying the origin, dynamics and function of intracellular organelles. They are specifically interested in proteins that make up the membranes of the Golgi, lysosomes and secretory vesicles. These workers have obtained specific monoclonal antibodies against integral membrane proteins from secretory granules to study the traffic of these organelles in secreting cells. The traffic of secretory granules most likely results from the sorting of their membrane proteins in different sites of the cell. One of these monoclonal antibodies, 5G10, has been extensively used for this purpose in studies carried on in RBL cells. The antibody recognizes an 80 kdalton membrane glycoprotein in RBL cells metabolically labeled with [^{35}S]methionine. In non-secreting cells, the protein is exclusively located in the membrane of secretory granules, as shown by their reaction with granules stained for the secretory product heparin with Wright's reagent. Furthermore, the exposure of the 5G10 epitope on the cell surface during secretion has confirmed the localization of the 5G10 antigen in the membrane of secretory granules, and has allowed study of the insertion of these membranes into the plasma membrane during the release of secretory products by exocytosis. The correlation between membrane insertion and secretion has been demonstrated by the increase in the levels of 5G10 antigen expressed on the surface of cells stimulated to secrete with either DNP-BSA (i.e., allergen)-activated IgE or the Ca^{++} ionophore A23187, as well as by the failure of irrelevant IgE to stimulate the surface expression of the antigen and the interruption of that expression following the dissociation of the DNP-BSA-anti DNP IgE with DNP lysine. Furthermore, the insertion of the membranes from secretory granules into the plasma

membrane is a very fast process, as shown by the expression of maximal levels of 5G10 antigen on the cell surface only 5 minutes after the stimulation of secretion.

The regulated secretion displayed by RBL cells has also allowed study of the retrieval of the inserted membranes independently from insertion. Sandoval and his colleagues have calculated from the rate of surface disappearance of the antigen, measured in cells whose secretion was stopped, that its half-life in the plasma membrane is about 10 minutes. That the disappearance of surface 5G10 antigen is due to internalization has been directly shown by measuring the internalization of iodinated 5G10 antibody by secreting RBL cells. Interestingly this rate is comparable to that of surface ligands internalized by receptor-mediated endocytosis. Monitoring of cells continuously stimulated to secrete has revealed that the expression of the 5G10 antigen on the cell surface is sustained for 45 min, after which it declines slowly. This sustained expression probably results from the continuous insertion of the membranes from secretory granules into the plasma membrane and their rapid removal by endocytosis. This membrane retrieval is most likely carried on by the cell to maintain the constancy of the chemical composition of the plasma membrane.

Immunoelectron microscopy studies, using antibody 5G10, have revealed that the internalization of the membranes of secretory granules is initiated via coated-pits. Furthermore, immunofluorescence microscopy studies show that the internalized antigen stays briefly in vesicular organelles located in the cell periphery, probably in endosomes, before moving to the area containing the Golgi apparatus, and is finally found in secretory granules. Study of the transit of the antigen through the Golgi apparatus, a step that could be required to repair and reprogram the protein, is being performed by analyzing the glycosylation of deglycosylated antibody internalized by endocytosis. The recycling of the 5G10 antigen back to secretory granules seems to be a very effective process, as no degradation of the radioiodinated 5G10 antibody bound and internalized with the antigen has been detected. Electron microscopy experiments are being carried out to characterize each one of the organelles traversed by the 5G10 antigen in its route from the plasma membrane to secretory granules.

The significance of these studies on immune response receptors, iron metabolism and physiology, and the structure and function of cellular organelles can be summarized as follows: The activation of T cells is the central event in immune responses. The molecular mechanisms of this process are, however, entirely unknown. An attempt to define the detailed nature of the molecules involved in T cell activation is thus an essential prerequisite to elucidating this processes. Furthermore, defining the biochemical and enzymatic mediators of activation signals may allow for rational intervention in abnormal immune responses in human problems ranging from immunodeficiency to autoimmunity.

With respect to the transferrin receptor, it should be noted that this system is one of the better characterized endocytic systems. All proliferating cells utilize transferrin receptors to acquire iron and it is instrumental in a wide variety of essential biochemical processes including energy production and cell division. The regulation of the acquisition of iron is apparently accomplished by modulation of the expression and function of the transferrin receptor, and the studies conducted here on the molecular events and mechanisms underlying this modulation should add significantly to the understanding of these processes. Moreover, insights gained regarding the dynamics of the transferrin receptor and regulation of its gene should be applicable to membrane protein

movement and gene regulation in general. It is also evident that the regulation of intracellular iron is essential to the control of proliferation and normal metabolic function. The finding that the regulation of intracellular ferritin by iron in turn regulates the cellular handling of delivered iron allows a molecular model for iron homeostasis. Illumination of the details of how this mechanism is encoded will be of great value in understanding cellular control. Hereditary hemochromatosis is a common genetic disorder present in approximately one of four hundred Americans. These laboratory studies, which in all likelihood relate to the molecular basis of this disease, should lead to an early understanding of the basic defect in patients with this life-threatening disorder. (The CBMB, in fact, directs an outpatient clinic for the study and management of hereditary hemochromatosis.)

Finally, the use of specific antibodies against integral membrane proteins from secretory granules is helping these workers to gain new insight into the mechanisms of cellular secretion. Understanding of these mechanisms should be useful in determining the defects in the secretory pathway that may be responsible for the development of a number of hereditary endocrine, immunological, and neurological diseases.

Laboratory of Developmental and Molecular Immunity--
John Robbins, M.D., Chief

This Laboratory conducts research into the developmental and molecular biologic aspects of "natural" and immunization-induced immunity. The areas of interest include: 1) The immuno-pathogenic mechanisms of various infant and childhood bacterial diseases, development of vaccines for their prevention, and characterization of the age-related immuno-regulatory mechanisms of the young host; 2) Class I transplantation antigen genes, an essential system for the recognition of cell-associated antigens, which in this Laboratory are modified by site-specific mutagenesis in order to determine the structural basis for the immunologic polymorphism and function of these antigens at the DNA level (the activation and expression of paternal Class I transplantation antigen genes is also being studied in developing embryos); 3) Lastly, the immuno-regulatory functions of lymphoid cells, and the effect upon these activities which is exerted by bacterial toxins of biological interest (this is being studied in vitro using hybridomas constructed of T-cells at various stages of differentiation).

During the past year, **J. Robbins** and **R. Schneerson** continued their work on the pathogenesis and prevention of invasive diseases caused by encapsulated bacteria. Their emphasis has been primarily on Haemophilus influenzae type b and pneumococci. These two bacteria remain a serious cause of morbidity and mortality in infants and young children, despite the availability of effective antibiotics and supportive therapy.

Recently, an H. influenzae type b polysaccharide vaccine, initially developed by these investigators in the early 1970's, was licensed and recommended by the American Academy of Pediatrics and the U.S. Public Health Service for "universal immunization of all children at 2 years of age". Since its development, however, clinical studies have established the age-related and T-independent immunogenicity of this vaccine which limits its use in young infants (who are at greatest risk for H. flu complications). Methods were therefore developed

to covalently bind the H. influenzae type b polysaccharide to proteins in order to increase its immunogenicity and confer the properties of T cell-dependence. The H. influenzae type b polysaccharide-tetanus toxoid conjugate was the first of a series of prototype conjugate vaccines to be synthesized and evaluated in this Laboratory. Following an extensive study of these conjugates in laboratory rodents, and in juvenile as well as infant primates, it was concluded that the conjugates had both increased immunogenicity and T-cell dependent properties, and could induce protective levels of antibodies in infant primates after an immunization course, in a dosage consistent with that used for routine infant immunization in the United States. (This was of particular importance because of lesser reactivity of this non-human species toward this class of antigens as compared to humans). During the past year, two clinical studies were conducted with these conjugates. The first was in young adult volunteers at Davidson College in North Carolina. Two injections of the conjugates were given 3 weeks apart, and the antibodies measured by radioimmunoassay and a class-specific ELISA in order to determine both the isotype and subclasses of the polysaccharide antibodies induced by these vaccines. All volunteers responded with a 4-fold or greater increase in type b antibodies, which rose to about 180-fold above their pre-immunization level. These levels declined to about one-half of the original values 6 months after immunization. This represents about a 20-fold increase in immunogenicity over that of polysaccharide alone. The conjugate-induced antibodies had all of the biologic properties associated with immunity toward H. influenzae type b and pneumococcus infection. The H. influenzae type b conjugate was also given to young adults in Goteborg, Sweden with defined immunodeficiency disorders involving one or two subclasses of IgG. Seventeen of the eighteen immuno-deficient patients responded with levels of antibodies indistinguishable from those achieved in the adult volunteers. Only one, an individual with a combined subclass and IgA deficiency, failed to respond with a 4-fold or greater increase in antibodies. Clinical studies in infants, ages 18 months to 24 months, are planned. The animal data and the volunteer studies strongly suggest that the conjugates will induce protective levels of antibodies in infants, unlike the newly licensed H. flu vaccine. Accordingly, epidemiologic information has been collected in Goteborg, Sweden, in preparation for a double-blinded study in which the nearly synthesized H. influenzae type b polysaccharide conjugate vaccine (as well as the new pertussis toxin vaccine) will be studied for immunogenicity and effectiveness.

Pneumococcal infections cause otitis media, a common and often chronic infection of infants which is a major cause of acquired hearing loss in the United States. Clinical trials with pneumococcal vaccine have shown that immunization-induced capsular polysaccharide antibodies confer protection against otitis media. Yet, the current pneumococcal vaccine is not satisfactory for prevention of otitis media in infants and children, the age group with the highest attack rate, because of its age-related immunogenicity and its failure to induce a booster response (T-independent). Two approaches have been used to solve this problem of inducing pneumococcal immunity. The first was to devise a species-specific, rather than a type-specific, pneumococcal antigen. A covalent conjugate between a species-specific surface antigen, C-polysaccharide, and several carrier proteins was achieved using a novel method for binding these two different macromolecules. The C-polysaccharide was chosen because of previous reports indicating that antibodies to phosphocholine (PC) could protect mice against pneumococcal infection. The C-polysaccharide protein conjugate was injected into rabbits and induced antibodies to the otherwise non-immunogenic C-polysaccharide. Antibodies to the C-polysaccharide, as

well as other components of pneumococci, were induced by unencapsulated pneumococcus with an unusually long C-polysaccharide chain. The C-polysaccharide antibodies, induced by the C-polysaccharide protein conjugate, had no reactivity with PC. (PC was present in the conjugate as shown by its reactivity with monoclonal PC antibody.) The C-polysaccharide antibodies induced by this vaccine did not protect mice. However, mice were protected by passive immunization with antibodies against unencapsulated bacteria. Yet, absorption of this sera and bacteria with the purified C-polysaccharide failed to maintain its protective activity. These experiments showed that the PC-containing structure that induced protective immunity was probably not the C-polysaccharide, and a search for other PC-containing structures is under way. Another approach is to create a more immunogenic capsular polysaccharide, of an optimum molecular weight and homogeneous polysaccharide, for synthesis into protein conjugates. A smaller, more homogeneous, polysaccharide has now been synthesized in protein conjugates and its immunogenicity and other serologic properties are being defined.

Typhoid fever is another example of an invasive disease due to encapsulated bacteria, Salmonella typhi. The capsular polysaccharide of this bacteria is the Vi antigen. The immunopathogenic role (and its potential for a vaccine) of the Vi antigen has been studied by Robbins' group. On the basis of this study, two clinical lots of a Vi polysaccharide prepared under non-denaturing conditions were evaluated in volunteers. The degree of adverse reactivity was shown to be related to the amount of lipopolysaccharides (LPS). The serum antibodies elicited by these two Vi polysaccharides were shown to be equal to those observed after convalescence from typhoid fever, and equal to those elicited by the current whole cell vaccine. In addition, the availability of a pure Vi polysaccharide has prompted reinvestigation into the serologic response to disease. It has been found that determination of the Vi antibody level provides a reliable and rapid method for identifying asymptomatic carriers of S. typhi. Asymptomatic carriers are difficult to identify by conventional methods of culture. This diagnostic method has now permitted the identification of carriers and, therefore, more effective control of typhoid than heretofore possible. A new method for covalently binding the Vi polysaccharide to carrier proteins, including cholera toxin, has been devised. This Vi-cholera toxin conjugate has superior immunogenic properties to the Vi polysaccharide alone, and its clinical use is being evaluated. Two clinical trials of a Vi polysaccharide with low LPS content should be initated in early 1986.

The age-related development of protective antibodies to the capsular polysaccharides of common encapsulated bacterial pathogens, such as meningococcus, pneumococcus, and H. influenzae type b, has been shown to be largely stimulated by a continual interaction with non-pathogenic flora of the respiratory and gastrointestinal tracts. The origin of Group A meningococcal capsular polysaccharide antibodies, however, has never been clearly identified. In collaboration with Egyptian investigators, two Escherichia coli strains with capsular polysaccharides (K antigens) were shown to be capable of inducing bactericidal and precipitating antibodies to the Group A polysaccharide. The structures of these two E. coli were identified. It is of interest that the more cross-reactive one, the E. coli K93 capsular polysaccharide, has no obvious structural relation to the Group A polysaccharide. The stereochemistry of these two related polysacharrides, E. coli K93 and Group A meningococcus, is currently being investigated.

Pertussis remains a major health problem despite its rarity in the United States. Control of pertussis has been achieved by mass immunization with vaccines composed of whole inactivated Bordetella pertussis. While these vaccines have been effective in virtually eliminating pertussis, they have come under increasing scrutiny because of the serious adverse reactions sometimes associated with their use. **R. Sekura** has recently developed methods for the large scale production of pertussis toxin (PT), an extra-cellular protein excreted by B. pertussis. Investigations have indicated that serum anti-PT has an enzymatic action, ADP ribosylation, similar to that of other bacterial strains. The binding of B. pertussis to mammalian cells was studied using the glycoprotein, fetuin, as a model compound. PT binding to fetuin was shown to be due to its interaction with an asparagine-linked oligosaccharide. A novel method was devised in order to inactivate the PT enzymatic activity while retaining at least one-third of its antigenicity. The detoxified PT was shown to induce protection against intracerebral challenge with B. pertussis and to protect the immunized animal against the lethal effects of PT. A series of assays to monitor the retained antigenicity, immunogenicity, and toxicity was devised that could be used for standardization of a PT vaccine for clinical use. In addition, serologic methods for both the absolute measurement and biologic assay of antibodies induced by the vaccine, and those induced by the disease per se, were established. It is now planned to study this novel toxoided PT in sequential clinical trials involving first adults and then, infants and children. Ultimately, the pertussis toxoid vaccine will be used in a double-blinded trial along with the H. influenzae type b vaccine in Goteborg, Sweden, to characterize its effectiveness in preventing these two common serious diseases of infants and children.

B. pertussis is only a pathogen for humans; pertussis has never been established in an animal species. B. pertussis secretes a variety of biologically active extracellular materials, including adenylate cyclase and an activity referred to as heat-labile (HLT) or dermonecrotic toxin. This latter activity has never been characterized. Sekura and his associates have isolated this HLT from sonicates of B. pertussis cells. They have found that HLT is an unstable protein and has extraordinarily high toxicity, with an unexplained propensity to cause involution of the spleen of suckling mice. The structure-function relation, and its immunopathogenic role, are under scrutiny by a variety of techniques, including studying the effect of passive immunization to HLT with polyclonal and monoclonal antibodies.

The Class I major histocompatibility antigens are cell surface polymorphic membrane glycoproteins required for recognition by T-cells. They are complex molecules composed of at least three domains. **K. Ozato's** group has studied the structure-function relationships of these Class I antigens by site-directed mutagenesis, in which the DNA sequence of a Class I gene is changed at desired positions by recombinant DNA technology. A mutant lacking the disulfide bridge in the domain most proximal to the plasma membrane (external domain) was incapable of expressing the Class I antigen in this membrane. The antigen was synthesized, as evidenced by its accumulation within the cell, but could not be expressed in the plasma membrane. This observation, and kinetic studies with other mutant antigens, has led Ozato to propose that the disulfide bridge in the third domain is essential for transport of the Class I antigen from the endoplasmic reticulum to the plasma membrane. Another mutant, lacking all of the endolinked glycosylation sites, was produced by three-step mutagenesis. The resultant mutant antigen was found to be expressed on cell surfaces, although at a reduced level. The expressed antigen was, however, reactive with both

cytotoxic T-cells and a panel of antibodies specific for the Class I wild-type antigen. These data indicate that carbohydrate moieties are not essential for the immunogenicity and immune function of Class I antigens. This experimental approach, using site-directed mutagenesis and DNA recombinant technology, will be used to identify completely the structure-function relations of the Class I antigens and will be extended to other histocompatibility antigens.

The ontogeny of Class I histocompatibility antigens has been the subject of numerous investigations. However, the experimental data resulting from these studies, which is based upon evidence with polyclonal antisera, has been contradictory and not generally accepted. Ozato and her colleagues have now shown that the Class I antigens are first expressed at about 9 to 10 days of fetal development in the mouse. This conclusion was based upon embryo studies using a well-defined panel of monoclonal Class I MHC antibodies and several immunochemical assays. Homozygous mice, differing only at the Class I MHC locus, were used. It was also found that MHC expression can be accelerated by interferon-alpha, beta or gamma. The expression of paternally inherited Class I antigens after exposure of the fetus to appropriate antibody was also studied. Paternal MHC Class I antibodies were administered to the fetus via the placental circulation by passive immunization of the mother. The cellular distribution of these passively administered monoclonal antibodies was studied; they were detected in various fetal tissues and inhibited the expression of these Class I antigens. This finding, using highly specific antibodies and quantitating their interaction with cell antigens, provides the basis of a powerful tool for studying the ontogeny of these structures and their effect upon immune function.

Another aspect of ontogeny of Class I antigens was studied by using early embryo and embryonal teratocarcinoma cells, as well as a model of retrovirus infection. It has been known that production of retroviruses does not occur in early embryo or teratocarcinoma cells. These teratocarcinoma cells and embryonic cells, from as early as 8 days after fertilization, could be productively infected by some mouse leukemia viruses of various groups. There was no relation between the expression of the Class I antigens and susceptibility to infectivity. However, the administration of interferon, as was observed in vivo, resulted in the expression of the MHC Class I antigens in these in vitro cells. It is now planned to study the conditions for an experimental system in which Class I gene expression can be inhibited. Several approaches are under consideration, including the construction of anti-sense DNAs for Class I genes which produce transcripts complimentary to the Class I gene message. These anti-sense DNAs will be introduced into fertilized mouse embryos in order to obtain transgenic mice in which Class I gene expression can be inhibited throughout the animal's life. In addition, the effect of passive immunization, with paternal histocompatibility monoclonal antibodies, upon the expression of the Class I genes will also be studied.

E. Hanna's group studies the regulation and control mechanisms in immune systems and how microbial products, such as the streptococcal exotoxin (SPE) and pertussis toxin (PT), influence or deregulate these immune systems. Investigations are under way to: 1) Understand the mechanisms by which thymus-derived T-cells regulate antibody formation and secretion by interactions with bone marrow-derived precursors (B-cells) of antibody forming cells, and 2) to understand the interrelationships between the various T-cell phenotypes, from the precursor stage through development of helper and suppressor functions. A number of monoclonal lines and hybridomas which express regulatory T-cell func-

tions (both helper and/or suppressor) have been constructed from fractionated spleen cells of wild type mice and nude mice. Some of these hybridomas, from nude mouse splenocytes, express surface molecules (Thy 1, Tla, Lyt 1, Lyt a, and Lyt 3) and functional phenotypes which classify them as model precursors of regulatory T-cells.

To understand how effector or cytotoxic T-lymphyocyte (CTL) responses are regulated, and whether other T-cells which regulate Ig formation by B-cells may also regulate CTL, a number of CTL clones have been derived by repeated stimulation with the antigen and in the continuous presence of T-cell growth factor (TCGF). Some of these CTL clones are restricted in their recognition of H-2K^{k}-TNP and others are restricted in their recognition of alloantigens.

Hanna's experiments are also directed towards understanding how microbial agents such as SPE and PT divert thymus-derived T-cells towards different phenotypes or different functions. Previous experiments suggested that SPE deregulates the down regulation of T-cell dependent antibody responses. Current experiments, using monoclonal T-cell lines, show that the effective targets of SPE responsible for the observed activity are precursor T-cells, rather than mature suppressor T-cells. Pertussis toxin is also suppressive for the development of B-cell (PFC) responses. Cytotoxic T-lymphocyte responses to alloantigens are also suppressed by both SPE and pertussis toxin. Since CTL-cloned lines are not suppressed by either SPE or PT, it is suggested that either CTL precursors or regulators of the CTL responses may be the site of action by these toxins. Although SPE and PT suppress CTL responses, experiments to date suggest that these two toxins may have different cellular sites of action in the generation of CTL suppression.

Developmental Endocrinology Branch--
D. Lynn Loriaux, M.D., Ph.D., Chief

The endocrine concomitants of normal and abnormal human growth, development, and differentiation are examined in this clinical research Branch. Specific areas of study include the mechanisms underlying the initiation of puberty; the regulatory physiology and biochemistry of the glycoprotein hormones; the roles of sex steroid hormones, growth hormones, and other growth factors in bone growth; and the physiology, biochemistry, and mechanism of action of hypothalamic releasing hormones. Clinical research on male and female reproductive disorders is also a major interest of this Branch.

The primary goal of current studies by **Loriaux, Cutler**, and their colleagues is to understand the relative roles of the steroid hormones and the various growth factors in regulating human skeletal growth and epiphysial maturation. The role of the somatomedins in linear growth and epiphyseal maturation is controversial. Precocious puberty, with its early growth acceleration, provides an experiment of nature in which the endocrine concomitants of growth can be examined. These investigators compared somatomedin C (SmC) levels in 41 children with precocious puberty, 87 age-matched controls, and 110 normal pubertal children. SmC levels were significantly greater in patients with precocious puberty when compared to age-matched controls. SmC levels correlated with pubertal stage in both normal children and children with precocious puberty. The treatment of children with precocious puberty using the LHRH analog,

D-Trp6-Pro9-Net-LHRH (4 µg/kg/d), decreased both the growth rate and the SmC levels. However, the decrease in growth rate was about 50%, while the decrease in SmC was only about 5%. Changes in growth correlated strongly with changes in plasma sex steroid concentrations, but weakly with changes in SmC. These findings suggest that SmC is not the principle modulator of pubertal growth, but that SmC plays a permissive role in this process.

The role of the steroid hormones in skeletal growth has been examined in several studies. This group has previously demonstrated that the dose of ethinyl estradiol giving optimal growth in girls with Turner's syndrome is 100 ng/kg/d. This dose is considerably lower than the currently recommended dose. The DEB studies, however, were of short duration. These investigators have now examined whether this growth stimulation is sustained during a 6 month treatment period, and whether the bone age is advanced at an accelerated rate. Sixteen girls with Turner's syndrome, ages 5 to 15 years, were randomly assigned to a 6 month treatment period with 100 ng/kg/d of ethinyl estradiol or placebo. Lower leg growth rate, height, and bone age were measured. Ethinyl estradiol at the dose of 100 ng/kg/d for 6 months produced sustained growth without undue bone age advancement. This finding suggests that ultimate stature in patients with Turner's syndrome may be improved with low dose estrogen treatment, and that the currently popular regimen of almost 10 times the dose employed here may actually reduce ultimate height. If so, this represents an important advance in the treatment of this disorder.

During the past year, it was found that some lots of human growth hormone, as prepared from the pituitary glands of cadavers, may have been contaminated with the infectious agent(a slow virus) of Jacob-Kreutzfeld disease (a fatal degenerative encephalopathy). As a result, human growth hormone is no longer available for clinical use. This lends urgency to studies attempting to identify treatment alternatives for children with short stature. Many children with apparent growth hormone deficiency respond to a single dose of growth hormone-releasing hormone (GHRH) with an acceleration in growth. This suggests that repeated doses of GHRH might be used to treat growth hormone deficiency. To examine the effects of GHRH on growth, the GHRH response rate, and what factors might predict response, GHRH was administered, 1 µg/kg every 3 h for 10 days, to 8 children with growth hormone deficiency. Five of these patients were also studied during a placebo injection period. GHRH stimulated growth in 4 children. These four had elevations of growth hormone in response to GHRH and a variable rise in somatomedin-C. In these four, GHRH accelerated growth more than human growth hormone given at the standard recommended dose. The growth response to GHRH seemed to correlate inversely with bone age and directly with the growth hormone rise after GHRH. These investigators concluded that GHRH may be an effective alternative to growth hormone in selected patients with apparent growth hormone deficiency.

New results of **Sherins** and his colleagues relevant to the reproductive process center about findings in infertile men and the demonstration of a potential new contraceptive for women. The commonest cause of male infertility is idiopathic. The sperm appear normal in number, morphology, and activity. They are, however, unable to fertilize an ovum. Recent work suggests that proteins coating the head of the sperm serve as the recognition site for the sperm-egg interaction. Several of these proteins, of epididymal origin, have been isolated by J. Blaquier, a Visiting Scientist from Argentina. He has generated polyclonal antibodies against these proteins, and can now map the distribution of several of the proteins on spermatozoa. Sherins and Blaquier have found

that in a high proportion of men with idiopathic infertility, the distribution of these proteins over the sperm head is abnormal, and the abnormality can be qualititive or quantitative. This finding may be important in the effort to clarify the mechanism underlying idiopathic male infertility, and suggests possible immunological approaches to therapy.

Effective contraception continues to be a problem of world-wide importance. Current methods are either incompletely effective or carry a significant health risk. Theoretically, an antagonist of progesterone should be effective as a contraceptive agent. DEB investigators have examined such a compound for safety and efficacy. RU 486, a newly developed antiprogestin supplied by Roussel-UCLAF, was initially examined for toxicity in a man with adrenal carcinoma. Twenty mg/kg/day for 12 weeks yielded no evidence of toxicity, but 5 mg/kg was completely effective as an antiprogestin. When given as a single 5 mg dose to a group of volunteer women, menses was induced in all women in the luteal phase of the cycle. This finding strongly supports the antiprogestational action of this drug. A single monthly dose of RU 486, 5 mg/kg, prevented pregnancy in a group of 20 fertile rhesus monkeys. A placebo-treated group had 15 pregnancies in the same period. Thus, this compound holds promise as a once monthly contraceptive agent that appears to be of very low toxicity. These findings also suggest that this agent may serve as the long-sought "day after" contraceptive agent.

Studies by **G. Chrousos** and his colleagues in the past year have centered about the use of corticotropin releasing factor (CRF) in the differential diagnosis of Cushing's syndrome. A prospective comparison of CRF with the standard dexamethasone suppression test showed the CRF test to be quicker, simpler and less expensive than the dexamethasone suppression test. The sensitivity and specificity of the test was as good or better than the standard dexamethasone suppression test. It was efficient in differentiating ACTH-dependent Cushing's syndrome from ACTH-independent Cushing's syndrome, as well as being able reliably to separate patients with the ectopic ACTH syndrome from those with ACTH-secreting pituitary microadenomas. CRF also has proven useful in clarifying the pathophysiology of the hypercortisolism associated with psychiatric disorders. The response to CRF administration in these disorders is blunted compared to the response in both normal subjects and patients with Cushing's disease. These findings support the notion that the hypercortisolism in Cushing's syndrome is CRF-independent, while that associated with psychiatric disorders is CRF-dependent. This result suggests that a test might be devised that will differentiate the hypercortisolism of psychiatric disorders from that of ACTH-dependent Cushing's syndrome early in the course of either disorder.

The role of glucocorticoids in allowing man to tolerate stress has also been reexamined by this group. Current dogma has it that man requires increased plasma concentrations of cortisol to sustain severe stress such as surgery or systemic infection. No biochemical basis for this need, however, has been identified. An experiment was accomplished in which adrenalectomized cynomolgus macaque monkeys were subjected to surgical stress at varying levels of glucocorticoid replacement. It was found that no advantage was gained by replacement at doses greater than the basal secretory rate of cortisol in this species. This suggests that the increased cortisol levels may be an epiphenomenon related to the stress response. The increased cortisol production may reflect increased endogenous opiate production, for example, as the primary event.

DEB studies of the hypothalamic-pituitary-gonadal axis over the past year have centered on developing rational therapies for the less common causes of isosexual precocious puberty. Both the McCune-Albright syndrome and familial male isosexual precocious puberty have been found to be independent of gonadotropin secretion. Hence, LHRH analogue therapy is ineffective in these patients. These workers have shown that the aromatase inhibitor, testolactone, diminishes plasma estrogen concentrations in patients with the McCune-Albright syndrome, and as a consequence, slows the progression of secondary sexual characteristics in these subjects. The antiandrogen spironolactone has been used to treat boys with familial male precocious puberty. This approach has been effective in halting the progression of male sexual characteristics, but has been ineffective in slowing the accelerated rate of growth characteristic of the condition. Interestingly, the addition of the aromatase inhibitor testolactone did slow the accelerated growth. These findings point to the importance of estradiol for the pubertal growth spurt in both girls and boys.

Studies by **Nisula** and his co-workers have as their objective an improved understanding of the basic endocrinology of the glycoprotein hormones. Hormones under study include hCG, LH, FSH, and TSH. Studies in the last year have emphasized work on the hCG molecule. Abnormal forms of hCG and their metabolites are of clinical interest as molecular markers of the malignant transformation of the placenta (trophoblast). Normal forms of hCG (produced by the embryo) may serve as markers of the earliest stages of pregnancy, even before the missed menstrual period, and therefore be very useful in epidemiological studies of early pregnancy loss. Previous work conducted by this group demonstrated that patients with gestational trophoblastic neoplasms (carcinoma arising from the placenta) frequently excrete into their urine forms of hCG deficient in sialic acid, as well as carboxyterminal fragments of the hCG b-subunit. Neither of these unusual molecules is excreted in the urine of healthy pregnant women. The occurrence of caboxyterminal peptide fragments in association with hCG forms bearing an altered carbohydrate structure suggested a precursor-product relationship. To address this hypothesis, healthy human subjects were given infusions of either native hCG or desialylated hCG and their urines analyzed for carboxyterminal fragments. Subjects given desialylated hCG, but not those given native hCG, excreted carboxyterminal fragments in urine. These results indicate the existence of a peripheral metabolic pathway that cleaves carboxyterminal fragments from desialylated hCG and allows their excretion in urine. Thus, the frequent presence of carboxyterminal peptide fragments in the urine of patients with gestational trophoblastic neoplasia can be accounted for in part, if not entirely, by the peripheral metabolism of forms of hCG bearing an abnormal carbohydrate structure.

As indicated above, forms of hCG deficient in sialic acid are prevalent in the urine of patients with gestational trophoblastic neoplasia, but not in subjects infused with native hCG or in healthy pregnant women. It has been widely believed that serum glycoproteins, like hCG, are principally metabolized via a pathway in which desialylation is the initial step. Once desialylated, such glycoproteins would be taken up by hepatic receptors for galactose-terminated glycoproteins. Ashwell and Morell were the first to show that removal of sialic acid from the carbohydrate chains of glycoproteins drastically reduces their survival in the circulation, and demonstrated that this was due to accelerated uptake by receptors located on hepatocytes. Nisula's observations suggest that desialylation may not play a significant role in the normal metabolism of sialylated serum glycoproteins <u>in vivo</u>. To examine this question, the metabolism of hCG was studied in rats. Rats were given infusions of

either native hCG or desialylated hCG, with or without desialylated fetuin which blocks hepatic receptors for galactose-terminated glycoproteins. Blockade of the hepatic receptors did not impede hCG turnover in the circulation, impair hepatic uptake or catabolism of hCG, or lead to the accumulation of desialylated products of hCG in plasma. These findings demonstrate that there is negligible catabolism of glycoproteins, such as hCG, via a pathway that involves peripheral desialylation and subsequent uptake by hepatic receptors for galactose-terminated glycoproteins. These results have impact in two areas. First, the role of the hepatic receptors for galactose-terminated glycoproteins needs to be reexamined. Their role, in the rat at least, does not appear to be the disposal of desialylated circulating glycoproteins of the CG-type or the disposal of secreted forms of glycoproteins that are incompletely sialylated or desialylated intracellularly prior to secretion. This construct gives new insight into the meaning of the prevalence of desialylated hCG in gestational trophoblastic neoplasia. The results imply that the abnormal carbohydrate structure of hCG reflects abnormal glycosylation mechanisms in malignant trophoblastic tissue, a notion previously suggested by studies of choriocarcinoma cells in vitro.

Endocrinology and Reproduction Research Branch--
Kevin Catt, M.D., Ph.D., Chief

The research programs of this Branch are directed at the elucidation of cellular mechanisms involved in hormone secretion and action, and at the investigation of normal and disordered function of the hypothalamic-pituitary system and its effects upon gonadal and adrenal function. These programs include studies on the characterization of peptide hormones and their cellular receptors; the structure-function relationships of peptide and glycoprotein hormones; the regulation of hormone biosynthesis and secretion; and the mechanisms of peptide hormone action in endocrine target cells. Of particular interest are the analysis of pituitary-gonadal and pituitary-adrenal regulation, the control of ovarian activity during the reproductive cycle and pregnancy, and the participation of hormone receptors in the regulation of pituitary, gonadal, and adrenal function. In the current year, research has been pursued in several areas of hormone secretion and action, and on the receptor-mediated processes that are responsible for the control of steroid production in endocrine target cells. The role of hormones in cellular regulation has also been examined in selected areas of normal and disordered human endocrine function, and in appropriate animal model systems for the analysis of peptide secretion and the stimulatory and inhibitory control of target-cell function. The staff of the ERRB share common interests in the secretion and mechanisms of action of peptide and glycoprotein hormones, the role of neuropeptides in hypothalamic-pituitary regulation, the control of gonadal and adrenal function by pituitary hormones, the renin-angiotensin system and aldosterone secretion, and the role of phosphorylation in metabolic regulation.

The section led by **Kevin Catt** carries out research on the control of endocrine target cells by peptide hormones, in particular the characterization, regulation, and activation mechanisms of membrane receptors for gonadotropins, angiotensin II, gonadotropin-releasing hormone (GnRH), and corticotropin-releasing factor (CRF). The receptor-mediated actions of gonadotropin-releasing hormone (GnRH) and other regulators of pituitary hormone secretion are studied in

cultured anterior pituitary cells. In the gonadotroph, it was found this year that occupancy of GnRH receptors and activation of gonadotropin secretion is followed by receptor turnover and subsequent up-regulation of receptor number. In contrast, potent GnRH antagonists dissociated slowly from the GnRH receptor and did not cause down-regulation of receptors and desensitization of the gonadotroph. The bound antagonist remained localized at the cell surface for much longer periods than bound agonist analogs, suggesting that receptor activation is necessary to initiate internalization of the hormone-receptor complex. GnRH was found to enhance phosphatidylinositol (PI) turnover, with increased formation of phosphatidic acid (PA) and arachidonic acid (AA). In studies on the actions of these compounds on LH release, PA stimulated cGMP formation and LH release in a calcium-dependent manner, suggesting that endogenous PA may function as a calcium ionophore in the gonadotropic action of GnRH and its agonist analogs. Protein kinase C was shown to be abundant in purified gonadotrophs and to be activated by phorbol esters and diacylglycerols, which also stimulated LH release. GnRH-stimulated release of luteinizing hormone (LH) from gonadotroph-enriched cells was accompanied by a rapid and dose-dependent decrease in cytosolic protein kinase C and by a corresponding increase in the particulate enzyme. Retinal directly inhibited the activity of cytosolic protein kinase C and also attenuated the release of LH from GnRH-stimulated gonadotrophs. These findings, and the ability of GnRH to cause rapid translocation of cytosolic protein kinase C to a membrane-associated form, suggest that hormonal activation of protein kinase C is an intermediate step in the stimulation of pituitary LH secretion by GnRH.

The hormonal control of gonadal endocrine function is studied in ovarian and testicular target cells. In the ovarian granulosa cell, which is a model of hormone-induced cellular differentiation, these investigators found that FSH causes early and delayed increases in cAMP production, with induction of protein kinase, peptide hormone receptors (LH, PRL) and steroidogenic enzymes from 18-24 hr of culture. The RNA and protein required for differentiation are synthesized largely during the second day of culture, and endogenously produced estrogen is essential for the full expression of LH receptors. Granulosa cell maturation is also induced by cAMP generators such as choleragen and forskolin, and is modified by GnRH, EGF, PDGF, adenosine, calcium agents, and estrogens. Most of these agents act rapidly to influence granulosa-cell differentiation, but their effects are largely manifested during the second day of culture. Inhibition of granulosa cell maturation by GnRH is highly calcium-dependent, similar to its stimulatory action in the pituitary, and is reproduced by elevation of cytosolic calcium. GnRH also attenuates the FSH-induced rise in Type II protein kinase, initially decreasing enzyme activity and later reducing the synthesis of the RII cAMP-binding subunit. In the adult testis, GnRH receptor blockade decreased PRL (prolactin) receptors and testosterone production but did not alter the desensitization response to exogenous gonadotropin, showing that testicular GnRH does not mediate the down-regulating effects of elevated gonadotropin on testicular function. The neonatal rat testis was found to be relatively immune to the inhibitory effects of estrogen, and to show largely positive responses to elevations in plasma gonadotropins and PRL, instead of the receptor loss and desensitization seen in the adult. These features of the fetal-neonatal population of Leydig cells may serve to ensure optimal androgen production during the critical period of sexual differentiation.

G. Aguilera investigates the physiological and pathological aspects of the renin-angiotensin system in rodent and primate models, with emphasis on the role of angiotensin II (AII) in the regulation of aldosterone secretion and

circulatory homeostasis. AII mediates the increases in aldosterone secretion during sodium restriction, but the adrenal response to the peptide also depends on the sensitivity of the glomerulosa cell to AII. Aguilera has found that adrenal sensitivity to AII is increased during sodium restriction, and previous studies in the rat have indicated that up-regulation of adrenal AII receptors contributes to the enhanced adrenal sensitivity to AII. However, in the primate the increased adrenal sensitivity to AII has been found to depend on increased 18-hydroxylase activity rather than AII receptor regulation. The recently characterized cardiac peptide, atrial natriuretic factor (ANF) is a potent inhibitor of aldosterone production in vivo and in vitro, being more effective in inhibiting AII-than ACTH-stimulated steroidogenesis. Studies are in progress to determine the possible role of ANF in the regulation of adrenal sensitivity to AII in several physiological conditions. The action of AII is highly calcium-dependent, and studies with the dihydropyridine calcium channel agonist, Bay K 8644, have indicated that voltage-dependent calcium channels are involved in the mechanism of action of AII. The participation of calcium-dependent protein kinases in the control of adrenal glomerulosa function was suggested by the preferential location of calcium-calmodulin and calcium-phospholipid dependent protein kinases in the zona glomerulosa of the adrenal cortex, and by the ability of AII to decrease cytosolic calcium-calmodulin dependent protein kinase activity in isolated adrenal glomerulosa cells. The central receptors and actions of AII are being further studied in the brain, to characterize the interactions of ANF with the neural effects of AII, and to determine the localization of AII receptors in the primate brain.

The neuroendocrine components of homeostatic regulation are also studied, with emphasis on the mechanisms of stress responses and the hypothalamic control of corticotropin (ACTH) release. The receptors and actions of corticotropin releasing factor (CRF) have been characterized in the pituitary gland and nervous system. The increases in ACTH secretion that follow adrenalectomy are accompanied by marked increases in basal ACTH release in isolated pituitary cells, and by decreases in pituitary CRF receptors and CRF-stimulated adenylate cyclase activity. Studies are in progress to determine the mechanisms of receptor desensitization and maintenance of elevated ACTH secretion after adrenalectomy and stress. Analysis of neuropeptide release from median eminences in vitro showed a decrease in CRF release and an increase in vasopressin (VP) release after adrenalectomy, suggesting that VP is a major factor in the control of ACTH release. Autoradiographic analysis of frozen brain sections has shown that CRF receptors are prominently located in the cerebral cortex and limbic system-related areas. Brain CRF receptors are coupled to adenylate cyclase, and in contrast to the pituitary receptors are not down-regulated after adrenalectomy. Functional CRF receptors were also found in sympathetic ganglia and in the adrenal medulla, indicating that CRF is involved in the peripheral response to stress. These findings have revealed that CRF exerts receptor-mediated actions within the nervous system at sites involved in the behavioral and autonomic responses to stress, as well as on the pituitary-adrenal secretion of stress hormones.

M. Dufau investigates the molecular basis of peptide hormone action, with particular emphasis on the characterization of gonadotropin receptors, activation of steroid biosynthesis in gonads and adrenal, and analysis of the biological activity of circulating gonadotropins. Recent studies on the properties and purification of solubilized prolactin receptors have given new information on receptor structure and have led to the isolation of small amounts of purified receptor. Crosslinking studies with ^{125}I-hGH have shown

the existence of subsets of high (81 and 91K) and low (31 and 37K) binding subunits in the gonads. The high and low Mr species were also found in mammary gland, liver, and kidney. The presence of subspecies of similar Mr in the gonads reflects differences in glycosylation or phosphorylation within the subunit population. The smaller (37K) species appears to represent a disulfide-linked portion of the larger (91K) component. The ovarian receptor was purified by affinity chromotography to a specific activity of 20 pmol/µg, close to that expected from its apparent Mr, with retention of hormone binding activity. The purified ovarian receptor contained 88K and 95K variants of the high Mr subunit, and a single 41K subunit. Both of the purified receptor subunits were biologically active as shown by hormone binding to the free subunits after trans-blotting to nitrocellulose. This approach has permitted the isolation of active lactogen receptors from the ovary in microgram amounts, and has provided the first evidence for the presence of a high MW binding component in the purified free receptor.

In studies on gonadotropin action, Dufau and her colleagues extended the previously observed effect of LH on guanyl nucleotide binding and protein phosphorylation in Leydig cell plasma membranes by further analysis of the GTP-dependence of the calcium-sensitive phosphorylation of a 44K protein that may be related to the guanyl nucleotide regulatory protein family. The mechanisms of gonadotropin-induced desensitization of Leydig cell steroidogenesis were further analyzed to clarify the post-stimulatory depression of androgen production, and the role of endogenous estrogens in this process. Such steroidogenic lesions, which are independent of changes in LH receptors or protein kinase activation, are prominent in the adult testis but minor in the fetal-neonatal population of Leydig cells. Recent studies have shown that as a result of gonadotropin action, estrogen-mediated desensitization is initiated by an early cyclic AMP-dependent activation of aromatase, which is followed by a significant rise in estradiol formation due to increased substrate availability. Leydig cells are the major site of estradiol synthesis in the adult rat testis, and the low aromatase activity observed in immature rat Leydig cells could explain the lack of desensitization observed in fetal and early life. Estrogen action that precedes desensitization includes increased synthesis of a 27K estradiol-regulated protein. This protein was found to be immunologically similar to a major estradiol-regulated 27K protein of MCF-7 tumor cells, and provides a sensitive probe for detection of estrogen action. Antibodies against the MCF-7 protein will be employed for functional and structural characterization of the 27K protein and for studies of the nuclear actions of estrogen in the Leydig cell. There is a continuous basal supply of steroidogenic cholesterol in the mitochondrion regardless of the presence of gonadotropins, a process probably regulated by the levels of endogenous steroids. The early lesion is not due to an inappropriate concentration of precursors, since the levels of cholesterol in the inner mitochondrial membrane are increased. A heat-labile inhibitory protein factor was identified in mitochondria and shown to be markedly increased by hCG treatment. This factor, which competitively inhibits cholesterol side-chain cleavage activity, could contribute to the early steroidogenic lesion and may also serve as an endogenous modulator of steroid hormone biosynthesis.

Further studies on LH bioactivity by Dufau have provided new insights into the regulation of pituitary-gonadal function of man, rhesus monkey and rat. Modulation of the frequency and bio:immuno ratio (B:I) of plasma LH pulses provides an important physiological mechanism for regulating the concentrations of bioactive LH available to the gonads. The bioactivity of circulating LH is modu-

lated by gonadal steroids (normal men have higher B:I than cycling females; castration in rats decreases the B:I), possibly via changes in glycosylation. The decrease in B:I of LH in castrated animals could also be related to increased LH secretion rate. Older men with prostatic cancer have low B:I ratios, indicating the potential importance of changes in B:I with age and sickness. With advancing age, B:I and plasma testosterone are inversely related, and ill men over age 40 have lower B:I ratios. The qualitative nature of LH varies as a function of aging and illness in men, and the secretion of LH with low B:I may be etiologically relevant in patients with impotence and normal immunoreactive pituitary hormones and LH periodicity, and low normal testosterone levels. During GnRH or clomiphene therapy in a patient with impotence and pituitary calcification, a rise in B:I indicated a relationship between pituitary GnRH action and the potency of secreted LH. The endocrine consequences of reversible endogenous estrogen excess on the pituitary-gonadal axis in man were analyzed in a patient with an estrogen-producing adrenal tumor, in whom hypogonadism was attributable to selective reduction in bioactive LH and low B:I ratio. The latter changes could result from estradiol action at the hypothalamic level to reduce GnRH secretion, and from direct effects on pituitary LH processing. It was also demonstrated that the adenine analog (4-Aminopyrazolo-(3,4-d)-Pyrimidine) has an inhibitory action on GnRH release from the hypothalamus, and the relation of this effect to the mechanism of neurohormone secretion is being further analyzed.

C. Strott investigates the physiology and regulation of adrenal steroidogenesis, by characterization of cellular steroid binding proteins and soluble factors which mediate steroidogenic responses to ACTH, as well as analysis of cellular mechanisms of cholesterol utilization in steroid biosynthesis. The guinea pig, a cortisol-producing species, is employed as a model for studies on the regulation of steroidogenesis in the adrenal cortex, and the development and regulation of cellular zonation in the adrenal cortex. The guinea pig adrenal is relatively large, with a cortico-medullary ratio of 80:1, and is readily separated into glomerulosa-fasciculata and reticularis zones. The latter zone is of particular interest since little is known of its functional nature, steroidogenic capability, and specific regulation. The role of ACTH in regulating the zona fasciculata is well characterized; however its role in regulating the zona reticularis is unclear. Strott found that when dexamethasone was administered to animals to suppress secretion of endogenous ACTH, the zona fasciculata showed the expected atrophy and decrease in steroid production, but the zona reticularis was unaffected. In contrast to the zona fasciculata, lipoprotein receptor activity and steroid synthesis in the reticularis are not stimulated by ACTH. On the other hand, adenylate cyclase activity, ascorbic acid depletion, and hydrolysis of cholesteryl ester stores are promoted by ACTH in the zona reticularis as they are in the zona fasciculata. Thus, the zona reticularis has retained certain specific responses to ACTH while losing others.

The rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone, a process whereby cholesterol must be made available and delivered to, and pregnenolone removed from, the active site located in the inner mitochondrial membrane. To study this complex process in detail, adenylate cyclase activity and cyclic AMP production have been determined using isolated cells and membrane particles. Also, cyclic AMP-dependent protein kinase activity was found mostly in the soluble fraction (80-90%), composed of the type II isozyme, and 40% more active in the outer cortex. A study of soluble and particulate phosphoproteins is in progress. Other studies include analyses of

the binding of lipoproteins to membrane receptors, cellular uptake of cholesterol and cholesteryl esters, and the de novo synthesis and metabolism of cholesterol. A specific pregnenolone-binding protein is being further purified by high-performance liquid chromatography. Preliminary data suggest that the binding protein is phosphorylated by a cAMP-dependent protein kinase.

H. C. Chen's section conducts research on the analysis, synthesis, and structure-function relationships of biologically active peptides and proteins. This includes the identification and synthesis of unusual structures and sequences in amino acids and peptides, and the development of new techniques for peptide sequencing and synthesis. Correlations between peptide structure and function are analyzed in hypothalamic releasing hormones, including gonadotropin-releasing hormone (GnRH) and the recently discovered corticotropin-releasing factor (CRF). In studies on the human CRF molecule, ten peptides corresponding to sequences 9-41 to 17-41 of hCRF were synthesized and purified in order to test the effect of Arg^{16} (in the postulated α-helical form of the hormone) on the expression of agonist or antagonist activity. A 2-3 fold increase in binding to rat pituitary membrane receptor was observed on addition of Arg, with a general increase in binding on elongation. However, the shorter fragments 11-and 12-41 bind more strongly than the longer 9- and 10-41 peptides. Agonist activity is expressed in fragments of length 4-41, with antagonist activity peaking at 11- and 12-41, and reexpression of agonist activity in the still shorter fragments 14- and 15-41. These results indicate the importance of Arg^{16} as a critical residue for expression of the receptor binding and bioactivity of CRF.

In pursuit of more highly potent gonadotropin releasing hormone agonists, dimeric des-Gly^{10}-[$D\text{-}Lys^{6}$]-GnRH-NHEt analogs crosslinked at the ε-amino group of $D\text{-}Lys^{6}$ with -Gly-$COCH_2$-CO-Gly_n- (Ia, n=0; Ib, n=1; Ic, n=2) were synthesized, purified and evaluated for their biological activities. Such crosslinking of the agonist into dimers was found to enhance both in vivo and in vitro biological activities, and dimer Ib exhibited the highest antifertility effect ever reported.

In preparation for projected structure-function studies, two peptides of 14 and 18 amino acid residues corresponding to α helical and helix turn regions of the cytoplasmic portion of the human transferrin receptor were synthesized by Chen and his colleagues for use as immunogens. The antibodies are to be used in a study of the function of these domains in receptor dynamics and regulation. Also, a peptide of 22 amino acid residues in a protein expressed during gastrulation of Xenopus laevis was synthesized for use as an immunogen to be employed in developmental studies.

Recent studies on the biology of human chorionic gonadotropic (hCG), including the development of a simple procedure for collection, concentration, and a specific urinary hCG radioimmunoassay, were applied successfully in a pilot study for the detection of early fetal loss in a population of healthy women. In 86 menstrual cycles studied, 19 samples (one sample from each cycle) showed a positive hCG level. Among those hCG-positive women, eleven had no detectable level of hCG in the subsequent month, indicating early fetal loss. This 58% fetal loss rate, which is derived from a general population of normal subjects, may be more representative than those of other studies which were carried out in selected groups of women.

Further structure-function studies were performed on a chemically deglycosylated hCG derivative (HF-hCG) previously shown to possess enhanced receptor binding activity but reduced ability to stimulate cyclic AMP and steroid production in gonadal cells. HF-hCG also antagonized cAMP production in hCG- and FSH-stimulated granulosa cells as well as in hCG-stimulated Leydig cells. Addition of bivalent anti-hCG antibodies, but not their monovalent Fab fragments, to the inactive HF-hCG:LH-receptor complex in differentiated granulosa cells, significantly increased both cAMP and progesterone production. Since both conformation-specific and sequence-specific hCG-antibodies were equally effective, and antibody bivalency was required to induce the stimulatory effect, crosslinking and microaggregation of the hormone:receptor complex is the most likely cause of target-cell activation by anti-hCG antibodies after initial binding of the antagonist derivative.

The section led by **K.-P. Huang** studies the regulation and hormonal control of glycogen metabolism in normal and diabetic tissues, and the activities of glycogen synthase and phosphorylase kinase. Phosphorylation-dephosphorylation of enzymes that control rate-limiting steps is one of the most important mechanisms by which cellular metabolism is modulated by hormones and other regulators. Glycogen synthase and phosphorylase kinase are two such enzymes that are regulated by this mechanism in response to hormonal ligands. Huang has found that these enzymes can be phosphorylated and dephosphorylated, respectively, by multiple forms of protein kinases and phosphatases. It is not entirely understood how hormones affect the activities of the various protein kinases and phosphatases. Previous studies have defined the action of glucagon and β-adrenergic agonists via the pathway involving cAMP and cAMP-dependent protein kinase. However, the actions of other hormone-mediated phosphorylation systems have yet to be correlated directly with the actions of certain kinases. Tumor-promoting phorbol esters mimic the action of some hormones which regulate glycogen synthase activity in isolated hepatocytes. The pleiotropic responses elicited by these phorbol esters are presumably through binding and activation of their receptor, which has been provisionally identified as a phospholipid-dependent and calcium-activated protein kinase (protein kinase C). This protein kinase is ubiquitous in eukaryotes and seems to play a pivotal role in mediating the actions of ligands causing receptor-mediated breakdown of inositol phospholipids. To study the role of this enzyme in glycogen metabolism, protein kinase C from rat brain has been purified by Huang, et al. to near homogeneity in high yield. The purified enzyme phosphorylates glycogen synthase without causing its inactivation, in contrast to the effect of exposing intact hepatocytes to tumor-promoting phorbol esters. These findings indicate that the actions of other mediators in addition to protein kinase C must be necessary to express the effect of phorbol esters. Polyclonal and monoclonal antibodies against protein kinase C have been prepared for immunocytochemical studies, and the regulation of protein kinase C activity by autophosphorylation is under investigation.

Office of the Scientific Director--

- **Section on Viruses and Cellular Biology**
  Arthur S. Levine, M.D., Head

In this laboratory, DNA viruses are used to probe the developmental programs that regulate changes in the differentiation phenotypes of normal and transformed animal cells. The DNA viruses are also utilized as models in studies on mechanisms of eukaryotic DNA replication and mutagenesis.. In one project, Levine, Patch, and their collaborators have attempted to identify the phenotypic characteristics of hamster cells transformed by adenovirus 2(Ad2) or SV40 that correlate with their ability to form tumors in syngeneic animals. Through the use of somatic cell hybrids formed between Ad2 ("non-oncogenic")-and SV40 ("highly oncogenic")-transformed cells, it has been shown that Ad2 is dominant in regulating this phenotype. Cells transformed by Ad2 are rendered sensitive to in vitro lysis by unprimed immune effector cells, and fail to form tumors upon transplantation to adult syngeneic hamsters. However, cells containing only the SV40 genome are resistant to lysis in vitro and form tumors even in allogeneic hosts. In the past year, these workers found that the Ad2 phenotype correlates with the concentration of Ad2 encoded proteins. In a study of other phenotypic events that may be altered by transformation with DNA viruses, these investigators sought to determine whether there are differences in the autocrine growth factors elaborated by Ad2- or SV40-transformed cells, and found that the two cell phenotypes are identical with the exception that SV40-transformed cells secrete a soluble growth inhibitor. This factor is a 24 Kd peptide which is heat-sensitive (unlike TGF_{β} and Holley's BSC-1 factor) and more inhibitory to the growth of normal than transformed cells. All of these results point to the possibility that cells transformed by SV40 are less immunogenic than cells transformed by Ad2, and that SV40-transformed cells also secrete a factor which may inhibit the growth of immunocytes. Experiments in the coming year will address this possibility directly.

A group led by K. Dixon has found that tumors induced in hamsters by a small t-antigen deletion mutant of SV40 develop more slowly than do tumors induced by wild-type SV40, metastasize more frequently, and occur in different sites upon subcutaneous injection of the virus. These differences in behavior may relate to a difference in tissue tropism between the mutant and wild-type viruses, and this possibility will be examined. Thus, the SV40 system can be used to study the genetic basis of tumor latency, metastases, and in all likelihood, tissue tropism. In turn, these studies should provide further insight as to how cell proliferation and migration are regulated at the genetic level.

Another project in this group involves the study of mutagenesis, a process of great importance since mutations are the underlying cause of most inherited diseases, many developmental anomalies, and most tumors induced by environmental agents. Previous studies by these workers on replication of UV-damaged SV40 DNA have led to a well-defined model of how the mammalian cell replication machinery responds to DNA damage, and at what steps in the replication process mutations become irreversibly established. During the past year, this group constructed a shuttle vector which permits the induction of mutations in the mammalian system, and their identification in a bacterial system. The vector contains the origin of SV40 DNA replication, so that it replicates like the SV40 virus in mammalian cells. The vector also contains the PBR327 bacterial plasmid origin and replication functions, such that it can replicate in bacterial cells. Finally, the vector includes the gene for a suppressor tRNA which

provides the target for mutagenesis. After sequencing the DNAs of an extensive series of UV-induced as well as spontaneous vector mutants, it was found that there is a localized loss in fidelity of DNA synthesis (presumably during gap filling) such that adenine is preferentially inserted opposite UV-induced photoproducts in the template DNA and additional base substitutions are made nearby. Further studies using this shuttle vector should permit a characterization of the types of mutations induced by specific agents, and a correlation of the mechanism of mutation induction and fixation with specific types of DNA damage. Because of the mounting evidence that tumorigenesis, mutagenesis, and normal events in developmental biology are intimately related, this section's results should help to illuminate the mechanisms which regulate basic developmental phenomena.

° **Section on Growth Factors--**
Gordon Guroff, Ph.D., Head

This section is studying the mechanism of action of growth factors of neurochemical interest, and especially, the physiology of nerve growth factor (NGF). This factor is one of the best characterized of the large and increasing number of growth and differentiation factors already known. NGF is required for the survival and ultimate development of the sympathetic and sensory nervous systems and as well, has some action on the adrenal mudulla, the central nervous system, and on tumors arising from cells derived from the neural crest. A fuller knowledge of the mechanism of action of NGF should lead to a better understanding of the mode of regulation of the growth and differentiation of neurons. NGF is of particular interest also in that it may well be involved in the regrowth of damaged or severed neural tracts, an issue of great clinical significance.

NGF, a polypeptide, appears to control the expression of specific genes in target neurons, and thus to influence the development and differentiation of these cells. Guroff and his colleagues have been studying the intracellular events which follow the binding of NGF to its receptor, using PC12, a pheochromocytoma cell which differentiates in response to NGF. This group has developed three cell-free phosphorylation systems (cytoplasmic, nuclear, and ribosomal) which reflect the action of NGF on the cells. They have separated the components of the soluble systems, kinase and substrate, and purified each. The overall effect of NGF is to decrease phosphorylation due to a decrease in kinase activity rather than a decrease in substrate. However, an early step in the action of NGF on phosphorylation appears to involve an increase in soluble protein kinase C activity, a situation which is mimicked by treatment with tumor promoters (e.g., TPA). Moreover, the addition of protein kinase C to the cell-free system duplicates the effects of prior treatment of the cells by NGF, allowing the conclusion that protein kinase C is an early participant in the nerve growth factor-induced changes in phosphorylation. Clearly then, protein kinase C has an important and early role in the phosphorylation-dephosphorylation cascade initiated by nerve growth factor. Moreover, it is likely that changes in the phosphorylation of nuclear proteins may relate to alterations in the transcription of DNA. Several years ago, Guroff and his coworkers found that one consequence of NGF treatment of PC12 cells is an apparent decrease in the number of epidermal growth factor (EGF) receptors, such that as the nerve cell begins to differentiate under the influence of NGF, it loses its sensitivity to the proliferative stimulus of EGF. This group now has preliminary

evidence that there is a dramatic inhibition of the rate of transcription of the EGF receptor gene after treatment with NGF. In the coming year, appropriate probes will be constructed to permit studies on the specific changes in expression of the EGF receptor gene (as well as other genes) caused by NGF.

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collagen has a role.

Proposed Course of Project:

I. It is planned to study the structure of collagen isolated from subcellular fractions in order to obtain evidence concerning the mechanism of collagen synthesis.

II. Further studies on the biochemical events in calcification are in progress.

Part B included Yes