Individual amoebae of Dictyostelium aggregate to form a slug-like mass of cells which eventually differentiates into a sorocarp, a stalk and a mass of spores. One stage of this differentiation, the "Mexican hat stage," is shown in this picture obtained with a scanning microscope at a magnification of 100X. (Photo by Miller and Revel, on AMR instrument.)
A Report for the Year 1971–72
on the Research and Other Activities
of the
Division of Biology
at the
California Institute of Technology
Pasadena, California
RESEARCH REPORTS

Much of the research work summarized here has not yet been reported in print, in many instances because it is not yet complete. For that reason this report is not intended as a publication and should not be cited as such. Individual projects should be referred to only if specific permission to do so is obtained from the investigator responsible for the material. References are made here to published papers bearing on the projects reported. Publications by members of the Division, covering the period July 1971-June 1972, are listed separately, at the end of the research reports of each group.
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INTRODUCTION
California Scientist of the Year

Cottrell Award

Lasker Award Winners (Freis, BENZER, Brenner, Yanofsky)
THE FACULTY

The numerous honors and awards received by our faculty during the year are listed elsewhere in this volume, but five seem worthy of special note:

Professor Roger Sperry received the “California Scientist of the Year” award for 1972.

Professor Seymour Benzer received the Albert Lasker Award in basic medical research (together with Sydney Brenner, Edward Freis and Charles Yanofsky).

Professor (Emeritus) Arie J. Haagen-Smit received the first Cottrell Award for Environmental Quality from the National Academy of Sciences.

Professor William Wood and Visiting Associate Roy Britten (on leave of absence from the Carnegie Institution of Washington) were elected to the National Academy of Sciences. Eleven of the permanent members of our faculty and two of our Professors Emeriti are now members.
NEW BUILDING

Construction of the new Behavioral Biology Building was begun in February 1972. When completed (hopefully in August 1973) this building will provide the first major addition to the facilities of the Division since the completion of Alles Laboratory in 1960; it will denote the strong emphasis to be placed upon the field of behavioral biology among our chosen programs of investigation.

Dr. Arnold Beckman has generously provided a significant portion of the funds for construction of this building which will include some 47,000 sq. ft. of research and teaching space. Dr. Beckman was honored at a “ground-breaking” ceremony on May 8, 1972 and presented with a plastic model of a human brain as an “honorary neurobiologist.” Professors Sperry, Wiersma and Van Harreveld were similarly honored for their long-time contributions to the development of neurobiology and psychobiology at Caltech. Our former Chairman and present Trustee, Dr. George Beadle, was a principal speaker, talking about “The Evolution of the Biology Division at Caltech.”
LECTURESHIPS

The third Jean Weigle Memorial Lecture was presented on April 3, 1972 by Dr. A. Dale Kaiser, Professor of Biochemistry in the Department of Biochemistry at Stanford University Medical School. Dr. Kaiser who did his doctoral research with Jean Weigle and received his Ph.D. from this Division in 1955 spoke on “Gene Regulation in Phage Lambda.”

The third Albert Tyler Memorial Lecture was presented on May 3, 1972 by Mr. Robert Parke Jr., Deputy Director of the Presidential Commission on Population and the American Future. Mr. Parke’s subject was “U.S. Population Policy: Limits and Objectives.” Mr. Parke also presented a seminar on “Defining the Impacts of Population Growth in the United States.”

A. Dale Kaiser

Robert Parke Jr.

JOINT LECTURE SERIES

The Division and the Huntington Hospital Institute of Applied Medical Research joined to organize during the year a series of eight lectures concerning basic biological problems related to cancer. The speakers in this series and their topics were:

Dr. Karl E. Hellstrom, University of Washington School of Medicine: “Blocking antibodies and cancer.”

Dr. Walter Eckhart, The Salk Institute: “Polyoma gene functions for cell transformation.”

Dr. Stuart Aaronson, National Cancer Institute: “Recent advances in the biology of RNA tumor viruses.”

Dr. John Fahey, UCLA School of Medicine: “Therapeutic and diagnostic implications of advances in cancer immunology.”

Dr. David Baltimore, Massachusetts Institute of Technology: “The DNA polymerase of RNA tumor viruses.”

Dr. Max Burger, Princeton University: “Cell surface changes in transformed cells as monitored with agglutinins.”

Dr. Malcolm Bagshaw, Stanford University School of Medicine: “Implications of radiobiology for cancer radiotherapy.”

Dr. Charles Craddock, UCLA School of Medicine: “Some principles of the chemotherapy of leukemias.”

A similar series of lectures is planned for next year relative to problems in neuropathology.
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GENETICS
NEUROCHEMISTRY

Kerckhoff-Alles-Church
(Second Floor)
1. AN ATTEMPT TO ACCOUNT FOR GENETIC CHARACTERISTICS IN TERMS OF MEIOSIS-RELATED BIOCHEMICAL EVENTS BY "A THEORY OF GENETIC RECOMBINATION NOT REQUIRING PRECISELY MATCHED CHROMATID-BREAKS"

**Investigator:** Sterling Emerson  
**Support:** National Institutes of Health, Public Health Service  
**General Funds**

While reanalyzing Kitani and Olive's data of gene conversion (intragenic recombination) it became necessary to modify earlier models of recombination. By making use of the newer knowledge of biochemical processes specifically related to the different stages of meiosis it was possible to derive a general interpretation of genetic recombination which accounts for its known characteristics in a consistent way. A brief outline of the model follows.

Both intragenic and intergenic recombination are initiated by the separation from their complementary fixed strands of newly synthesized genomic fractions of DNA strands which have free ends due to the delay in replication of the terminal organelles of replicons. Restriction of initiation by newly synthesized strands to those identical in 3'-5' polarity prevents meiotic recombination between sister-chromatids. Strand separation is facilitated by the meiotic DNA-binding protein, permitting separation to proceed for a considerable distance. Restricting extensive complexing with the meiotic protein to single-stranded DNA of only one polarity makes it impossible for more than two chromatids to engage in recombination at any one point in the chromosome. Reassociation into double-helices is also facilitated by the meiotic protein. When reassociation occurs between a free strand and the fixed strand of its homolog, biparental DNA is formed, with heteroduplexes occurring at included heterozygous mutant sites. Delayed replication of terminal organelles during zygotene, and the union between them and the previously replicated genomic portions by early pachytene, secures the switched strands, preserving the regions of biparental DNA and the cross-connections (half-chromatid chiasmata) between chromatids. Inhibition of protein synthesis at the end of zygotene or beginning of pachytene prevents the union between newly replicated terminal organelles and genomic DNA, permitting the return of switched strands to their original partners under stresses due to chromatid separation during diplotene, and disrupts all connections between chromatids. The requirement for protein synthesis for this union suggests that a unique kind of bonding is involved. Intragenic recombination results from heteroduplex repair, which involves excision of a mispaired base, extensive degradation of the strand cut (accounting for coincident repair at two sites in one strand and for "map expansion"), resynthesis of the degraded strand with its complementary strand as template, and union with the undegraded part of the strand or with the terminal organelle. Cross-connections between chromosomes are cut and rejoined to restore chromatid continuity by the same repair process. Strands of the same polarity are cut and, depending upon which pair is cut, the restored chromatids do or do not become reciprocally recombinant. Complexing of enzymes involved in repair is postulated to account for the persistence of cross-connections when DNA synthesis is inhibited during pachytene. Production of 3-chromatid double-exchanges requires premeiotic recombination between sister-chromatids. Premeiotic and mitotic sister-chromatid exchanges result from strand realignment, after severance of the unique bond between terminal organelles and genomic DNA, at replicon junctures between the two sites of reciprocal exchange. Reversals in polarity can occur at the uniquely bonded juncture and can accumulate during all mitoses preceding meiosis. Alternatively, strand polarity may be preserved if breaks occur within the terminal organelles to initiate sister-chromatid exchange.

At one locus in *Sordaria fimicola*, strand separation starts at the proximal end. Regions of biparental DNA sometimes extend sufficiently far to include the sites of all known mutant alleles and are sometimes short enough to exclude the three most distal. In from one-fifth to one-third of all instances, biparental DNA occurs in only one chromatid and does not lead to intergenic recombination. When hybrid DNA is formed reciprocally in two chromatids, intergenic recombi-
nation accompanies intragenic in one-half of all instances. Frequencies of repair of heteroduplexes to wild type and mutant are sometimes quite different in two reciprocally-formed heteroduplexes.

References:


The following reports describe continuing research into the structure, function, and morphogenesis of the bacteriophage T4, a model system which is providing insight into the general problem of how biological ultrastructure is formed under genetic control. The phage is a nucleoprotein structure of intricate morphology, containing at least 30 different kinds of protein molecules. Conditionally lethal mutations affecting phage assembly permit experimental arrest of the morphogenetic process at any of approximately 40 stages. Since many assembly steps can be carried out in vitro, using precursor components derived from infected cell extracts made with appropriate mutants, it is possible to apply biochemical and genetic techniques as well as electron microscopy to the problem of how the phage particle is assembled. During the past year we have continued work on the assembly and function of the phage tail fibers, components of the viral absorption apparatus which are particularly amenable to study. The goal of this work is to understand the molecular role of each of the 7 phage-induced proteins required for tail fiber assembly and attachment. All of these proteins have now been identified and their molecular weights estimated by electrophoretic analysis in polyacrylamide gels containing the strong denaturant sodium dodecyl sulfate (SDS). Three of them have been shown not to be structural components of the phage; these are therefore candidates for catalytic roles in fiber assembly. Reactions involving two of these have been further studied with a view toward determining their mechanisms of action. Genetic and serological techniques have been combined with electron microscopy to determine the orientation in the tail fiber of the major structural proteins determining phage host range.

In other work, a number of bacterial mutants which block normal phage assembly have been isolated and characterized with the goal of investigating the role of host cell factors in the process of phage morphogenesis.

In the following reports, defective extracts prepared from restrictive host cells infected with amber (am) mutant phage are designated by the number(s) of the defective gene(s) in the mutant used for infection: e.g., 23:24-defective extract. Proteins synthesized under the control of mutationally identified phage genes are designated as P followed by the gene number: e.g., P36. A genetic map of T4 showing gene numbers, relative gene locations, and the phenotypic consequences of various gene defects is described in the first report.

General References:

2. A REVISED PHYSICAL MAP OF T4 GENES

Investigator: William B. Wood

Support: National Institutes of Health, Public Health Service

The map of T4 (page 14) has been revised to include recent information from a number of laboratories on gene functions, sizes, and relative locations. Functions of essential genes (t, e, and all numbered genes) can be inferred from the consequences of the corresponding gene defects, indicated by the boxed diagrams or abbreviations in the outer circle. Gene 11 and 12 mutants produce a normal number of non-infectious, non-killing particles with tail fibers. Gene 2 mutants produce a lower number of killing particles with tail fibers, in addition to the unattached components shown. Open hexagons indicate heads which appear empty of DNA in
electron micrographs; solid hexagons represent filled heads. Functions or designations of nonessential genes (all others) are as follows: rII-III, rapid lysis; u, UV sensitivity; ipI-III, internal head proteins; tRNA, 8 transfer RNAs; cd, dCMP deaminase; denA, endonuclease A; nrdA-C, nucleoside diphosphate reductase; td, thymidylate synthetase; frd, dihydrofolate reductase; ac, acridine resistance; dexA, exonuclease A; sp, rapid lysis (spackle); a%gt, DNA-glucosyl transferases. Gene sizes where shown are based on gene product molecular weights estimated by polyacrylamide gel electrophoresis in the presence of SDS. Assuming the genome size of $1.66 \times 10^5$ nucleotide pairs (n.p.) determined by Kim (1972), 1° equals 460 n.p. (corresponding to 17,000 daltons of protein). Gene positions indicated by lines on the inside of the circle have been determined by a physical mapping procedure independent of recombination frequencies (Mosig, 1968, and personal communication). In the 0 to 60° sector of the map these positions have been corrected to conform to the rIIB-e interval of 145° determined by electron microscope-heteroduplex mapping (Kim, 1972).
Two other intervals measured by this method are rIIA - 38 and e - tRNA. Positions of the remaining genes relative to the physically mapped loci have been estimated from recombination frequencies. Co-transcribed genes are indicated by arrows showing transcription direction (Stahl et al., 1970; L. Gold, K. Hercules and W. Sauerbier, personal communications). Mutationally identified genes can now account for approximately 50% of the phage genetic material.

References:

3. STRUCTURE AND ASSEMBLY OF T4 TAIL FIBERS

Investigator: Robert C. Dickson
Support: National Institutes of Health, Public Health Service

Tail fiber assembly in bacteriophage T4 requires the functioning of at least 6 phage genes. To further our understanding of how genes control the assembly of biological structures we are examining in detail the function of these genes. Only 4 gene products are found in whole tail fibers (Biology 1971, No. 35). The products of genes 34 and 37 (P34 and P37) are the major structural components of the A and BC' half-fibers respectively (see Figure 1). P36 is a small component of the BC' half-fiber. The additional 40,000-dalton component of BC' half-fiber has now been identified as P35. The remaining 2 gene products, P38 and P57, have been identified as bands which are removed from the electrophoretic pattern of infected cell lysates as the result of am mutations in genes 38 and 57, respectively. The molecular weight of P38 is 26,000 daltons, and that of P57 is approximately 10,000 daltons. These 2 proteins are not found in either whole phage, tail fibers, nor any of the tail fiber precursors. They may therefore act catalytically in fiber assembly. The P38 function is now being studied in vitro (see Biology 1972, No. 6), but the P57 function has so far been inaccessible to this approach. The action of P57 appears to be pleiotropic. It is required for the addition of P12 (58,000 daltons) to baseplates as well as for the assembly of P34 and P37 to form the A and C half-fibers, respectively (see Figure 1). In the absence of P57 all 3 proteins sediment with cell debris rather than remaining soluble. P57 could function by altering the structure of P12, P34 and P37, such as by modifying amino acid residues or by breaking or making peptide bonds. Experiments to test these notions are in progress.

References:

Figure 1. Assembly of Bacteriophage T4 Tail Fibers
4. SOME CHARACTERISTICS OF THE A HALF-FIBER ACTIVITY

Investigator: Gregory J. Del Zoppo
Support: National Institutes of Health, Public Health Service

The formation of whole tail fibers involves the attachment of A to BC' half-fibers. The current work is an attempt to investigate the attachment process in vitro. Preparations of A and BC' half-fibers are mixed and incubated with purified tail-fiberless particles under conditions such that the rate of formation of infective phage particles is a crude but consistent measure of the A half-fiber activity. Using this assay it has been shown that A half-fibers purified from an extract as described previously (Biology 1971, No. 37) have nearly the same activity per unit A antigen as A half-fibers in crude 18:23:27:37-defective extracts, contrary to an earlier report (Ward et al., 1970).

A study of the effect of the source and treatment of the crude A-containing extracts on A half-fiber activity has been undertaken. Extracts prepared from E. coli grown in Hershey broth are as much as 30-fold more active per unit antigen than extracts prepared using minimal medium. Efforts to denature the A antigen in crude extracts by a number of treatments have shown that loss of A activity is generally accompanied by loss or precipitation of the A antigen. A significant increase (up to 10-fold) in A activity per unit antigen was observed, however, when the A-containing extract was heated for several minutes at 48°C and dialyzed. EDTA, normally added to extracts at 0.001 M or less, exerts an inhibitory effect on A activity. These results are difficult to interpret because the assay depends upon tail fiber attachment to the baseplate as well as A to BC' attachment. A direct assay for the latter reaction is therefore being developed.

Reference:

5. GENETIC AND SEROLOGICAL ANALYSIS OF P37 CONFORMATION IN T4 TAIL FIBERS

Investigators: Steven K. Beckendorf, Ilga Lielausis, Jung-Suh Kim*
Support: National Institutes of Health, Public Health Service
American Cancer Society

The closely related bacteriophages T2 and T4 have diverged considerably in their host ranges, and in the corresponding recognition specificities of their tail fibers. Antiserum specific for the distal half-tail fiber of T4 shows little cross-reaction with T2 tail fibers, and gene products P37 and P38, which interact during conversion of P37 to a distal half-fiber precursor structure, are incompatible between the two phages. In crosses between T2 and T4, mutant in these genes, only low levels of viable recombinants are observed. There is no recombination at the end of gene 37 corresponding to the C-terminus of P37, which carries the host range determinant. In the rest of gene 37 there are two small regions with relatively high recombination and one region of low recombination.

When T2/T4 heteroduplex DNA molecules are examined in the electron microscope, four non-homologous loops are seen in the region of genes 37 and 38. Heteroduplexes between hybrid phages which have part of gene 37 from T4 and part from T2 allow approximate location of gene 37 mutations in the heteroduplex pattern. For more precise location, a physical map of gene 37 was constructed by determining the molecular weights of amber polypeptide fragments of P37 on SDS polyacrylamide gels. When the physical and heteroduplex maps are aligned, the regions of low recombination correspond to regions of non-homology between T2 and T4 while regions with relatively high recombination are homologous.

Different gene 37-hybrid phages were found to block different fractions of antiserum specific for T4 distal half-fibers, showing that there are at least 4 subclasses of antigen corresponding to different parts of P37. The locations of these antigens on the tail fiber structure were approximately determined from electron micrographs of hybrid phages complexed with antibody. The results show that P37 is oriented linearly in the distal half-fiber with its N-terminus near the joint between the two half-fibers and its C-terminus near the tip of the fiber.

*Division of Chemistry and Chemical Engineering, California Institute of Technology.
6. THE ROLE OF P38 IN THE ASSEMBLY OF THE BC' COMPONENT OF THE T4 TAIL FIBER

Investigator: Robert J. Bishop

Support: National Institutes of Health, Public Health Service

The assembly of the distal half-fiber (BC') of bacteriophage T4 requires the function of 5 genes (see Figure 1, page 15). Three of these genes (35, 36 and 37) control the synthesis of the structural components of the half-fiber. P38 and P57 are not found in the assembled phage and therefore play nonstructural roles in half-fiber assembly. All but one step (that controlled by gene 57) in the assembly of the BC' half-fiber can be carried out in vitro.

P38 and P57 are required for the appearance of the C antigen and the C half-fiber intermediate. Extracts of restrictive host cells infected with am mutants defective in either of these genes contain normal levels of P37 but little or no antigen capable of cross-reacting with antibodies directed against the BC' half-fiber. No C or BC half-fibers are visible in electron micrographs of these extracts.

P38 activity can be assayed in vitro (Biology 1971, No. 36) either by the production of infectious phage or by the appearance of C antigen in mixtures of 37-defective extracts. 37-defective extracts from which the cell debris have been removed by centrifugation have no P38 activity; P38 activity is released from the cell debris in the presence of 1.5 M NaCl. In 37:57-defective extracts P38 activity is found in the supernatant fraction.

The rate of the reaction is proportional to the concentrations of both P37 and P38. Because of the thermolability of P38 it has not yet been possible to determine whether it acts catalytically or is consumed in the course of reaction. The reaction is temperature-dependent and appears to require no dialyzable factors. Further studies into the nature of P38 function are underway.

7. THE SITE OF P63 ACTION IN T4 TAIL FIBER ATTACHMENT

Investigators: William B. Wood, M. Patricia Conley, Robert C. Dickson

Support: National Institutes of Health, Public Health Service

We have previously shown that P63 promotes tail fiber attachment, the terminal step of T4 assembly (Wood and Henninger, 1969). Its action is catalytic, but its mechanism remains obscure (Biology 1969, No. 191). Serological evidence, reported in Biology 1970, No. 91, suggests that T4 tail fiber attachment involves a reversible interaction between the distal (BC') half of the fiber and the phage head, as well as the irreversible attachment of the proximal (A) half of the fiber to the phage baseplate. To determine which of these interactions involves P63, we have studied each independently of the other, taking advantage of a finding that A half-fibers will attach inefficiently to tail-fiberless particles in the absence of BC' half-fibers. The particles produced by a 37-defective mutant (unable to form BC' half-fibers; see Figure 1, page 15) were purified by equilibrium sedimentation in a CsCl gradient and found to carry up to 20% of the normal amount of A antigen. Electrophoretic analysis confirmed that these particles contain P34, but not P37, and electron micrographs showed particles with half-fibers attached to the baseplate. Particles produced by a 37:63-defective double mutant were found to carry less than 5% of the normal amount of A antigen, suggesting that P63 promotes the inefficient in vivo attachment of A half-fibers.

Similar results were observed in vitro. When purified A half-fibers are incubated with purified particles and partially purified P63, antigen attachment proceeds linearly at about 1/50 the rate of whole tail fiber attachment under the same conditions. Omission of P63 from the reaction decreases the attachment rate by about 5-fold.

Purified noninfectious particles with attached A half-fibers can be converted to infectious phage by incubation with a source of BC' half-fibers. This reaction is stimulated less than 1.5-fold by P63. We conclude that in normal tail fiber attachment, P63 promotes the interaction of the proximal A half-fiber with the baseplate, and is probably not involved in the reversible interaction between the distal BC' half-fiber and the head.

Reference:
8. ISOLATION AND CHARACTERIZATION OF BACTERIAL MUTANTS UNABLE TO PROPAGATE BACTERIOPHAGE T4

Investigators: Helen R. Revel, Barbara L. Stitt, Ilga Lielausis, Marie Beale*

Support: National Institutes of Health, Public Health Service
American Cancer Society

The development of bacteriophage T4 in Escherichia coli depends upon the coordination of several processes: (1) preexisting bacterial metabolic pathways and protein-synthesizing machinery; (2) specific phage modification of existing host components such as RNA polymerase and tRNA; (3) phage-directed de novo synthesis of specific enzymes, nucleic acids and structural proteins; and finally (4) the assembly of the mature phage particle. An unanswered question is whether all the structural information for the assembly process is encoded in the phage DNA or whether some is supplied by the host bacterium. A study of the role of host cell components in T4 development may allow new insights into phage assembly and regulation.

As an approach to this problem we have isolated 250 host-defective (hd) bacterial mutants from Escherichia coli K12 (10 each from 25 independent cultures) after nitrosoguanidine mutagenesis and challenge with a mixture of T4 and T6 phage. Bacterial mutants are selected as cells that are infected and killed by T4 but produce no infectious progeny phage.

Phage mutants (called go), isolated by plating high concentrations of T4 on hd hosts, can overcome the block to T4 development. Testing the ability of various go mutants to grow on all hd bacterial strains has permitted subdivision of the defective bacteria into at least six classes.

In the majority of the hd mutants (96%), T4 development is blocked at an early step in phage head assembly. These mutants include classes hd A-D (90%), hd D (4%), hd A (1%) and hd B (1%). In these hosts, T4 infection leads to normal phage DNA synthesis and lysis of the cell at 30 minutes without the production of viable progeny. T4 go mutants (go A and go D), which were selected on hd A and hd D hosts, respectively, and also grow on hd A-D strains, have been shown by complementation tests to carry an alteration in gene 31, a gene known to control an early step in T4 phage head assembly (Laemmli, Beguin and Gujer-Kellenberger, 1970). In addition, the block in T4 development in hd D and hd B hosts can be overcome by go mutants which give 1.0 to 1.5% recombination with am B17 in gene 23, a gene which codes for the major head protein of T4. These results suggest that some host factor, altered in these hd hosts, must interact with both phage gene products P31 and P23 to make a functional complex which acts early in T4 phage head assembly. The following report, on characterization of a similar mutant, lends further support to this view.

The remaining bacterial hd mutants, hd C (1%) and hd F (1%), are blocked at an earlier stage of T4 development. T4 infection of hd C and hd F strains leads to delayed phage DNA synthesis: synthesis is initiated at the normal time at a slow rate and eventually (in minimal medium but not in broth) achieves a faster rate of synthesis comparable to that in T4 infection of the hd* host. A go mutant which grows on hd F hosts has been mapped close to (or in) gene 39, a gene in which am mutations also cause the phenotype of delayed DNA synthesis. The delay in DNA synthesis may result from an altered bacterial component involved directly in DNA replication or its reinitiation, or may possibly be due to some perturbation of an earlier step in phage development. The hd C and hd F strains provide additional tools to probe the nature of the 4 DNA delay genes of T4.

In an attempt to find other classes of hd mutants the selection procedure was repeated using T4 go A phage in place of T4*. This should eliminate the most common hd class (hd A-D), and thereby increase the probability of obtaining rarer mutant types. Of the mutant bacteria which survived the selection, 32% are hd F, 3% hd C, 45% hd D, and 20% are as yet unclassified. Thus far only the F type bacteria have been examined closely. T4 go F mutants which will grow on the hd F bacteria are being characterized; 3 out of 5 T4 go F mutants have been found to contain mutations which lie in or close to gene 39 by recombination mapping; the other two carry mutations which lie far from gene 39.

Reference:

*Student Assistant.
9. CHARACTERIZATION OF A BACTERIAL MUTATION BLOCKING T4 HEAD ASSEMBLY

Investigators: William B. Wood, Ilga Lielausis

Support: National Institutes of Health, Public Health Service
         American Cancer Society

We have continued the characterization of a bacterial mutant, similar to some of those described in the preceding report, which was originally selected as unable to propagate bacteriophage λ and then found to be host-defective for T4 as well (Biology 1971, No. 42). The mutant, designated groEA44, is infected and killed at the normal rate by T4, but less than 0.5 progeny phage per cell are produced. The rate of phage DNA synthesis and time of cell lysis are approximately normal. Electron microscopy shows that the infected cells produce normal appearing tails and tail fibers but no recognizable head-related structures. In vitro complementation experiments confirm the presence of active tails and tail fibers but no active heads in extracts of T4-infected groEA44 cells, indicating that the bacterial mutation blocks an early step in phage head formation.

The blocked step is that normally controlled by phage gene 31, based on the following evidence. Phage mutants, designated T4e, selected for ability to grow on groEA44, still propagate normally on wild-type bacteria, but have lost the ability to grow on groEB515, a second mutant strain host-defective for λ but not for T4 wild type (see Table 1). Complementation tests with T4 am mutants using groEB515 as host showed that 25/25 independently-isolated T4e mutants are altered in gene 31. Mapping by 2- and 3-factor crosses confirms that the e mutation occurs in a single small region between two known gene 31 am mutations. Moreover, the consequences of the groEA44 host mutation in T4 wild-type infection appear identical to those of a phage gene 31 defect: the normal head protein cleavages are blocked, and the major head protein P23 accumulates in amorphous aggregates or lumps associated with the bacterial membrane.

Uninfected groEA44 cells are temperature-sensitive; when a growing culture is shifted from 37° to 42°, the cells cease to divide and form long filamentous structures up to 20 cell-lengths long. Spontaneous ts revertants of groEA44 have regained the ability to propagate both T4 and λ, suggesting that a single mutation causes both the host-defectiveness of the mutant strain as well as its temperature-sensitive growth properties. The simplest explanation for these results is that some component of the bacterial cell, altered in groEA44 and groEB515, must interact with P31 to allow the proper organization of the head protein P23 into head precursor structures. The bacterial factor may be a membrane component, since its alteration in groEA44 causes a defect in cell division.

This work was done in collaboration with C. P. Georgopoulos, R. W. Hendrix and A. D. Kaiser, Department of Biochemistry, Stanford University Medical School, Stanford, California.

Table 1. Growth of λ and T4 wild-type and mutant phage on wild-type and host-defective E. coli K12 bacterial strains

<table>
<thead>
<tr>
<th>Host bacteria</th>
<th>Phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKB178 (hd*)</td>
<td>λ</td>
</tr>
<tr>
<td>groEA44</td>
<td>-</td>
</tr>
<tr>
<td>groEB515</td>
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PUBLICATIONS


Living organisms have a high and, within narrow limits, apparently irreducible requirement for water. For example, it has been found recently that the dry valleys of Antarctica—cold desert valleys where the mean relative humidity is in the neighborhood of 45%—are abiotic owing to the shortage of water (Horowitz, Cameron and Hubbard, 1972). These valleys are at least 10,000 years old and have been exposed to a fallout of microorganisms from the ocean and from lakes within the valleys presumably since the valleys originated, yet no mutant type capable of occupying the region has appeared in this time. In many other parts of the world, also, water is the life-limiting factor. Yet little is known about what determines the water requirements of organisms. The following report by Charlang et al. (Biology 1972, No. 10) summarizes recent work in our laboratory on the water requirements of Neurospora crassa. As we showed previously, loss of the germination factor is one of the first effects of the reduction of water activity in the medium. It appears to be a new growth-factor-like substance.

The major energy source for prebiotic organic synthesis on the primitive earth was the sun. The distribution of energies in the solar spectrum is such, however, that very little of this energy can be absorbed by the simple gaseous molecules thought to have been present in the primitive atmosphere (CO, CH₄, N₂, NH₃, H₂O, H₂). We previously showed that organic compounds are produced from CO and H₂O in a novel surface-catalyzed reaction in the presence of long-wavelength ultraviolet (λ >2000 Å) (Hubbard, Hardy and Horowitz, 1971). The effective wavelengths are much longer than those absorbed by CO and H₂O and are abundant in the solar spectrum. In the following report by Golub et al. (Biology 1972, No. 11), experiments using methane as a substrate for this reaction are described.

General References:

10. NEUROSPORA GERMINATION FACTOR

Investigators: Gisela Charlang, George Horn, Norman H. Horowitz
Support: National Aeronautics and Space Administration

Purification of the germination factor (GF) described in last year's report has continued. The present purification procedure involves the following steps: extraction with hot water; isoelectric precipitation of proteins; precipitation successively with ethanol and lead acetate; chromatography on ion exchange resins and polyacrylamide gel. The resulting product is active at concentrations of less than 0.2 µg/ml. This represents an approximate 500-fold purification over the deproteinized crude extract. Since we estimate that the specific activity of GF approximates that of biotin (Charlang and Horowitz, 1971), it appears that the current preparations are still less than 10% pure.

The nephelometric assay mentioned in last year's report has proved to be unreliable, and we have gone back to the original assay, based on dry weight measurements of cultures. Failure of the nephelometric method to discriminate between GF and other nutritional factors led to some erroneous conclusions regarding GF. The following are the known properties of the substance, based on experiments using the dry weight assay: It is a water-soluble, uncharged substance with a molecular weight in the range 1000 to 2000. It is thermostable in neutral solution, but is easily destroyed by acid or alkali. It is stable in the presence of chitinase, α-amylase, pronase, neuraminidase, cellulase, and hyaluronidase. It is found in Neurospora, Aspergillus, Penicillium, Phycomyces, and edible mushrooms. Traces of activity are found in E. coli and the seaweed Macrocystis. No activity is found in liver, spinach, milk, or yeast. Present evidence thus
suggests that GF is limited to filamentous fungi.

Reference:

11. SYNTHESIS OF ORGANIC COMPOUNDS FROM METHANE WITH LONG-WAVELENGTH ULTRAVIOLET LIGHT

Investigators: Ellis E. Golub, Jerry Hubbard*, Norman H. Horowitz

Support: The President's Fund
National Aeronautics and Space Administration

When fractured Vycor glass particles of high surface area, dried at 720°C for 16 hours, are irradiated with a high-pressure mercury arc (λ>2500 Å) under an atmosphere of 14CH4 and N2, a compound tentatively identified as formaldehyde, plus other unidentified organic compounds, is produced. The organic products are deposited on the glass particles and are removed by hot water extraction. Little or no CO2 is detected in the gas phase. The reaction is absolutely dependent on irradiation and exposed surface. Silica gel or alumina can also function as solid substrata for the reaction.

After approximately 72 hours of irradiation the reaction stops, and no further photocatalytic synthesis occurs. If the gas phase from a reaction which has stopped is transferred to a chamber containing fresh surface, a new burst of synthesis occurs which again stops. Transfer of the gas phase to a third chamber containing fresh surface again leads to a new burst of synthesis. This and other experiments show that the surface is the limiting factor in the synthesis and that it is exhaustible.

The atmosphere of the primitive earth is presumed to have been reducing, and reducing conditions are required for gas-phase syntheses of organic compounds from simple gases. However, Brinkmann (1969) has recently shown that up to 25% of the present atmospheric level of O2 could have been present in the prebiotic atmosphere. Investigation of the photocatalytic reaction described above in the presence of up to 6% O2 has shown that normal yields of organic products are obtained. Under these conditions, the unreacted methane is quantitatively converted to CO2.

Preliminary experiments with NH3 show that new compounds, presumably containing nitrogen, can be produced. Identification of these remains to be accomplished. N2 is apparently inert in this system.

The possibilities for new and exciting applications of this fascinating reaction have only barely been examined. Clearly, this process which can take advantage of the abundant energy available from the sun may have played a role in the synthesis of biologically important materials on the primitive earth; and it may have relevance for present considerations of the chemical balance of the earth, including hydrocarbon pollution of the lower atmosphere.

Reference:
*Jet Propulsion Laboratory.

PUBLICATIONS


Assistant Professor: Richard L. Russell
Research Fellows: Randall C. Cassada, Randle W. Ware
Graduate Students: John L. Carrigan, David H. Hall, Carl D. Johnson
Research Assistant: Lauren L. Hollen

The following reports describe continuing research into the formation of synaptic contacts between nerve cells, using two model systems—the small soil nematode *Caenorhabditis elegans* and the electric eel *Electrophorus electricus*. Our approach with the nematode is genetic, taking advantage of this animal's short generation time and ease of mutant isolation. We have isolated a large number of behaviorally altered mutants, and we are trying to discern in which of these there are genetically determined differences in the "wiring pattern" by which neurons are synaptically connected. This task is made somewhat easier by the relative simplicity of the *C. elegans* nervous system—about 200 cells in all—but it still requires the development of some new anatomical techniques, as described below. Our hope is that some of the mutant differences will shed light both on the ways in which genes determine the specificity that characterizes synaptic contacts and on the way in which the *C. elegans* nervous system mediates the animal's behavior.

Our approach with the electric eel is biochemical—we use it as a convenient source for the enzyme acetylcholinesterase, a component of most synapses where acetylcholine is the chemical transmitter between neurons. Because acetylcholinesterase is specifically incorporated into neuronal membranes at regions of synaptic contact, we are interested in the mechanism by which this incorporation occurs. Because we suspect that the multiple molecular forms of acetylcholinesterase found in the nervous systems of various animals are related to this incorporation process, we are purifying these forms from the electric eel and studying their properties. We hope that these studies will give some insight into the molecular events accompanying synapse formation.

12. LARGE SCALE ISOLATION OF CAENORHABDITIS ELEGANS MUTANTS


*Support:* National Institutes of Health, Public Health Service
National Science Foundation
Sloan Fund for Basic Research

As described in last year's annual report (*Biology* 1971, No. 52), the attraction of the small nematode *Caenorhabditis elegans* to its food bacterium *Escherichia coli* can be used to select mutant nematodes with various behavioral defects. Last summer (1971) this method was scaled up in a large group effort to provide a sizable pool of mutants for neuroanatomical and biochemical study. Nematodes were mutagenized, grown to permit mutant expression, selected for inability to migrate well toward a bacterial lawn, and then examined for behavioral alterations. Eight microscope observers, working for eight days, examined 838 selection plates containing the progeny resulting from about $10^6$ mutagenized gametes. 4103 possible mutants were picked; 475 of these survived as confirmed mutants and 336 of these were independent isolates. The phenotypes of these mutants are diverse, but most fall into a few major classes, as shown in Table 1. The numbers in each class are a function of at least two variables; the frequency with which such mutants occur, and the ease with which they are detected. Subjective impression (only) suggests that the rather high proportion of dumpies is due to their relatively easy detection. Relative numbers in the other classes might be taken as more indicative of relative frequencies of occurrence.

Some of these mutants, to judge by their phenotypes, seem particularly promising for anatomical studies of neuromuscular control and nervous integration in the simple nervous system of this animal. These mutant phenotypes are described in somewhat more detail below.

**Rollers:** Normal *C. elegans* lies on its side on a flat surface, with its sagittal plane parallel to the surface; when it moves, the sagittal plane remains parallel to the surface. By contrast, roller mutants roll continuously about their own long axis as they move, and their cuticles are twisted about this axis.
as well. The rolling is unidirectional and its sense is genetically determined; about three-fourths of the independently isolated roller mutants describe only left-handed helices, while the remainder describe only right-handed helices. The basis of the rolling behavior is not understood; however, the strength and frequency of muscle contractions appear normal, suggesting that the patterning of these contractions may be altered. We are currently searching for anatomical evidence concerning this possibility.

**Blebs:** These mutants have fluid-filled blisters at variable positions over their surfaces. These blisters apparently inactivate sensory receptors when they cover them, and can thus be used to localize the position of these receptors on the animal’s surface. Although the origin of the blisters is unclear, they appear to arise by aberrant forms of the normal cuticular molting process. Since there is evidence from other nematodes that molting may be controlled by neurosecretory cells, blebs mutants may have neurosecretory defects.

**Twitcher:** In twitcher mutants, the normal orderly pattern of muscle contraction which produces organized sinusoidal movement is overlaid by extra muscle contractions of low intensity, which may occur as isolated contractions of (apparently) individual muscle cells or as very fast-moving, low-amplitude “ectopic” waves. The general impression is of hyperexcitability. In other nematodes there is some evidence that muscle cells may generate their own action potentials and be regulated in a negative (inhibitory) way by their neuronal inputs. If this is true in *C. elegans*, twitcher mutants might have defective neuromuscular connections. As with rollers, we are searching for anatomical evidence concerning this possibility.

**Stiffhead:** Although most of the bodies of these mutants move normally, their heads are never capable of independent movement; if sufficiently turgid, the head may be pushed around like a stiff anterior appendage, and if not, it frequently folds back limply and is simply dragged along by the rest of the body. Electron micrographs show that the anterior (head) muscle cells of these mutants are nearly entirely devoid of both of mitochondria and of the thick and thin (presumably contractile) filaments normally found in *C. elegans* muscle. The defect is confined to the anterior muscle cells, and immediately adjacent muscle cells are apparently normal. The neuromuscular junctions involving the defective muscle cells are currently being examined to see whether the defect affects them as well.

Besides these behavioral mutants, the isolation also yielded some temperature-sensitive mutants described in Biology 1972, No. 13. All of these mutants are currently being maintained either by continuous culture, or by liquid nitrogen storage (see Biology 1972, No. 14), and they are available on request.

*Volunteer Assistant.  
**NSF Summer Trainee, 1971.*

<table>
<thead>
<tr>
<th>Mutant phenotype</th>
<th>Total isolates</th>
<th>Independent isolates</th>
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<tr>
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13. TEMPERATURE-SENSITIVE MUTANTS OF CAENORHABDITIS ELEGANS

**Investigator:** Randall C. Cassada

**Support:** National Institutes of Health, Public Health Service

Behavioral and morphological mutants previously selected here (see Biology 1972, No. 12) were tested for growth at 16°C and nongrowth at 26°C. From several hundred strains tested some 25 strains showing clear temperature sensitivity (ts) have been obtained. At least an equal number, while apparently somewhat ts, were either too leaky at 26°C or too sick at 16°C to be useful. The best ts mutants can be classified as to stage of arrest by transferring developing eggs from permis- sive to restrictive temperature. In most cases, the resulting worms developed to maturity but were sterile as adults. In the remaining cases, growth ceased at a particular stage or over a range of
stages between hatching and maturity. None appeared to arrest embryological development (within the egg) either immediately or in the following generation. Further characterizations are in progress to determine the time of thermosensitivity as well as the (potentially later) stage of developmental arrest following temperature shift. Such an ordered series of conditional (ts) developmental mutants should prove useful for a study of development in C. elegans.

14. FREEZING CAENORHABDITIS ELEGANS FOR LONG TERM STORAGE

Investigators: Lauren L. Hollen, Richard L. Russell

Support: National Institutes of Health, Public Health Service
          Sloan Fund for Basic Research

In order to maintain several hundred mutant stocks of C. elegans it has been necessary to use a long term storage procedure. Starting from a procedure developed by S. Brenner (personal communication), a liquid nitrogen freezing technique was developed which eliminates many man-hours spent in routine transfers and cuts down on space needed for storage. Worms from E. coli-seeded agar plates are suspended in 12.5% DMSO in phosphate buffer and, in capped ¼-dram glass vials, are lowered about 2°C per minute from room temperature to -70°C in a dry ice-ethanol bath. Stoppers are removed and the vials are transferred directly into liquid nitrogen. For thawing, vials are removed from liquid nitrogen and allowed to stand at room temperature. Worms are then transferred to petri plates for normal growth. Survival of worms seems unrelated to age or original plate conditions although with different mutants varying success is achieved. Survival for wild-type C. elegans is occasionally as high as 50%, but more typically is 5 to 10%. We are currently trying to improve survival rates. This technique has now been used as the technique of choice for maintaining most of the mutants described in Biology 1972, No. 12.

15. FEULGEN STAINING OF CAENORHABDITIS ELEGANS

Investigators: Edward Hedgecock*, Lauren L. Hollen, Richard L. Russell

Support: National Institutes of Health, Public Health Service
          National Science Foundation
          Sloan Fund for Basic Research

In analyzing the structure of the simple, approximately 200-cell nervous system of C. elegans, the primary tool must necessarily be the electron microscope, as discussed in Biology 1972, No. 16. However, it is also very useful, for purposes of mutant screening, to have a technique which will easily provide information on some gross aspects of C. elegans neuroanatomy, albeit at lower resolution than the electron microscope provides. Feulgen staining of whole mounts of C. elegans is apparently such a technique; although all cell nuclei stain in such preparations, it appears that neuronal nuclei have a characteristic condensed morphology by which they may be distinguished from the others. Furthermore, because C. elegans is relatively thin (about 50 microns), whole mounts may be easily scanned in the light microscope and nuclear positions may be determined with minimal optical interference.

We have examined a number of Feulgen-stained C. elegans to see whether the resulting information on number and position of neuronal nuclei could be useful in scanning behavioral mutants for gross neuroanatomical changes. We have concluded that such changes should be detectable in at least three regions of the nervous system: (1) the ventral nerve cord, which contains 57 to 60 neuronal nuclei aligned in sequence along the ventral surface, (2) two symmetrically placed lateral ganglia of five nuclei each, lateral to and slightly behind the nerve ring, and (3) two symmetrically placed tail ganglia of eight nuclei each. We expect that better preparation techniques will allow changes to be detectable in several other parts of the nervous system. We are currently screening some of the behavioral mutants described in Biology 1972, No. 12, for changes in those three areas where changes can be found, with the intent of pursuing any detected changes by electron microscopy.

*NSF Summer Trainee, 1971.
16. STRUCTURAL ANALYSIS IN THE SENSORY NERVOUS SYSTEM OF CAENORHABDITIS ELEGANS

Investigators: Randle W. Ware, Lauren L. Hollen

Support: National Institutes of Health, Public Health Service
        Sloan Fund for Basic Research

One possible approach to the study of genetic factors governing the specificity of synapse formation is to investigate the structure and development of mutant animals which fail to establish some of the normal synaptic connections. To study such mutants effectively, it is necessary to use the resolution provided by the electron microscope, since many synapses are well below the limit of resolution of light microscopes. Since only rather thin sections (~1000 to 1500 Å) can currently be viewed by standard transmission electron microscopy, it is clear that to obtain a complete synaptic catalog, even of a simple nervous system like that of C. elegans, requires producing, viewing, photographing, and reconstructing from a very large number of serial thin sections. This task can be facilitated to some extent by semiautomated techniques developed by one of us (RWW) for use in another system (Levinthal and Ware, 1972). However it still seems wise, at least initially, to concentrate attention on a small part of the whole C. elegans nervous system, so that the task is further restricted.

We have chosen to concentrate on the sensory aspects of C. elegans, primarily because the receptors and the associated ganglia are well identified, rather circumscribed structures which are convenient for structural analysis, and because mutants specifically defective in sensory reception or its immediate transmission should, as far as we know, be completely viable. There are two known sensory inputs in the anterior tip of the normal hermaphroditic nematode. One is tactile and is thought to be conveyed to the central nervous system, or nerve ring, by 6 observed nerves, each containing about 6 fibers, arranged hexadially about the animal. We have found that these nerves do not enter the nerve ring in the same way. The dorsal pair, for example, send their fibers into the central portion of the nerve ring where they form their first synaptic contacts, whereas the ventral pair send their fibers first to the esophagus under the ring and partly encircle it before forming synapses.

Of greater interest to us at this time is chemoreception, and we have chosen to examine a rather nonspecific behavior, "attraction" or "avoidance," due to a rather specific stimulus, a known chemical, in the hope of being able to locate a synaptic alteration in the central nervous system. The mediators of chemoreception in the nematode are thought to be 26 fibers contained in two bundles of 13, one on either side of the animal. These fibers arise from bipolar cells which form two ganglia, the amphidial ganglia, slightly posterior to the nerve ring. These ganglia have been found to contain no synaptic connections themselves, but are merely, localized aggregations of cell bodies. One fiber of each cell ends in a small opening at the snout of the animal; the other travels to the posterior portion of the nerve ring, the ventral ganglion, where it forms its first synaptic contacts. We have just begun to work out the synaptic connections of the amphidial nerves to the main nerve mass in the wild-type animal using extensive 3-dimensional reconstruction at the level of the electron microscope (Levinthal and Ware, 1972). This is being facilitated by the use of a computer graphics setup which enables an operator to record information directly from a sequence of aligned electron micrographs into a computer. Information immediately of interest to us is the exact 3-dimensional pathway of any given cell and its connectivity, by means of synapses, with other cell fibers in the nervous system. We also expect that the techniques of counter-current separation developed by David Dusenbery (see Biology 1972, No. 18) should soon provide mutants, defective in chemoreception, which can be scanned for synaptic defects in this part of the nervous system.

Reference:
17. THE MECHANISM OF CHEMOTAXIS IN A NEMATODE

Investigator: David B. Dusenbery

Support: National Institutes of Health, Public Health Service

Initial studies of chemotaxis (Biology 1971, No. 51; S. Ward, personal communication) have demonstrated that the nematode C. elegans can detect and respond to a variety of chemical gradients, but they have not elucidated the mechanism of detection. It seems that the nematode must be able to compare detected concentrations of a chemical either at different points in space or at different points in time. The three most likely possibilities seem to be: (1) a comparison of concentration at the head with that at the tail; (2) a comparison of concentration at one lateral extreme of the undulatory motion with that at the opposite extreme; and (3) a comparison of concentration in the vicinity of the worm at one time with that in the vicinity of the worm at some earlier time.

I have investigated this problem by determining whether the worms could respond to a purely temporal stimulus. This was done by holding an individual nematode by the tail in a stream of flowing liquid and recording its movements. The chemical composition of the stream in the vicinity of the worm could be altered in a few seconds. If the alteration was to a less attractive solution, the nematode would usually respond within a few seconds by greatly increasing its amplitude of undulation. In addition it would often execute a series of vigorous “kicks,” probably identical with a response observed in unrestrained nematodes which causes them to turn end-for-end. This increase in activity often lasts for at least a minute, even though the change in composition takes only a few seconds. Thus it seems clear that the nematode can respond to a purely temporal change in chemical stimulation.

A test of the second possibility mentioned above was performed in a similar apparatus in which the nematode was held at the boundary between two adjacent streams in such a way that its undulatory motion would carry its head back and forth between the two streams. If one of the streams was made attractive with respect to the other there was no indication that the nematode favored that side. This suggests that the nematode does not compare concentrations at the opposite sides of its undulatory motion, at least in liquid, where this motion is rapid (2 waves/sec).

18. ANALYSIS OF CHEMOTAXIS BY STEADY-FLOW COUNTER-CURRENT SEPARATION

Investigator: David B. Dusenbery

Support: National Institutes of Health, Public Health Service

My continuing analysis of chemotaxis in the nematode, C. elegans, has been greatly aided by the development of a new method of counter-current separation. In this method two solutions in contact with one another are made to flow in opposite directions, and the compositions are varied to make one solution more attractive than the other. When nematodes are injected between the two solutions, they tend to spend the majority of their time in the attractive solution and hence they are moved away from the point of injection in the direction of flow of the attractive solution. With this technique it is possible to identify attractants (or repellents), compare their relative strengths, and determine the concentration ranges over which they are active.

Thus far it has been found that with two exceptions all salts tested between 1 and 50 mM are attractive throughout this range. The exceptions are CO₂ (or one of its hydrated forms) and H₃O⁺, both of which repel the worms at concentrations of 1 to 10 mM. In addition to this type of analysis of chemotaxis the method has great potential for selecting mutants which have altered chemotactic responses, and I will soon explore this application.

19. MULTIPLE MOLECULAR FORMS OF ACETYLCHOLINESTERASE

Investigator: Carl D. Johnson

Support: National Institutes of Health, Public Health Service

The electric organs of the electric eel (Electrophorus electricus) have long been used as a source of proteins associated with synaptic transmission mediated by the neurotransmitter acetylcholine. Acetylcholinesterase (AChE), the enzyme which hydrolyzes acetylcholine following its postsynaptic
action, can be extracted from electric organ tissue by homogenization in solutions of high ionic strength (1.0 M NaCl). The molecular properties of eel AChE extracted in this way are significantly different from those of the widely used and commercially available form of the enzyme (prepared by a lengthy autolysis of the organ under toluene). From fresh extracts three forms of AChE may be separated by high ionic strength sucrose gradients. They are called A, 9.6S; C, 14.4S; and D, 18.3S. In crude extracts the larger two forms (C & D) aggregate in low ionic strength solutions whereas the smallest form (A) does not. The commercially available form of the enzyme has a sedimentation coefficient of 11.5S in both high and low ionic strength gradients.

Forms A, C and D have been purified ~150-fold (~35% pure) by (1) ammonium sulfate precipitation, (2) chromatography on benzyl-DEAE-cellulose, and (3) chromatography on cellulose phosphate. Following separation of the three forms on sucrose gradients, it was found that all three forms (A, C & D) were insoluble in solutions of low ionic strength. They do not enter either polyacrylamide gels (pH 4.5 and 8.9; 7.5% to 2.5% acrylamide) or starch gels.

We are now attempting to purify the three forms in sufficient amounts to allow determination of subunit structure and hope to investigate how they are related to the process of assembling AChE into synaptic structures.
The cells of the cellular slime mold \textit{Dictyostelium discoideum}, can be induced to develop synchronously by starvation. Then the solitary amoebae join to form an organism with $10^5$ cells. These cells differentiate into morphologically and biochemically distinct stalk or spore cells in 24 hours. During development there is no growth and very little cell division but new RNAs and proteins are made. Some of these gene products appear to be directly related to development. For this reason we are studying the nuclear RNA polymerase of \textit{D. discoideum}. It has been purified to homogeneity from amoebae and developing cells. There is no change in the size or number of its subunits and we are presently looking for other chemical changes in the enzyme. During development the cells become cohesive. A good deal of evidence suggests that changes in the plasma membrane are responsible for this cohesiveness and for other cell interactions during development. Moreover some evidence suggests that there is functional and spatial differentiation of the plasma membrane. We have begun to examine the slime mold membrane biochemically and genetically in order to understand the mechanism of some intercellular interactions.

We are also studying \textit{Chlamydomonas reinhardi}. It contains two major genomes in its cell, a chloroplast genome (genetic complexity equal to T4) and a nuclear genome (whose genetic complexity is 10 times that of \textit{E. coli}). There are a number of distinctive gene products associated with the chloroplast, and the formation of chloroplast and cytoplasmic gene products is closely coordinated. Attempts have been made to study the site of synthesis of chloroplast macromolecules and the regulation of chloroplast and cytoplasmic macromolecular synthesis. Most of the results of these investigations are in conflict however because antibiotics with important side effects were used in them. So in an attempt to alleviate this problem we are isolating and characterizing mutants which are conditionally defective in macromolecular synthesis. Some of these are described in the following reports.

20. DICTYOSTELIUM DISCOIDEUM FORM B RNA POLYMERASE

\textbf{Investigator}: Brian Seed*

\textbf{Support}: National Institutes of Health, Public Health Service

Nuclei of the cellular slime mold, \textit{Dictyostelium discoideum}, possess two forms of RNA polymerase (Biology 1971, No. 54). The α-amanitin-sensitive enzyme has been purified to homogeneity by ion exchange and adsorption chromatography, and density gradient centrifugation. The enzyme consists of 4 subunits of molecular weight 210,000, 145,000, 26,000 and 13,000. Degradation of the largest subunit to an intermediate band of MW 185,000 has been observed and can be partially inhibited by phenylmethylsulfonyl fluoride.

We are investigating this enzyme to see whether developmental alteration of polymerase structure might be responsible for some of the transcriptional control observed in this organism.

*Undergraduate, California Institute of Technology.

21. THE ISOLATION OF DEVELOPMENTAL MUTANTS OF DICTYOSTELIUM DISCOIDEUM WHICH ARE SENSITIVE TO pH

\textbf{Investigator}: Daniel McMahon

\textbf{Support}: National Institutes of Health, Public Health Service

The methods with which cells recognize each other are largely undetermined. The Tyler-Weiss hypothesis proposes that intercellular recognition occurs through complementary interactions between molecules on adjacent cells (in a manner similar to the reaction between antibody and antigen). In spite of the length of time which has passed since this hypothesis was offered, no direct evidence has been offered in support of it, although a considerable amount of circumstantial evidence supports it. Since intercellular recognition and communication are integral and important parts of development and differentiation, we have been attempting to isolate mutants which might be
conditionally defective in these processes.

These processes are distinct from most other cellular functions because they occur on the outside of the plasma membrane. Therefore we have looked for an environmental parameter to use in the selection of mutants which could be varied primarily outside the cell. Many cells are extremely well buffered, and maintain a relatively constant internal pH when the external pH is changed. Therefore we investigated D. discoideum in this regard. The cells develop normally at pH 6 and pH 8. In addition they maintain an internal pH of 6.7 even when exposed to quite different external pHs. Three independent methods have been used to determine their internal pH: it has been measured on the extract from homogenized cells; also we have measured it by determining the partition of dimethylxazolidinedione between the inside and outside of the cell (measured by Charles Romeo) and we have measured the average ionization state of phosphate ion inside the cell by measuring the chemical shift of the $^31$P nucleus in phosphate with nuclear magnetic resonance spectroscopy (measured by Richard Moon).

We have isolated mutants whose development is sensitive to pH. One of these mutants forms very few sorocarps under nonpermissive conditions and those which are formed have abnormally thick and short stalks. As the $H^+$ ion concentration is increased development becomes more normal. However Ca$^{++}$ antagonizes the effects of $H^+$ at concentrations more than 100 times lower than those which affect wild-type cells. The developing cells become progressively more resistant to the nonpermissive pH during development but they only show completely normal culmination when kept at the permissive pH until the beginning of culmination. In other experiments we have shown that this mutant aggregates together with wild-type cells but that it apparently sorts abnormally in differentiation so only a small fraction of the mutant cells become spores. This “normality” of sorting is sensitive to pH and Ca$^{++}$ ion concentration. We plan to analyze the membranes of this and other pH mutants for an altered component. This should be considerably facilitated because many of the altered gene products should have differences in net charge which will facilitate their identification by electrophoresis.

References:


22. LABELING AND ISOLATING SLIME MOLD MEMBRANES

Investigators: Sharon L. Spivak*, Welcome W. Bender, Daniel McMahon

Support: National Science Foundation

We have been attempting to label the cell membranes of intact slime mold amoebae with colored or fluorescent dyes. We plan to use the color, which will distinguish the plasma membrane from other cell components, to facilitate isolation of the plasma membrane from disrupted cells, and to follow the sorting of living cells in the developing organism. We intend to characterize the proteins of the membrane at different stages of slime mold development. So far, we have had some success in labeling cells with isothiocyanate derivatives of fluorescein and rhodamine. We have not been able to color cells intensely enough to enable us to see single labeled cells mixed with unlabeled cells, partially because the unlabeled cells are themselves slightly colored and fluorescent. We have begun isolating labeled membranes by homogenization of cells and centrifugation on sucrose gradients. Two bands from the gradients containing membranes (as demonstrated by electron microscopy) have 5’-nucleotidase activity; this enzyme is found in the plasma membrane. These bands have little or no contamination by other cell components, as tested by assays for other marker enzymes. Only one of these bands shows significant labeling with fluorescein.

*Undergraduate, California Institute of Technology.

23. MATURATION OF RIBOSOMAL RNA IN CHLAMYDOMonas REINHARDI

Investigator: Mark J. Miller

Support: National Institutes of Health, Public Health Service

The unicellular alga, Chlamydomonas reinhardtii, contains both cytoplasmic and chloroplast ribosomes. The RNA species isolated from these ribosomes (rRNA) have sedimentation coefficients
of about 25S and 18S for the cytoplasm, and 22S and 16S for the chloroplast (Hoober and Blobel, 1969), with molecular weights of 1.3, 0.67, 1.05 and 0.56 x 10^6 daltons respectively. I have recently been successful in isolating presumed precursors of these rRNAs. The isolation procedure used involves rapid lysis of cells in a detergent-containing buffer, followed by phenol deproteinization. It is necessary that a mutant without a cell wall (the gift of Dr. D. Roy Davies) be used in this procedure, because the cell wall of wild-type C. reinhardi has proved impossible to break without the loss of all presumed precursor rRNA. The rRNA is separated by polyacrylamide-agarose gel electrophoresis and the S_r (the electrophoretic sedimentation coefficients) and molecular weight values are determined as described by Robberson et al. (1971).

During 5- and 10-minute pulses of ^32P, label first appears in three high molecular weight species of 2.4 x 10^6 (34S_r), 1.4 x 10^6 (26S_r) and 1.1 x 10^6 (23S_r) daltons. Most of the label appears in the 34S_r peak, but with increased labeling periods more counts are found in the other precursor species and in the mature rRNAs. After 30 minutes of labeling, the largest rRNA species now has a molecular weight of 2.1 x 10^6 daltons with an S_r value of 33. This, and the fact that the peak is broader than would be expected for a single RNA species, indicates that this highest molecular weight peak is made up of multiple species of RNA, ranging from 2.4 to 2.1 x 10^6 daltons. This could be the result of the multiple rRNA genes producing precursors of different sizes, but the progression of the S_r to a lower value with longer labeling times indicates a processing of the 34S_r size. Since all eukaryotes which have been investigated synthesize their rRNA as a single, high molecular weight precursor, the following scheme for rRNA maturation in C. reinhardi seems likely.

\[
\begin{align*}
(34S \rightarrow 33S) & \rightarrow 26S \rightarrow 25S \\
(33S \rightarrow 18S) & \rightarrow 23S \rightarrow 22S \\
(?) & \rightarrow 16S
\end{align*}
\]

It is not likely that the 23S species is the precursor of mature 18S cytoplasmic rRNA, since the combined molecular weight of the 23S and 26S molecules is greater than that of the 34S precursor.

References:

24. INVESTIGATION OF A MUTANT OF CHLAMYDOMONAS REINHARDI CONDITIONALLY DEFECTIVE IN RIBOSOMAL RNA SYNTHESIS

Investigator: Mark J. Miller

Support: National Institutes of Health, Public Health Service

Actinomycin D is an inhibitor which exerts its effect by inserting into the DNA helix and so blocking the transcription of RNA. Work done in this laboratory has shown that actinomycin D treatment will kill cells of the unicellular alga, Chlamydomonas reinhardi, at a rapid rate, as well as cause a depression in RNA biosynthesis. In hopes of studying the mechanism of actinomycin D killing, as well as the synthesis of RNA in C. reinhardi, a search for temperature-sensitive mutants was begun, selecting with actinomycin D. Mutagenized cells were exposed to the inhibitor at 33°C and were then screened for temperature-sensitives in a method analogous to that used by McMahon (1971) to isolate cells temperature-sensitive in protein synthesis.

One mutant isolated in this way proved to be defective in RNA biosynthesis. These cells will cut off incorporation of labeled precursors into their RNA after 4 to 6 hours at 33°C, while protein synthesis remains largely unaffected. They are about 100 times more resistant to actinomycin D killing than are wild-type cells at both 22°C and 33°C. The inhibitor will depress the synthesis of RNA, but not to as great a degree as in wild type. Extraction of RNA from cells grown at 33°C without actinomycin D and separation of the species by polyacrylamide-agarose gel electrophoresis shows that there is a general depression of incorporation of labeled adenine into ribosomal RNA (rRNA), but that the effect is much more pronounced in the high molecular weight species of both the cytoplasmic (25S) and the chloroplast (22S) rRNAs, than in their respective smaller species (18S and 16S). Work is currently under way to determine whether the lesion lies at the
transcriptional level, or with the maturation process of the rRNA.

Reference:

25. MUTANTS IN CHLOROPLAST MACROMOLECULAR SYNTHESIS

Investigator: John W. Cross Jr.

Support: National Institutes of Health, Public Health Service

Little is known about the synthesis of chloroplast components. Much of the confusion in this field is due to reliance on inhibitors of unknown specificity, and we have shown that many inhibitors of cytoplasmic protein synthesis in vivo also inhibit chloroplast protein synthesis. Moreover, temperature-sensitive mutants having lesions which show Mendelian inheritance appear to be affected in both chloroplast and cytoplasmic protein synthesis. It appears that disturbances in cytoplasmic protein synthesis closely control chloroplast activities. Inhibitors of chloroplast protein and RNA synthesis are not known to produce such alterations in cytoplasmic or nuclear activities. However, most such experiments have been performed under mixotrophic conditions where such regulation might be expected to be of less importance. It is interesting to note that no Chlamydomonas mutant “cured” of plastid DNA is known. This could indicate a closer dependence of the Chlamydomonas cell on chloroplast products than is known in Euglena. Thus, chloroplast regulation of cytoplasmic activities is a possibility.

To study this problem with precision, I am attempting to isolate Chlamydomonas mutants conditionally defective in chloroplast macromolecular synthesis. For this work it is vitally important that mutants be isolated for which a specific lesion can be demonstrated. Mutants are known with altered chloroplast ribosomes or decreased rRNA, but no specific lesion has been demonstrated, and the mutants are not conditionally defective.

Conditionally-defective mutants with reduced synthetic activities would help answer questions concerning chloroplast development in y-1, the sites of synthesis of chloroplast components, and the dependence of the cell on chloroplast products in heterotrophically-grown cultures.

26. ATTEMPTS AT SELECTING MUTANTS RESISTANT TO HIGH INTENSITY LIGHT

Investigator: John W. Cross Jr.

Support: National Institutes of Health, Public Health Service

I have shown that continuous exposure to high intensity white or red light in the presence of oxygen results in killing green Chlamydomonas cells. Dark-grown y-1 cells having little chlorophyll exhibit continuously increasing sensitivity. Therefore, mutagenized y-1 cells recently greened at 33°C were used for mutant selection.

Cells surviving exposure at 33°C were plated at 22°C and replica-plated at 33°C on heterotrophic medium. Colonies were picked which appeared more lightly pigmented at 33°C than 22°C. In a control experiment unselected cells were plated and light-pigmented colonies found. The frequencies of light-pigmented colonies were similar in both experiments. Most mutants exhibited a coordinate reduction in all major photosynthetic pigments.

Few mutants were thermosensitive; of those which were, a variety of phenotypes were demonstrated. Some were defective in acetate-utilization, others defective in unknown requirements for phototrophic growth, and there were several with thermosensitivity under both autotrophic and heterotrophic growth.

Since the selective process did not yield a higher percentage of lightly pigmented mutants than mass screening, it was concluded that it did not select for or against lightly pigmented mutants.

PUBLICATIONS


27. ENVIRONMENTAL MANAGEMENT

Investigator: A. J. Haagen-Smit

Support: General Funds

From his office in Kerckhoff, Dr. Haagen-Smit has continued to direct the efforts of the State of California to clear its air of pollution. In addition to being Chairman of the State Air Resources Board, he continued to serve on various committees involved in technical and legislative aspects of pollution control.

This year has seen further drastic control of pollution of new cars and a beginning has been made in the control of used cars. As a result, the monitoring stations in the Los Angeles basin have registered for the second year in a row a lowering of pollution levels. The same is true for the San Francisco basin. This decrease closely follows the measured decrease in automobile emissions and gives confidence that the control program will result in an acceptable air quality. In order to preserve the gains made in the control of automobiles and industry, plans have been submitted to the Federal government which specify the steps necessary to insure satisfactory air quality for a long time to come. These plans affect the present development of our metropolitan areas and include far-reaching proposals, such as land use planning and transportation management. This coming year will show if we can recruit support for this new deal in the battle for the healthy development of our cities.

PUBLICATIONS


Investigations in our group are concerned primarily with biochemical problems related to the regulation of development. We have made the greatest use of the fly, *Drosophila melanogaster*, as biological materials but other systems are used when appropriate. A good deal of effort has been directed toward exact definitions of enzyme or over-all metabolic systems which change during development. Examples are phenol oxidase, phenylalanine hydroxylase, chitinase, chitin synthesis enzymes, tyrosine metabolism related to cuticle formation, and peptide metabolism. Studies of such systems which are regulated during development are essential to an understanding of the specific means by which they are regulated.

A second approach to the regulation problem involves a search for natural substances which alter the normal course of development in a given system. Examples under study are dysin, a substance from *Drosophila* which has a phase-specific lethal action on the fly and the small polypeptides minimine and melittin from bee venom. In all of these cases biological action in regulation appears to be at the intercellular level through effects on membranes. Obviously this is a highly significant kind of regulation in higher organisms.

### 28. EFFECTS OF MINIMINE ON MITOCHONDRIA

**Investigators:** Herschel K. Mitchell, Peter H. Lowy  
**Support:** National Science Foundation  
Gordon Ross Medical Foundation

*Drosophila* larvae injected with minimine, a polypeptide (MW 6000) from bee venom, stop feeding, become inactive, and stop growing. However, metamorphosis does occur and miniature flies are produced. Among several possible explanations for this phenomenon, we considered it most probable that the peptide induces alterations of membrane permeability. In order to evaluate this hypothesis in a direct way, we studied the effects of minimine on isolated mitochondria. With mitochondria from *Drosophila* larvae, but more extensively with mitochondria from pigeon heart muscle, we found that the peptide does cause drastic alterations in permeability of the inner membrane. In the concentration range of $10^{-9}$ to $10^{-7}$ M minimine, swelling of mitochondria occurs in the presence of molecules as large as a tetrasaccharide but not in the presence of a 30-unit polysaccharide (inulin). At higher levels ($10^{-5}$ M) minimine causes disruption of mitochondria with the formation of small vesicles similar in appearance, by electron microscopy, to those obtained by sonication.

These results support the suggestion that altered permeability of membranes in general could explain the induction of miniature flies by minimine. The data also suggest that minimine may be useful in regulating cell leakage and thus growth in other systems than *Drosophila*. Preliminary experiments with HeLa cells in culture indicate that this is indeed the case.

**Reference:**  

### 29. THE ACTIVATION OF DROSOPHILA PHENOL OXIDASE

**Investigators:** William D. Seybold, Herschel K. Mitchell  
**Support:** National Science Foundation  
Gordon Ross Medical Foundation  
McCallum Fund

Phenol oxidase is the enzyme responsible for the tanning and hardening of the cuticle in *Drosophila*. The enzyme is latent, no activity being observed in fresh homogenates. Phenol oxidase
activity appears in a homogenate with characteristic kinetics following incubation. As a prelude to studies of the developmental regulation of phenol oxidase, extensive biochemical analyses of the enzyme have been carried out. Particular emphasis has been placed on understanding the nature of the activation process. Previous work (Mitchell, Weber and Schaar, 1967) has shown that a number of protein components are involved in the production of active phenol oxidase. This project has been concerned with the determination of the minimal number of components necessary for the activation process and the nature of the reactions between them. This has been accomplished through the partial purification of several of the components and kinetic studies of the reactions which they undergo. A model depicting the sequence of reactions believed to occur in the activation of phenol oxidase has been proposed and is depicted below.

\[
\text{Pre-S} \xrightarrow{S \text{ activation}} \text{S} \\
\text{P} \xrightarrow{S} \text{P}' \\
\text{P}' + A_1 \xrightarrow{\text{Phenol oxidase}} \text{Phenol oxidase}
\]

Further work will be concerned with the component composition of the active enzyme.

Reference:

30. TYROSINE DERIVATIVES IN THE PUPARIUM
Investigator: William J. Driskell
Support: National Institutes of Health, Public Health Service

At 120 hours of age, the Drosophila larva becomes immobile and forms within 2 hours a puparium, in which the fly is eventually formed. A rush of tyrosine derivatives goes into the forming puparium, where they probably cross-link protein; the effect is to tan and sclerotize the puparium. Radioactive tyrosine, dopa, and dopamine were injected into larvae at various times before puparium formation. Tyrosine tended to be partially converted to tyrosine-O-phosphate and small amounts of n-acetyldopamine prior to puparium formation. There is an indication that some of the tyrosine is stored in a polymeric form before it is hydrolyzed and incorporated into the puparium. Dopa and dopamine are quickly converted into n-acetyldopamine, which is incorporated into the puparium.

Another approach is to extract puparia and identify the extracted tyrosine derivatives. The extraction, of course, can alter the tyrosine derivatives so that it is difficult to infer their role in the puparium. Extraction with boiling 1 N HCl by the method of Andersen and Barrett (1971) produces a class of tyrosine derivative, the ketocatechols. The ketocatechols seem to be a major constituent of the puparium, contributing at least 50% to the OD_{280} extinction of the extract.

A comparison of the 1 N HCl extract of wild type Drosophila and the ebony mutant, which has a white puparium as compared to the tan puparium of wild type, is in progress.

References:

31. ACETYLCHOLINE ESTERASE FROM DROSOPHILA
Investigators: Herschel K. Mitchell, Lesley R. Dickson
Support: National Science Foundation
Gordon Ross Medical Foundation

We have previously reported that the bee venom peptide melittin is toxic to Drosophila and is a noncompetitive inhibitor of acetylcholine esterase from the electric eel. In order to assess further the effects of the peptide on Drosophila acetylcholine esterase, purification of the enzyme was undertaken and a homogeneous product has been obtained from fly heads. The enzyme from this source has a molecular weight of 135,000 by sucrose velocity gradients and it yields two subunits with molecular weights of 65,000 and 68,000 by sodium dodecyl sulfate (SDS) gel electrophoresis. The isoelectric point by electrophoresis is 4.7. Thus the fly enzyme appears to be half the size of the eel enzyme and to be made up of two subunits rather than four. The fly enzyme aggregates avidly with other proteins present in extracts of fly heads and this appears to be the basis for the multiple bands of enzyme activity that appear on standard and acrylamide gels.
prepared from crude extracts. The fly enzyme is inhibited by melittin and can be quantitatively precipitated by the peptide, but the precipitation is not specific for the enzyme. Thus the toxicity of melittin to *Drosophila* is probably due to interaction with a number of membrane proteins.

32. THE MOLTING PROCESS IN DROSOPHILA

*Investigators:* Thomas A. Cole, Julia E. Del Zoppo, Herschel K. Mitchell

*Support:* National Science Foundation
Gordon Ross Medical Foundation

Previous studies by electron microscopy have shown sequential changes in epithelial cells of *Drosophila* larvae during molting periods, that indicate alternation of production of groups of enzymes concerned with synthesis of cuticle and degradation of cuticle. A correspondence of chitinase activity in whole animals with the morphological changes has been established. For further work on enzymes of the degradative phase a method was developed for collection of molting fluid by continuous spraying of larvae during molting. Under usual circumstances the molting fluid is evidently ingested by the larvae at the time the cuticle is shed. Esterases and a phosphatase, as well as a variety of other proteins, were found in the molting fluid.

With regard to the cuticle synthetic phases, preliminary work on the enzymology has been carried out. Glucosamine-6-phosphate acetylase has been found to be a convenient marker for studies of initiation and termination of production of enzymes involved in synthesis. Methods have been developed for preparation of reasonably clean epithelial tissue from larvae on a scale large enough for enzyme work.

*References:*


**PUBLICATIONS**


The behavior of an organism is in large part determined by its genes, which dictate the development of the various components of the nervous system, their precise interconnections, and the biochemical features involved in their function. Alteration of specific genes may be used as a microsurgical tool to dissect the specific steps in these processes. Our research group is utilizing the fruit fly, Drosophila melanogaster, as a model system. Drosophila has a detailed nervous system and a broad repertoire of behavior, yet reproduces rapidly and lends itself readily to manipulation of its genetic material.

One application of genetic methods is to dissociate the sequential steps involved in sensory reception of the stimulus, integration in the central nervous system, and final motor output. Thus, a series of mutants has been isolated which have blocks at various steps in the phototactic response. Others have abnormal circadian rhythm, or uncoordinated locomotor behavior, or muscle defects, or drop dead suddenly. These genetic perturbations in behavior provide possible insights into the mechanism of the biological clock, the control of movement, and hereditary degenerative diseases.

Using classical genetic recombination methods, the locus of the mutant gene within the 1-dimensional chromosomal array can be precisely determined. It is another problem to identify the “focus” of the genetic alteration, i.e. the anatomical site at which the gene exerts its primary effect. This focus might be a particular region of the central nervous system, perhaps even a single cell, or it could lie in some distant organ whose malfunction influences behavior indirectly. A powerful method for pinpointing such a focus is the use of mosaics, i.e. composite individuals in which some tissues are mutant and the rest have normal genotype, thus making it possible to identify which part must be mutant to produce the altered behavior.

33. MAPPING OF BEHAVIOR IN DROSOPHILA MOSAICS

Investigators: Yoshiki Hotta, Seymour Benzer

Support: National Science Foundation

In studies of mosaics for behavioral mutations in Drosophila, it is sometimes observed that an effector organ (e.g., a leg) of mutant genotype shows normal behavior, or vice versa. This is not too surprising, since the function may be controlled, not by the organ itself, but by an internal element such as a motor neuron in the thoracic ganglion. In some mosaic individuals, the neuron could have a different genotype from the leg cuticle, since the two structures arise from different regions of a mosaic embryo. While the cuticle genotype can be identified with genetic markers such as yellow, the genotype of the ganglion is not evident on the surface. A method has been developed for constructing maps of internal behavioral foci with reference to externally visible body landmarks. Because of the correspondence between the positions of cells on the Drosophila blastula surface and the future body parts they will form, it is possible, using the observed probability that a mosaic boundary line will pass between a pair of sites, to construct a 2-dimensional fate map of the blastula and to locate a mutant behavioral focus on this map. From the position of the focus, the corresponding adult tissue may be inferred. Thus, certain visual mutants have been shown to behave abnormally because of defects that map to the part of the embryo that gives rise to the eye, others such as the drop-dead mutant map to the ectoderm which produces the central nervous system, and others such as wings-up mutants map to the mesoderm which produces the muscles.

Histological examination in some of these cases shows lesions in the corresponding structures of the adult fly that account for the behavioral anomalies observed. In addition to indicating where in the organism to look for a lesion, the
mapping technique identifies the primary focus of the mutant gene. That this turns out in some cases to correspond to the tissue related to the mutant behavior is reassuring, but it must be anticipated that this will not always be so. For instance, a histological anomaly of a flight muscle could conceivably be neurogenic. In such a case, however, the focus would map onto the area of the blastoderm destined to produce the nervous system. For the wings-up mutants analyzed, which have defective flight muscles, the fate map places the primary responsibility squarely onto the muscle itself. Since the map refers to the blastoderm stage, what the procedure in effect accomplishes is to dissect the complex relationship between nerves and muscles in the adult, unraveling them back to an embryonic stage in which they had not yet come together, so that each can be pinpointed separately.

Reference:

34. HISTOCHEMICAL MARKERS FOR THE ANALYSIS OF GENETIC MOSAICS

Investigators: Douglas R. Kankel, Jeffrey C. Hall
Support: National Institutes of Health, Public Health Service

In the use of mosaics for the analysis of the function and development of the nervous system in Drosophila, it would be extremely useful to have a cell marker for the genotype of the internal tissue. An enzyme could serve as such a marker provided it were present in sufficient quantity in the nervous system and a mutant lacking the enzyme were available.

Acid phosphatase (E.C.3.1.3.2), α-glycerophosphate dehydrogenase (E.C.1.1.1.8), isocitrate dehydrogenase (E.C.1.1.1.42), and acetylcholinesterase have been found in histochemically detectable quantities throughout most of the nervous system. The following is a brief description of the state of development of each of these markers: (1) Acid phosphatase. A small third chromosome duplication containing the wild-type allele of Acph-1 has been attached to the X-chromosome. The loss of this chromosome in an otherwise acid phosphatase-null background will allow the scoring of XX versus XO tissues in gynandromorphs. (2) α-glycerophosphate dehydrogenase. A small duplication of the second chromosome containing the wild-type allele of α-GPDH is being attached to an X-chromosome. This will be used as the X described above for acid phosphatase. (3) Isocitrate dehydrogenase. The third chromosome locus of IDH is defined only by electrophoretic variants. Deletions of the hairy locus (near that of IDH) have been made with X-rays and will be used for isolating induced null alleles of IDH. (4) Acetylcholinesterase. A search for the genetic locus or loci controlling this enzyme was conducted by screening the second and third chromosomes using a series of duplications and deletions. It was expected that a deficiency at an acetylcholinesterase locus would have decreased enzyme activity and a duplication increased activity. This has been shown to be true for several autosomally-linked enzymes of known genetic loci. Three segments on chromosome 3 repeatedly showed activity differences, whereby duplication>euploid>deletion. Disc acrylamide gel electrophoresis of fly head homogenates showed no qualitative differences in AChE banding pattern among duplications, deletions, and euploid flies.

Since acid phosphatase and α-glycerophosphate dehydrogenase have existing null alleles, it is possible to use these markers in conjunction with induced somatic recombination. Studies using these methods to define the relationship between the cells of the compound eye and the lamina are under way.

35. NEUROPHYSIOLOGICAL DEFECTS IN TEMPERATURE-SENSITIVE MUTANTS OF DROSOPHILA

Investigators: Obaid Siddiqi, Seymour Benzer
Support: Gosney Fund

Suzuki, Grigliatti and Williamson (1971) described a mutation para** on the X-chromosome of Drosophila that causes paralysis above 30°C. When the temperature is reduced again the fly recovers quickly. Such temperature-sensitive mutants, especially in conjunction with the available genetic
techniques for constructing mosaic flies, provide a potentially useful method of analyzing the neuro­motor system.

We have isolated a series of recessive temperature-sensitive mutants, involving different genes on the X-chromosome, and investigated the neuro­physiological basis of the paralysis in mutant and mosaic flies. Each mutant is paralyzed by a brief exposure above a critical temperature. Three of the mutants are 'allelic to \textit{para}^{44}' and have similar properties. A second group of three mutants involves a different gene, \textit{comatose}, located at 40 units on the X, which became paralyzed after exposure to temperatures above 38°C. While recovery of \textit{para}^{44} occurs in a matter of seconds, recovery of the \textit{comatose} mutants requires many minutes or hours, depending upon the length of exposure at high temperature. Normal flies require 42°C for paralysis and recover quickly.

The foci of the behavioral defects have been mapped by examining the behavior of mosaic flies, using the method of Hotta and Benzer. The foci of \textit{para} and \textit{comatose} map in a region of the blastoderm fated to become the ventral nervous system. There is a separate focus for each leg and these are located near the foci for another mutant, \textit{hyperkinetic}, which is known to involve the thoracic ganglion (Ikeda and Kaplan, 1970), but at different positions.

Neurophysiological studies have been made. Normal flies respond to electric stimulation of the cervical nerve with a strong jerk of the legs. \textit{para} continues to show this response well above its paralysis temperature. This shows that paralysis in these mutants is not due to a general defect in nerve conduction, neuromuscular junctions, or muscle excitability. More likely, high temperature blocks some normal synaptic pathway in the thoracic ganglion; artificial cervical stimulation may excite an alternative pathway not normally used for locomotion.

The \textit{comatose} mutant, on the other hand, after a short exposure above 38°C ceases to show leg jerks in response to cervical stimulation (at room temperature) until the long recovery period is over. Electromyograms recorded extracellularly from the legs show that during paralysis action potentials in the leg muscles are not elicited by cervical stimulation. As the fly recovers, the leg jerk response and the electromyogram return to normal.

This raises the question whether muscle function is affected. Intracellular recordings were made from the longitudinal flight muscles. The muscles of normal flies and \textit{para} maintain their resting potentials and continue to show action potentials in response to cervical stimulation at temperatures above 38°C. In the case of \textit{comatose}, however, the resting potential remains unchanged, but the muscle action potential ceases and all traces of neural input into the muscle disappear. After recovery, the postsynaptic junction potential and the action potential return. The excitability of the muscle itself was examined by intracellular injection of depolarizing current; the results show that the muscle itself is excitable under conditions where the fly is paralyzed. This indicates that the defect lies elsewhere. The \textit{comatose} mutant also differs from the other in that the electroretinogram is sensitive to temperature; the positive spike in the ERG is reversibly lost above 38°C. It is possible that, in this mutant, the neural input into muscles is blocked by a failure either in axon conduction or in synaptic transmission.

References:

36. CONDITIONAL BEHAVIOR IN DROSOPHILA

\textbf{Investigator:} William G. Quinn Jr.

\textbf{Support:} National Institutes of Health, Public Health Service
National Science Foundation

A paradigm has been developed in which \textit{Drosophila} shows conditioned behavior. The procedure involves training populations of flies to avoid an odor presented simultaneously with electric shock. Phototaxis stimulates flies to move from a darkened tube in which they are started towards an illuminated tube containing a grid on which they can be shocked. An apparatus devised by Benzer for behavioral countercurrent distribution is used; this permits the flies to be run from the same starting tube into 1 of 4 grid tubes. Odorant A is spread on the grids in tubes 1 and 3, and odorant B on grids 2 and 4. In the training phase the flies are run alternately into tubes 1 and
2. Ninety volts AC is applied to grid 1 so that the flies are shocked during their exposure to odor A. For testing, the flies are run alternately towards tubes 3 and 4. The populations are trained and tested in different tubes to eliminate odor cues laid down by the flies themselves. The number of flies avoiding each tube is recorded, and the flies are found to selectively avoid tube 3 containing odor A which was associated with shock during training (P<.01). If the entire experiment is repeated with a new population, except that the voltage is applied to grid 2, then during testing the flies selectively avoid tube 4 containing odor B. These results imply that the flies learn specifically to avoid the odor which was paired with shock during their training.

The experiments have been run “blind”; the results are the same. Permuting the alternation of odors during training or testing does not affect the results. Several odorants have been found which can be arbitrarily selected in pairs to use as odors A and B. Some learned behavior persists after 30 minutes. If two populations with different genetic markers are trained as above, one to avoid odor A and the other odor B, then mixed, each population avoids only the odor which was coupled with shock during its training. This suggests that the flies are acting as individuals rather than stampeding populations during these experiments, and makes possible the use of wild-type populations as “internal standards” in the isolation and characterization of mutants with altered properties.

The availability of this conditioning paradigm for Drosophila may permit the isolation of mutants with altered learning properties and the application of genetic methods to the study of memory acquisition and storage.

Reference:

37. USE OF MOSAICS IN THE ANALYSIS OF PATTERN FORMATION IN THE RETINA OF DROSOPHILA

Investigators: Thomas E. Hanson, Donald F. Ready, Seymour Benzer

Support: National Science Foundation
National Institutes of Health, Public Health Service

The retinular cell pattern in the upper half of the Drosophila eye is a mirror image of the lower half, the two areas being separated by an anterior-posterior equator. This raises the question whether symmetrical halves arise from two separate cell clones. This was tested in mosaic flies using cell markers. A mutation on the X-chromosome causing degeneration of photoreceptor cells was employed to produce mosaics by X-chromosome loss. Mosaics with small patches were also made by X-ray-induced somatic crossing-over. In the mutant white, the tiny pigment granules normally present within the photoreceptor cells are absent, so that this gene can be used to mark both these cells as well as the pigment cells. Many mosaic patches were found in the equatorial region which included both of the mirror image patterns. The establishment of the equator is not due to cell lineage.

Within a given ommatidium, the eight photoreceptor cells are arranged in a precise, asymmetric pattern. It has been suggested (Bernard, 1937) that these eight cells are derived from a single cell that undergoes three divisions, i.e. an ommatidium is derived from a single clone. There also exist other possible cloning patterns for retinular cells, which would create regularities in the types of cells involved in a single cross-over event. This was tested in mosaic flies utilizing the white marker. In mosaic patches, no strict relationship was found in the genotypes of the various possible combinations of retinular cell types. Thus, clonal origin is not involved in establishing the retinular cell types or patterns. It was found that along the borders of large mosaic patches the genotypes of retinular cells and pigment cells correspond closely. This indicates that both types of cells are ultimately derived from the same stem cells and that there is relatively little cell mixing or migration during development of the eye.

Reference:
38. NEUROGENESIS IN THE EYE AND OPTIC TRACTS OF DROSOPHILA

Investigator: Thomas E. Hanson

Support: National Science Foundation

We have examined the eye and optic tracts of Drosophila throughout their developmental history, concentrating attention on the critical events in establishing the specific neural wiring pattern. Outgrowth of axons from the eye imaginal disc directed through the optic stalk toward the brain occurs during the latter half of the third larval instar. Reconstruction of developing fields of retinular axons indicates that the sequence of axon outgrowth from the retina is highly ordered in time and controls precisely the projection of retinotopic order to the lamina. In the larval brain second- and third-order cells, originating in a similar sequential fashion, form connections with retinular axons such that the retinotopic order is automatically propagated throughout the optic ganglia. Retinular wiring patterns at the periphery of the adult eye indicate that second-order cells may exist even in situations in which they are utilized by only a single retinular axon rather than the normal group of six. Degenerate, excess second-order cells at the periphery of the developing lamina do not occur, indicating that programmed death of unutilized second-order cells is not the mechanism producing coincidence of first- and second-order fields. Rather, the set of second-order cells seems to be induced by the presence of the retinular axons in the developing lamina. The geometry of the developing optic ganglia allows the axon of a retinular cell and its second- and third-order cells to make transient contact at the same time and place, so that formation of connections between these three groups of cells is essentially a single event.

Wiring mechanisms such as those observed in the optic tracts are probably utilized repeatedly throughout the developing nervous system. Preliminary observations indicate that the development of the lobula and lobular plate are similar in mechanism to that of the lamina-medulla complex. Also the development of the ocellar connections is in general similar to that of the retina.

39. GROWTH CONE DYNAMICS IN THE LAMINA OF DROSOPHILA

Investigators: Thomas E. Hanson, Yu-Hwa Jiang, Jen-Yu Lee

Support: National Science Foundation

Evidence, both from our work and others, suggests that the specificity of retinular axon connections in the lamina derives in some way from the relative orientation of retinular cells in the retina (Horridge and Meinerzhagen, 1970; Horridge, 1972). We have found that many mutants having derangements of retinular cell pattern also have derangement of lamina neuropil structure. EM reconstruction of the lamina of one such mutant has shown that the pattern of retinular axon connections is almost randomized, although the general ultrastructure is normal. The nature of these wiring errors indicates that the axons of the outer six retinular cells are chemically capable of establishing a wide variety of abnormal connection patterns. The normal patterning may depend on mechanisms involving the mechanics of growth cone movement rather than differential labeling of growth cone membranes.

Due to the regularity of the lamina neuropil at most stages of its development it is possible to observe by EM reconstructions the behavior of identifiable retinular axon growth cones in the process of establishing predictable connections. Contacts are made during the 48 hours following pupation, and the formation of recognizable synapses between retinular axons and second-order cells occurs at 72 hours after pupation. The essential feature of the progression of retinular growth cones toward their targets is that on the surface of the growth cone microspikes are produced in random orientations, the cone initially expanding in all directions. In so doing it makes physical contact with many target areas, but over a period of 30 hours the cone slowly elongates preferentially in the direction of the correct target. Nevertheless, it persists in extending microspikes at random along its entire surface into incorrect target areas.

At the earliest stage observed, the start of pupation, growth cones belonging to a given ommatidium in the retina are connected to each other by a particular type of junction, forming a ring that circles a set of second-order axons. At 48 hours the cones have progressed to their targets and have partially acquired axonal morphology. At
this point the growth cone junctions have disappeared from most of the axons. At the same time junctions have formed, in the target area, between the axons and their new partners which will make up an optic cartridge.

In the adult a bundle of retinular axons from the same ommatidium twists 180° before it enters the lamina neuropil. Achievement of an exact 180° twist is probably essential since it determines the orientation of the growth cone junctional group. This twist is observed in the lamina at the 12-hour stage and may exist earlier. At distal levels in the developing lamina, the bundles of retinular axons lie on the side of their set of second-order axons further from the equator. As the combined bundle approaches the lamina neuropil the retinular bundle spirals around the second-order axons to the equatorial side. The spiral is such that the individual fibers in the retinular bundle are twisted 180° with respect to each other. The force responsible for the spiral could come from either an interaction between retinular and second-order axons or between retinular growth cones and an unknown constituent in the neuropil.

References:

40. ENTRAINMENT OF NORMAL AND MUTANT CIRCADIAN RHYTHMS BY LIGHT AND TEMPERATURE CYCLES

Investigator: Ronald J. Konopka
Support: National Science Foundation
National Institutes of Health, Public Health Service

A circadian rhythm normally persists with a defined period under constant environmental conditions. By introducing a cyclic change in some environmental variable, however, it is possible to entrain the rhythm to the period of the environmental cycle. The circadian eclosion rhythm in Drosophila pseudoobscura can be entrained by light and temperature pulses (Pittendrigh, 1960); single brief light pulses can permanently reset the eclosion rhythm, advancing or delaying it by as much as 12 hours. Single temperature pulses, on the other hand, produce spectacular temporary perturbations in the rhythm, but the phase of the rhythm eventually stabilizes at a point only 1 to 3 hours away from its original position. These results have been interpreted to mean that there are two oscillators in the Drosophila clock, a light-sensitive driving oscillator, and a temperature-sensitive driven oscillator.

If this is indeed the case, a mutation in the clock mechanism might affect the driving oscillator, the driven, or both. Three mutations affecting the clock of D. melanogaster have previously been described (Konopka and Benzer, 1971); since these mutations alter the steady-state period of the eclosion rhythm, it can be inferred that the driving oscillator is indeed affected. To determine whether the 2-oscillator model is appropriate for D. melanogaster, as well as to determine what part of the clock mechanism is affected by the mutations, the behavior of wild-type and mutant flies in light and temperature cycles was studied. Three fly strains were used in the experiments; one was rhythmically normal, one had a short period of 19 hours, and one was arrhythmic in constant conditions. The rhythm studied was the eclosion rhythm in adult flies from the pupal cases.

First, the three strains were exposed to a 24-hour light-dark cycle (LD 12:12). Both the normal strain and the 19-hour mutant strain showed a period of 24 hours, but the arrhythmic mutant remained arrhythmic. The three strains were then exposed to a temperature cycle (approximately 12 hours of 18°, 12 hours of 25°). When this was done in constant light, all three strains entrained to a 24-hour period. In constant darkness, however, only the normal flies and the arrhythmic flies entrained to a 24-hour period; the short-period flies had a period of about 21 hours (except when the temperature step-down coincided with the next projected eclosion peak, in which case an additional 9-hour delay was produced). When the three strains were exposed to a temperature cycle in constant light, then transferred to constant temperature and constant darkness, the normal and short-period strains reverted to their free-running periods, while the arrhythmic mutant reverted to its arrhythmic state.

These results may be interpreted in terms of the 2-oscillator model for the Drosophila clock. In the case of the arrhythmic mutant, the light-sensitive driving oscillator is effectively eliminated.
by the mutation, so that the mutant shows no rhythm under constant conditions or in a light-dark cycle. The temperature-sensitive driven oscillator is intact, however, and may be entrained by an exogenous temperature cycle, thus producing an eclosion rhythm. When the temperature cycle is subsequently eliminated, the rhythm disappears, since the driven oscillator is not self-sustaining. In the case of the short-period mutant, the light-sensitive driving oscillator is present, although its steady-state period is altered. The eclosion rhythm can, however, be entrained to a 24-hour light-dark cycle, since the driving oscillator is not completely eliminated. A 24-hour temperature cycle is insufficient to entrain the rhythm in constant darkness; the driving oscillator is relatively insensitive to temperature, so the required 5-hour phase delay cannot be produced, and the rhythm shows a period of about 21 hours. In constant light, the driving oscillator is stopped, and the driven oscillator, which is sensitive to temperature, can be entrained by the exogenous temperature cycle to a 24-hour period.

References:

PUBLICATIONS

Every type of differentiated mammalian cell utilizes surface receptor molecules for a variety of functions. These protein molecules serve as highly specific sensors and transducers which convey information to the cell concerning its environment. The receptors of our sensory neurons serve to detect light, odors, flavors, etc. Other cell surface receptors react specifically with any one of more than 15 polypeptide hormones. Still another class of receptors senses neural transmitters and other neurosecretory compounds such as the hypothalamic factors. Among the most fascinating of cell surface receptors are those responsible for the process of specific cell recognition during embryogenesis. These molecules control the recognition processes in the morphogenesis of all organs including the most complex of all, the human brain.

We are engaged in a systematic study of membrane receptor molecules starting with a simple receptor protein, rhodopsin. The study of the primary structure of such molecules is but one aspect of a program in which we hope to understand more of the molecular mechanisms by which membrane receptors function. We are particularly interested in looking for evolutionary and functional relationships which may exist between different molecules and between different classes of membrane receptors. The eventual goal of this program is to elucidate the genetic and molecular basis for cell recognition during morphogenesis.

As a second objective, we are working on the development of new methods for the fractionation of membrane proteins, procedures for refolding membrane proteins following fractionation in denaturing solvents, and procedures for studying the antigenic and functional properties of such highly purified and refolded membrane molecules. We will be aided in this program by a sensitive, new-generation peptide sequenator which we have developed which permits us to work with very much less material than is required for conventional commercial instruments. Several other highly sensitive methods for studying proteins and peptides will also be developed further. These methods and instrumental approaches, while important in their own right, are needed in order to make it possible to study classes of membrane receptor molecules which are present in extremely small amounts and which are at the same time exceedingly difficult to fractionate by means of contemporary techniques.

Our interest in membrane receptors has evolved from our studies of antibody molecules in the immune system. Although a great many questions remain to be answered, many immunologists are now in agreement as to the general nature of the molecular events underlying differentiation of the various cell lines seen in the immune system. Our contributions to this problem have been summarized in several publications. Briefly, we concluded from studies of the immune system that each antibody chain results from the expression of two genes which have been "spliced" together during the determinative events which precede the differentiation of an immunologically competent cell. The two classes of genes involved are referred to as "variable genes" and "common genes." Genes in one of these classes, the variable genes, appear to be carried in large numbers in the germ line of each vertebrate. According to this hypothesis, a precise, programmed chromosomal rearrangement occurs during cell determination which unites two previously separated stretches of genetic information. The new arrangement, which combines a variable gene with a common gene, then becomes a genetically stable property of the newly determined somatic cell line. Many thousands of distinct and stable somatic cell lines result from these programmed developmental events. Studies of normal antibody-producing cells, as well as myeloma tumor cell lines, indicate that a committed and differentiated cell is capable of synthesizing only one molecular species of antibody molecule. The antigen plays a selective role in triggering cell division and differentiation in only those clones of antibody-producing cells which have complementary surface receptors. In other words, the decision as to which antibody a given cell can synthesize is made before an antigenic stimulation. The antigen, a specific chemical shape on the surface of foreign molecules, then serves as the inducer or hormone-like stimulating agent which causes these cells to undergo further cell division and differentiation steps leading to the immune response.

The general acceptance of these concepts among immunologists has encouraged us to redirect our program to encompass questions of membrane recognition processes involved in the differentiation of other specific
cell types. In addition to programming the specificity which is found on the surface of cells in the immune system, chromosomes must also program the cell surface specificity found on differentiated cells in other complex systems. There is, of course, no certainty that the genetic and molecular phenomena which underlie the production of membrane receptors in the immune system are in any way related to those responsible for membrane functions of other cell lines. However, evolutionary considerations lead us to believe that there may well be certain common denominators.

Our long term goal of understanding the genetic and molecular basis of cell recognition during morphogenesis is clearly a formidable task. For this reason, we are working out the methodological problems in simple model systems before making major investments in the analysis of more complex membranes. Thus, the abstracts which follow describe work on (1) fractionation of proteins from bacterial membranes, (2) membranes and morphogenesis in the cellular slime molds, (3) structure and function of the visual receptor membrane protein, rhodopsin, and (4) development of methods for use in studies of membrane proteins.

General References:

41. A NEW PROCEDURE FOR ION EXCHANGE CHROMATOGRAPHY OF MEMBRANE PROTEINS

Investigators: John E. Smart, Richard S. Newman*

Support: National Institutes of Health, Public Health Service
National Science Foundation

The fractionation and characterization of membrane proteins has been severely retarded by the fact that they are not generally soluble in the common aqueous systems. One of the more general techniques which has been developed to circumvent this problem involves treating the water-insoluble proteins with detergents, such as sodium dodecyl sulfate. The water-soluble detergent-protein complexes are then fractionated by molecular size sieving. Because this technique is limited to fractionation on the basis of molecular size, we have developed a new procedure for the general solubilization and monomerization of membrane components which permits them to be subsequently fractionated by ion exchange chromatography in urea solutions. The initial solubilization involves exposing a concentrated suspension of membranes to an equal volume of cold 100% formic acid for 30 minutes. Enough urea and β-mercaptoethanol is added to yield a final concentration of 10 M urea, 25% formic acid, and 5 mM β-mercaptoethanol. This solution is stirred at room temperature until the urea is dissolved (ca. 1 hour). An ion exchange resin (usually DEAE or SP Sephadex) is added to the membrane solution. The slurry is then poured into a dialysis tube and dialyzed with frequent agitation (to keep the suspension fairly uniform) against 6 M urea, 10 mM trisma base, 5 mM EDTA, and 5 mM β-mercaptoethanol until the desired pH is obtained. It is worth noting that to a large extent the proteins which bind to the column can be regulated by appropriate choice of the final pH of the ion exchanger-membrane complex. The slurry is then packed into a column which is then developed with a sodium chloride gradients.

Using this procedure we have obtained several preparations of homogeneous membrane proteins (Smart, Biology 1972, No. 42; Smart and Tuchman, Biology 1972, No. 44). We have observed that without exception the proteins which are eluted from the column are soluble in 6 M urea solutions. This may be due to the absence of lipids in most of the fractions. In any case, the fact that membrane proteins are soluble in 6 M urea after the initial fractionation allows one to use many classical separation techniques in their further purification, e.g. isoelectric focusing, chromatography on other exchange resins, polyacrylamide gel electrophoresis, etc. Experiments are currently in progress to automate the initial steps using automatic titration devices coupled with amicon hollow fiber dialysis units.

*NSF Summer Trainee, 1972.
STUDIES ON A FAMILY OF RELATED PROTEINS FROM BACTERIAL MEMBRANES

Investigator: John E. Smart
Support: National Institutes of Health, Public Health Service

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretic analysis of E. coli K12 membranes which have been denatured in 1% SDS + 1% β-mercaptoethanol at 100°C for 5 minutes shows a major protein component at ca. 36,000 molecular weight (MW) as well as several minor components. If these same membranes are exposed to less severe denaturing conditions 37°C for 3 hours), essentially the same electrophoretic pattern is obtained for the minor components, whereas no major protein band is observed at ca. 36,000 MW, and instead a new major peak is observed at ca. 25,000 MW. If the species which exhibits the 25,000 MW under mild conditions is eluted from the gel and denatured more severely it then behaves as a 36,000 MW species. Ion exchange chromatography of bacterial membranes under the conditions described by Smart and Newman (Biology 1972, No. 41) separates the components of the membrane into several distinct peaks.

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<th>Residue #</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>5</th>
<th>6</th>
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<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>Ala</td>
<td>1/3 Pro</td>
<td>Lys</td>
<td>Asn</td>
<td>Asp</td>
<td>Thr</td>
<td>Mixture</td>
<td></td>
</tr>
<tr>
<td>Fraction 2</td>
<td>Ala</td>
<td>Glu</td>
<td>Val</td>
<td>Tyr</td>
<td>Asp</td>
<td>Thr</td>
<td>Asp</td>
<td>Gly</td>
</tr>
</tbody>
</table>

Thus it appears that this family of proteins has maintained certain amino acid sequences constant while allowing certain residues to vary. Other homogeneous 36,000 MW fractions are currently being characterized.

AN ASSAY FOR THE DEVELOPMENTALLY CONTROLLED CONTACT RECOGNITION IN CELLULAR SLIME MOLDS

Investigators: John E. Smart, Jessica Tuchman
Support: National Institutes of Health, Public Health Service
National Science Foundation

The study of the molecular events underlying cell contacts and changes in shape of multicellular systems during development (morphogenesis) represents one of the most important and potentially rewarding areas of current scientific investigation. An ever-increasing amount of data suggests that the particular cellular organelle which provides the decisive control in this type of cellular behavior is the cell surface membrane. Because of the extreme difficulties in developing a simple and reliable assay for contact recognition in mammalian cell cultures, we have chosen to perform our initial investigations on a simpler model system. The cellular slime mold, Dictyostelium discoideum, has well defined unicellular and multicellular phases during its life cycle that appear to necessitate a sorting-out mechanism during their morphogenesis. Several workers have found that cell surface antigens, composed of protein and carbohydrate, differ between the same species at different developmental stages and between different species at the same developmental stage (Gregg, 1960; Takeuchi, 1963; Kahn, 1964; Sonneborn, Sussman and Levine, 1964; Gerisch, 1969).

It is the purpose of this study to fractionate and characterize the membrane components of Dictyostelium discoideum which are responsible for the developmentally controlled, species-specific
cell aggregation. To do this we have developed a simple assay which measures the ability of membrane fractions to directly inhibit the aggregation and further development of the organism. The preparation under investigation is mixed with the appropriate concentration of myxamoebae and then plated. The ability of the preparation to inhibit the aggregation and further development of myxamoeba is monitored photographically at appropriate time intervals throughout development. Our studies have shown that the assay displays at least some of the development and organismic specificities of the intact organism. For example: (1) Membranes isolated from aggregating cells (aggregation phase membranes) of D. discoideum are 2 to 3 times more effective than membranes prepared from the unicellular myxamoebae (vegetative phase membranes). (2) Aggregation phase membranes are at least 10 times more effective than membranes isolated from HeLa cells (the highest obtainable concentration of HeLa cell membranes did not inhibit aggregation at all).

Additional observations are: (1) A large part (ca. one half) of the activity of aggregation phase membranes is heat-stable, whereas that in vegetative membranes appears to be completely heat-labile. (2) The activity in aggregation phase membranes is not sensitive to the addition of excess magnesium or calcium ions (membranes are not simply chelating divalent cations from the assay medium). (3) Neither DNase nor RNase destroys the activity of aggregation phase membranes. (4) Aggregation phase membrane components which have been totally denatured and dissociated by treatment with formic acid and urea and then dialyzed into assay conditions, are still active in the assay. (5) Each plate contains 5 x 10^7 cells, which would yield approximately the same amount of membrane as is required for inhibition of aggregation by aggregation phase membranes.

Our data support the model in which developmentally controlled carbohydrate cell surface antigens interact specifically with protein cell surface binding sites during the aggregation and further morphogenesis of cellular slime molds.

References:
45. STUDIES ON THE MECHANISM BY WHICH ISOLATED MEMBRANE FRAGMENTS INHIBIT THE DEVELOPMENTALLY CONTROLLED CONTACT RECOGNITION (AGGREGATION) OF DICTYOSTELIUM DISCOIDEUM

Investigators: Jessica Tuchman, John E. Smart

Support: National Institutes of Health, Public Health Service

Gerisch (1969) has observed that univalent antibodies which are directed against the cell surface of aggregating Dictyostelium discoideum are capable of inhibiting the species-specific, developmentally controlled aggregation. This type of interference is presumably due to a specific coating of the cell surface with antibodies which then sterically interfere with the cell-cell recognition process. In another report (Biology 1972, No. 43) we have described an assay which measures the ability of isolated membrane components to direct the aggregation and further development of the organism. This assay involves mixing isolated membrane components with vegetative (0 hours development, aggregation incompetent) cells. We have observed that if the cells are allowed to develop normally for 0, 3, or 6 hours before mixing with the isolated aggregation phase membranes, the aggregation and further development of the cells is inhibited (in these experiments approximately two times the minimal inhibiting concentration of membranes was used). However if the cells are allowed to develop normally for 9 or 12 hours (at 9 hours loose aggregates have formed; at 12 hours aggregation is complete) before mixing with isolated aggregation phase membranes the cells are not inhibited in their aggregation or further development. These observations suggested that the activity of aggregation phase membranes in this assay is due to more than simple coating of the cell surface and subsequent steric interference with the cell-cell recognition process.

In an attempt to determine some of the internal changes which occur after exposure of the myxamoebae to aggregation phase membranes, we have monitored several enzymes which are induced at specific times during the aggregation and further development of D. discoideum. The enzymes studied increase in activity during three main time periods: Early, before aggregation; Middle, after aggregation, but before sporulation; Late, during and after sporulation. Three of the six enzymes studied exhibit an activity after treatment with aggregation phase membranes which does not significantly differ from that of vegetative cells (0 hour cells). These are: uridine diphosphoglucose pyrophosphorylase (middle), tyrosine transaminase (middle), β-glucosidase isozyme I (early), and isozyme II (late). N-acetyl glucosaminidase (early) is totally repressed by treatment of the cells with aggregation phase membranes. Interestingly, we have found that one early enzyme, threonine dehydrase, is induced as an early enzyme even when all morphological development has been inhibited. Most importantly, alkaline phosphatase, a late enzyme, is prematurely induced (appears as a middle enzyme approximately 12 hours ahead of when it appears in normal development) when the cells are treated with aggregation phase membranes. The same enzymological results are obtained with heat denatured aggregation phase membrane components (120°C for 30 minutes).

Our data support the hypothesis that the species-specific, developmentally controlled contact recognition in this organism involves the interaction of a heat-denaturable component (presumably protein), which is always present on the cell surface, with a developmentally controlled heat-stable component (presumably carbohydrate) of the cell surface. Moreover, studies on the appearance of developmentally controlled enzymes suggest that a heat-stable component(s) of aggregation phase membranes directly affects the levels of expression of several enzymes. The data are consistent with the concept that proper contact between cell surfaces results in a hormonal-like induction or repression of certain enzymes, thereby, perhaps, setting the cell onto a new morphological pathway.

Reference:
46. PHOSPHORYLATION OF RHODOPSIN BY ATP: A KINASE-MEDIATED DARK REACTION OF RECEPTOR PROTEIN AFTER EXPOSURE TO LIGHT

Investigators: Hermann Kühn, James H. Cook, William J. Dreyer

Support: Deutscher Akademischer Austauschdienst
National Institutes of Health, Public Health Service

The retinal rod cell is a specialized neuron containing a receptor system triggered by light. The photoreceptors are located in the rod outer segment (ROS), a highly specialized membrane system containing the protein rhodopsin as a predominant component (Dreyer, Papermaster and Kühn, 1972). The receptor molecule, rhodopsin, can be isolated relatively easily in amounts that are useful for biochemical and protein chemical studies. Thus it serves as a useful model system for the study of membrane receptors.

The visual receptor (MW = 30,000 to 40,000) resembles other systems such as the acetylcholine receptor (MW = 40,000) in that it involves interaction of a small molecule (cis-retinal) with a specific membrane protein (opsin). Photoreceptor function differs from the usual system in that the primary triggering event is a light-induced isomerization of an 11-cis-retinal molecule already bound to the receptor protein, thus forming all-trans-retinal. The original light sensitivity of rhodopsin is restored when, through a cycle of reactions, 11-cis-retinal is again linked to the protein. Absorption of one single photon in the outer segment of a rod cell suffices to trigger a synaptic event at the other end of this neuron (Hagins, Penn and Yoshikami, 1970). Little is known about the primary event which signals the triggering of a photoreceptor, though secondary events involving changes in current flow through the outer cell membrane have been studied (Hagins, Penn and Yoshikami, 1970). In the case of the acetylcholine system it has been shown that receptor action leads to the opening of a channel which permits a flux of both sodium and potassium through the membrane (Thesleff, 1970). It is not known whether a similar molecular event occurs in the discs during photoreceptor function.

The rod cell is able to respond to light flashes in a wide range of sensitivity (about 8 orders of magnitude) without saturation. Efficient control mechanisms ("light/dark adaptation") must thus be involved in regulating the sensitivity; details concerning the molecular basis of this process are also virtually unknown. We have found a reaction that may play a role in this process. When bovine ROS membrane suspensions were incubated with γ-32P-ATP and MgCl₂ and exposed to light, rhodopsin became phosphorylated (Biology 1971, No. 82). Under optimum conditions, about one mole of phosphate was bound per mole of rhodopsin. 32P-serine and, to a lesser extent, 32P-threonine, could be identified after mild acid hydrolysis as phosphorylation sites.

Exposure of the membrane to light during or prior to the incubation with AT32P is a necessary condition for phosphorylation; dark-adapted rhodopsin is phosphorylated only to a very low extent. The enzyme that effects phosphorylation (rhodopsin-kinase) was found to be water-soluble and to be active independent of light. It is also able to phosphorylate certain other proteins, histones and protamine. These proteins were phosphorylated in dark as well as in light, whereas rhodopsin must first be bleached to become available for phosphorylation.

A series of in vivo experiments are in progress to determine whether this reaction, observed so far in vitro at the isolated photoreceptor membrane, also occurs in vivo as a part of the process of light/dark adaptation or vision.

References:

47. AN INVESTIGATION OF THE PRIMARY STRUCTURE OF BOVINE RHODOPSIN

Investigators: Paul A. Hargrave, Charles V. Barber, Ronald W. Siemens, William J. Dreyer

Support: National Institutes of Health, Public Health Service
American Cancer Society

Determination of the primary structure of a protein is an important step in attempting to correlate structure with protein function and in studying evolutionary relationships among proteins with related functions. Rhodopsin, a protein found in the membrane of a neuron, is a receptor for light. We expect that insights gained from the study of function in this specialized system will aid in understanding membrane receptor function generally.

Rhodopsin is the only receptor protein known to us which can be readily obtained in quantities sufficient for structural studies using existing methods. It is the principal protein of the rod outer segment disc membrane and contains the chromophore 11-cis-retinal bound at one of its active sites. Upon absorption of one quantum of light energy the 11-cis-retinal is converted to trans-retinal. The protein, opsins, then becomes a substrate for a protein kinase found in rod outer segments and undergoes another light-dependent reaction, a specific phosphorylation (see Biology 1972, No. 46). Our goal is to determine the primary structure of rhodopsin and to localize in the sequence the sites of phosphorylation, attachment of retinal, and other specific portions of the molecule which can be tagged (see for example Wu and Stryer, 1972). We are presently engaged in separating and sequencing various rhodopsin peptides by the use of standard and novel techniques.

Reference:

48. STUDIES ON THE REFOLDING OF MEMBRANE PROTEINS AFTER THE REMOVAL OF SODIUM DODECYL SULFATE

Investigator: Robert S. Molday

Support: American Cancer Society
National Institutes of Health, Public Health Service

The isolation and characterization of functionally active membrane proteins is essential for a detailed understanding of membrane systems. Previous studies on a number of different biological membranes have emphasized the difficulties inherent in the solubilization and fractionation of membrane proteins in their functional state (Razin, 1972). In particular, organic solvents such as formic acid or ionic detergents such as sodium dodecyl sulfate (SDS) which adequately solubilize and disaggregate membrane components are also potent protein denaturants; on the other hand, nonionic detergents such as Triton X-100 which maintain many of the functional properties of membrane proteins do not effectively disaggregate membranes into their components.

An alternate approach to the problem of membrane protein fractionation, currently under investigation, involves (1) solubilization of membrane proteins with powerful dissociating agents such as SDS; (2) fractionation of the protein components using preparative gel electrophoresis and/or other techniques (see Biology 1972, No. 41); and (3) the removal of the dissociating agents and subsequent refolding of the proteins into their native conformation in the presence of a suitable lipid matrix. Preliminary model studies on the refolding of the water-soluble proteins, lysozyme, cytochrome c, and alcohol dehydrogenase, after the removal of SDS by anion exchange chromatography, indicate that a substantial percentage of the functional (enzymatic) activity can be recovered after denaturation by SDS. Complete enzymatic activity is restored in the case of lysozyme. Efforts are now being directed toward the complete removal of SDS from SDS-solubilized membrane proteins and comparison and correlation of the physico-chemical, immunochemical, and functional properties of the refolded pure membrane proteins with those measured in the original membrane system. Membrane antigens or receptors, for example, could then be fractionated by means of relatively simple and powerful methods, yet could be readily assayed after refolding.

In addition to serving as a possible general method for fractionation of membrane components, such studies on the refolding of membrane proteins and the reconstitution of membrane
systems should yield deeper insight into the molecular events underlying the assembly of membrane structures.

References:


49. STUDIES ON THE PROTEIN KINASE CATALYZED PHOSPHORYLATION REACTION IN THE ELECTRIC ORGAN OF THE TORPEDO AND ELECTROPHOROUS ELECTRICUS

Investigator: Robert S. Molday
Support: American Cancer Society
National Institutes of Health, Public Health Service

Many studies have indicated that protein kinase catalyzed reactions involving the transfer of the γ-phosphoryl group from ATP to proteins are often a part of regulatory and control processes (Walsh, Perkins and Krebs, 1968). The recent observation in this laboratory that the visual photoreceptor, rhodopsin, is phosphorylated by ATP only after bleaching by light (Biology 1972, No. 46), raised the possibility that other neural receptors may be phosphorylated in an analogous fashion.

In order to assess the possible importance of such phosphorylation reactions in the control of other neurophysiological functions, such as in synaptic transmission, a study of protein kinase catalyzed reactions is currently being carried out on the cholinergic receptor system of the electric organ of the Torpedo and Electrophorous electricus. Preliminary results, however, indicate that membrane fragments high in acetylcholine receptor activity, as measured by α-bungarotoxin binding, are not phosphorylated by γ-32P-ATP even in the presence of cholinergic agonists. However, a protein kinase is present in the 20,000 g supernatant of homogenized electric tissue which catalyzes ATP-dependent phosphorylation of the exogenous protein substrate, histone II, and two major endogenous protein substrates of subunit molecular weights 44,000 and 53,000. These values were determined by 32P counting on SDS polyacrylamide gels after electrophoresis. Further studies are now in progress to elucidate the functional role of this phosphorylation reaction and to survey other receptor systems.

References:

PUBLICATIONS

BEHAVIORAL BIOLOGY
BIOLOGICAL SYSTEMS ANALYSIS
DROSOPHILA GENETICS
NEUROPHYSIOLOGY
PSYCHOBIOLOGY

Earle M. Jorgensen Laboratory of Information Science
Kerckhoff-Alles
(Third Floor)
This group is concerned with information processing in the human visual system. The specific areas of interest are: the functional properties of the oculomotor system, the interaction of eye scanning patterns and perception, and the processing of visual information in the cortex.

50. THE OCULOMOTOR SYSTEM OF THE HUMAN EYE

Investigators: Antony W. Goodwin*, Derek H. Fender

Support: National Institutes of Health, Public Health Service

The 2-dimensional tracking properties of the human eye movement control system have been investigated by using a target motion consisting of a sinusoid in either the vertical or horizontal direction, and low-pass filtered Gaussian random motion of variable bandwidth (and hence information content) in the orthogonal direction. The random motion reduced the correlation between the sinusoidal component and the eye tracking motion. However, the correlation was only slightly dependent on the bandwidth of the random motion. Thus the system may be considered as two independent informational channels with a small amount of mutual cross-talk.

These target motions were then rotated to test whether the system is capable of recognizing the 2-component nature of the target motion. That is, the sinusoid was presented along an oblique line (neither vertical nor horizontal) with the random motion orthogonal to it. The system did not simply track the vertical and horizontal components of motion, but rotated its frame of reference so that its two tracking channels coincided with the directions of the two target motion components. This recognition occurred even when the two orthogonal motions were both random, but with different bandwidths.

The time delays of the oculomotor system were also measured in response to various periodic signals, various bandwidths of narrow-band Gaussian random motions and sinusoids. The relationship between the time delay and the average speed of the retinal image was linear.

51. HUMAN CYCLOFUSIONAL RESPONSE

Investigators: Andrew E. Kertesz*, Derek H. Fender

Support: National Institutes of Health, Public Health Service

Over the past few decades it has generally been accepted that torsionally disparate images as seen by the two eyes induce counter-rotation of the globes about the line of sight in the interest of binocular vision. The response is referred to as a cyclofusional movement and its existence was postulated on the basis of subjective experiments.

Objective measurement of human cyclofusional response was carried out, using a binocular recording technique. Increasing stimulus complexity was found to result in an increased cyclofusional amplitude. The greater cyclofusional amplitude resulting from increased stimulus complexity is realized by an increased central response, while compensatory eye movements, if they indeed occur, play a minor role. The above results imply the existence of a central mechanism that is able to identify and bring together corresponding portions of the visual field in the interests of binocular vision. Experiments are being carried out to explore some of the operating characteristics of this central pairing mechanism.
52. THE INTERACTION OF COLOR AND LUMINANCE IN STEREOSCOPIC VISION

Investigators: Cary Lu, Derek H. Fender

Support: National Institutes of Health, Public Health Service

Two major areas of vision research traditionally examined in isolation have been combined. The interaction between binocular vision and color vision is mapped to determine the way in which visual information based on differences in color and visual information based on differences in luminance are used to perceive depth.

There are three major physiologic cues to depth perception: the accommodation of the eyes, convergence of the eyes, and retinal disparity. Of these, accommodation can be a monocular cue, the others are binocular. Accommodation and convergence are not essential factors in seeing depth, but retinal disparity is important.

Single vision is also subserved by the ability of the central visual system to fuse the images of objects that are not exactly on the horopter. Retinal correspondence need not be exact for single vision, only close enough for there to be correlation between the two images. Point-by-point remapping of these differences in disparity by the central visual system would yield the cue for depth perception. We can postulate a rather simple remapping mechanism which would operate in the case of simple figures having much monocular form structure, such as the classical Helmholtz line drawings of stereo pairs. These drawings typically show a pyramid seen from above. Once the outlines of the bases of the pyramids in the two images are fused, depth information concerning the remainder of the pyramidal structure could be computed by noting the horizontal distance between each closely spaced pair of lines. However, the experiments of Julesz with random-dot stereo-pair patterns have shown that the process of extracting depth information in the human visual system must be much more complex than the previous speculation. With random-dot stereo patterns it is not sufficient merely to look for local displacements left or right for coincidence between dots, for such coincidences can occur randomly in either direction and over many increments of distance. Instead, the depth sense extracts a global solution rather than a local one. It detects that complete correspondence between certain areas can be established if whole areas are shifted laterally even if this conflicts with the point-by-point solution. Whether or not these two processes are actually operative in the visual system is still an undecided question and whether these processes are really distinct is doubtful. However, it is quite clear that in some respects the two processes are different. In the first case the contours which delineate form can be seen monocularly, whereas in the case of the Julesz patterns the contours which are generated by the depth percept cannot be seen monocularly.

In real life there are obviously many monocular contours available, and thus a large part of depth perception may depend on a process similar to the first described above. Conversely, there are cases that are much closer to the stimulus presented by the Julesz patterns; for example, viewing the leaves of a tree at a distance: in this case, the individual leaves and their contours cannot be matched individually between the left and right eye images, for within the limits of acuity one leaf may very well match several others with equal goodness of fit, and yet the 3-dimensional structure of the tree can be seen. In this case, the matching procedure is more likely to be akin to the global process of the Julesz type of patterns. We may therefore consider which parameters of the visual scene are used in the central matching process to fit together the two retinal images in a case such as this. If we refer to this as a correlation process, then the correlation between the two images may be performed on the luminance distribution of the two patterns only or the color distribution of the two patterns, or there may be a trade-off between these parameters.

This work has shown that the global stereopsis mechanism uses only the luminance parameter; depth cannot be seen in patterns delineated by color differences alone.
53. HUMAN VISUALLY EVOKED RESPONSES

Investigators: Derek H. Fender, Robert N. Kavanagh*

Support: National Institutes of Health, Public Health Service Sloan Foundation

Human visually evoked responses have been studied from the aspect of localization of equivalent generators in the cortex. In the electromagnetic model of the system the brain and its surrounding fluid are considered to be a homogeneous ellipsoid surrounded by two shells which represent the skull and scalp respectively.

A recording system has been built which allows simultaneous recording from up to 100 electrodes on the surface of the head, thus defining accurately the distribution of potential for the evoked responses.

The equations for the surface potential due to a multipole or several dipoles have been derived. A nonlinear least-squares parameter estimation technique produces inverse determinations; i.e., we have found the equivalent dipole moments which best fit an observed map of the evoked response potentials at millisecond intervals for 0.256 seconds following stimulus presentation.

At various times throughout the visual evoked response stable dipole solutions have been found. These are displayed as a computer-generated 3-D movie, which permits the visualization of the localization of activity. Present work is directed towards an understanding of the involvement of cortical areas 17, 18 and 19 in the processing of binocular information.

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54. FUNCTIONAL MORPHOLOGY OF THE VERTEBRATE RETINA

Investigators: Ken-Ichi Naka, Panos Z. Marmarelis*, Nancy Carraway*

Support: National Institutes of Health, Public Health Service

We have been continuing our study of the catfish retina (a horizontal cell preponderated retina), concentrating our effort on three levels: the morphological reconstruction, physiological reconstruction, and nonlinear analysis and synthesis.

Morphology: Using the classical Golgi staining and also intracellular dye injection technique, we have identified three types of horizontal cells and two major classes of bipolar cells in the catfish retina. A similar study has been carried out in the frog retina, an amacrine preponderated retina. We have concluded that in these two retinas no conspicuous difference could be found in the roles played by the major types of neurons.

Physiology: We have established a functional model of that part of the retina which serves the concentric receptive field organization. The model assumes that the bipolar cells compare two inputs, one local signal which is the direct input to a bipolar cell from a small number of receptors, and the other the integrating signal which originates in the S-space or Naka-Rushton model of the row of horizontal cells. We have also refined the mathematical treatment of the S-space and the theoretical predictions agree well with the experimental data obtained from dye-identified horizontal cells. A similar functional model was proposed in the rod-dominated dogfish shark retina.

Analysis and Synthesis: Applying the nonlinear analysis technique based on the white-noise theory we have successfully identified the nonlinear transfer functionals of neuron chains in the assumed functional model. We have extended the theory to multi-input cases and applied it to the concentric receptive field organization. An assembly of kernels or a mimesis is able to synthesize the nonlinear response of the catfish horizontal and ganglion cells. We will apply this technique to the bipolar and amacrine cells to complete the structural and functional modeling of the part of the neuron networks which underlie the catfish receptive field.

*Division of Engineering and Applied Science, California Institute of Technology.

PUBLICATIONS


The second order kernel of the neuron chain (horizontal cell → ganglion cell) in the catfish retina. The second order kernel together with the first order (linear) kernel can define completely the nonlinear, dynamic transfer characteristics of the neuron chain which forms the surround of the concentric receptive field first described by Kuffler in 1953. The z-axis, $h_1$, is the amount of nonlinear response. Two axes, $\tau_1$ and $\tau_2$, are in units of time and denote the time relationship of the nonlinear interaction of two portions of the stimulus signal.
Professor: Anthonie Van Harreveld
Senior Research Fellow: Eva Fifkova
Research Fellows: Alexander J. Baethmann, Janett Trubatch
Graduate Student: Lee-Ming Kow
Special Graduate Student: Frank C. Verhulst
Research Assistants: Ruth E. Estey, Jane Y. Sun

Work on current subjects was continued or extended. The effect of visual deprivation on the fine structure of peripheral and central components of the rat's visual system was studied. The investigation of the effects of a variety of amino acids on the isolated retina was completed. The mechanism of the propagation of spreading depression in this preparation was studied. The water distribution in brain made edematous by metabolic inhibitors and by adrenalectomy was investigated. The validity of the use of iodide as an extracellular marker for the brain was considered. The mechanism of the transmitter release of synapses in the frog brain was studied with freeze-substitution. The postulated transmitter function of glutamate in crustacean neuromuscular synapses was compared with that in the chicken retina. Work on asphyxia rigidity in the cat was continued.

55. PLASTIC REACTION OF RETINAL SYNAPSES TO TRANSNEURONAL DEGENERATION

Investigator: Eva Fifkova
Support: National Science Foundation
National Institutes of Health, Public Health Service

Recently the damaging effect of light on visual cells of albino rats had been demonstrated. The extent of receptor damage is dependent on the amount of light entering the eye, making it possible to obtain retinas with receptors degenerated to different degrees. Since the activity generated in the receptors must pass two synaptic levels (outer and inner plexiform layers) to reach the retinal ganglion cells, retinas with destroyed receptors seemed to be a convenient preparation in which the effect of transneuronal changes can be investigated. The inner plexiform layer (layer of neuropil rich in synaptic contacts) was studied with both the light and electron microscope.

Albino rats illuminated daily for 16 hours were compared with those illuminated for 8 hours. The latter time has been shown not to interfere with the viability of visual cells whereas 16 hours illumination causes receptor degeneration (Fifkova, 1972a). After 6 months the heads of the narcotized animals were perfused with glutaraldehyde and the retinas prepared for light and electron microscope observation. This procedure as well as the sampling of data were described elsewhere (Fifkova, 1972b).

As the receptors degenerate, the outer retinal layers (formed by the receptors) become thinner. The rate of this process varied considerably: in electron micrographs 2 out of 10 animals had completely degenerated receptors, in 3 rats one-third and in 5 one-half of the receptors had disappeared as compared with the control values. The inner retinal layers were surprisingly well preserved except for the two most severely affected specimens. Samples were compared of the inner plexiform layers of the experimental rats (total of 56,000 µ²) and of the controls (29,000 µ²). The 16 hours of illumination caused a 20% decrease in the over-all synaptic density in this layer. Evaluation of the density of different synaptic types showed that the loss of bipolar/dendritic contacts mainly accounted for this change. Since the bipolar cells are the only elements directly connected with the degenerated receptors, transneuronal changes in their terminals can be expected. From the other two types of synapses in the inner plexiform layer the density of amacrine/amacrine contacts remained unchanged, which agrees with the relative independence of the amacrine cells from the receptors. However, a considerable increase in the density of amacrine/dendritic contacts has been observed. This finding is interpreted as a plastic reaction of amacrine endings which, when deprived of the main site of their termination (the bipolar terminals), make new contacts with dendrites of ganglion cells. The selectivity with which amacrine endings make additional contacts with dendrites
and not with each other could point to different functions of the two types of contacts, since after visual deprivation the density of amacrine/amacrine synapses is increased (Fifkova, 1972b). Because of the variability and low rate of the destruction of the receptors in 16-hour-illuminated animals, it was not possible to establish the exact time needed for the translocation of amacrine contacts. The destruction of receptors could perhaps be better timed by using iodoacetate or 1,5-di(p-aminophenoxy)pentane dichloride which are selectively toxic for the receptors.

References:

56. CHANGES IN RETINAL SYNAPSES CAUSED BY VISUAL DEPRIVATION AND ENHANCED ILLUMINATION

Investigator: Eva Fifkova

Support: National Science Foundation
National Institutes of Health, Public Health Service

Monocular lid-closure caused an increase in density of amacrine synapses in the deprived eye of rats daily exposed to 8 hours light. In this increase the amacrine/amacrine synapses were mainly involved, the amacrine/bipolar contacts less, whereas the amacrine/dendritic synapses remained unchanged. In the companion open eye a decrease in density of amacrine/bipolar contacts occurred without a change in amacrine/amacrine and amacrine/dendritic synapses. Since in this open eye a mild loss of photoreceptors had been found, the decreased number of amacrine/bipolar contacts was interpreted as a consequence of receptor damage. This damage is believed to be due to an increased amount of light entering the open eye of monocularly deprived animals (Fifkova, 1972a,b). After prolonged illumination (16 hours per day for 6 months) which destroys most of the receptors, a large loss of amacrine/bipolar synapses has been observed together with a considerably increased number of amacrine/dendritic contacts (Biology 1972, No. 55). The translocation of amacrine endings from bipolar terminals to the dendrites occurs probably only after a considerable loss of receptors and bipolar terminals. It has been shown previously that the closed lids protect the receptors from damage during 8 hours of daily illumination, whereas in 16-hour-illuminated rats the receptors are partly damaged even in the closed eye. Transneuronal changes can therefore be expected in the latter eye. As the visual deprivation and transneuronal changes due to the receptor degeneration affect selectively different types of amacrine contacts (the first increases the amacrine/amacrine contacts, the second the amacrine/dendritic ones) the effect of both conditions applied simultaneously has been studied.

Monocularly lid-sutured albino rats were daily illuminated for 16 hours starting with the 15th day of age. After 2 months their retinas were prepared for light and electron microscopic observation. Samples of the inner plexiform layer (23,000 µ²) have been evaluated. The over-all synaptic density was higher by 20% in the deprived than in the open eye. This difference ranged from 20 to 30% for various types of contacts except for the amacrine/dendritic ones where no difference was observed. The differences between the open and the deprived eyes were 2.5 times greater when compared to those after 8 hours of daily illumination (Fifkova, 1972b). The absolute density of amacrine/bipolar synapses was 1.5 to 2 times lower, and those of the amacrine/dendritic contacts 3 times higher after 16 than after 8 hours of illumination in both the deprived and open eyes. These changes were in the same direction, although less severe than those after the prolonged illumination (Biology 1972, No. 55). The density of amacrine/amacrine contacts in the open eye was not affected by the 16 hours of illumination which also agrees with the long-lasting illumination. The 16 hours of illumination, however, suppressed partly the new formation of amacrine/amacrine contacts in the lid-sutured eye. This result is difficult to interpret since no direct connection between the receptors and amacrine endings has been described so far. One has, therefore, to assume an indirect influence via bipolar elements. The light-induced decrease of synaptic density in the lid-sutured eye points to the incomplete protection of the closed lids against light and agrees with light microscope observations of visual receptors (Fifkova, 1972a). Since light can apparently interfere with the effect of deprivation a series of monocularly lid-sutured
animals were kept in light of lower intensities; the retinas are being studied.

57. EFFECTS OF AMINO ACIDS ON THE ISOLATED CHICK RETINA

Investigators: A. Van Harreveld, Eva Fifkova

Support: National Institutes of Health, Public Health Service
National Science Foundation

Spreading depression which can be elicited in the isolated chicken retina causes an increase in transparency of the tissue. A similar change in optical properties of the tissue can be elicited by stimulation with glutamate (threshold 0.2 mM). This stimulus also causes a release of $^{14}$C-glutamate from retinas previously charged with the labeled amino acid (Van Harreveld and Fifkova, 1970). The effect of 30 common amino acids on the retina were examined in the present investigation. Furthermore, the effects of these amino acids in a concentration of 10 mM on the stimulation of the retina with 0.5 mM glutamate were examined. It was expected that by competitive inhibition the glutamate-induced transparency change or the release of labeled glutamate or both would be suppressed by one of the simultaneously applied amino acids.

A number of amino acids such as leucine, phenylalanine, methionine, lysine, arginine and others had no effect on the retina and did not affect the reaction to glutamate stimulation. Another group caused a transient transparency increase upon application to the retina, which was in some cases accompanied by a release of labeled glutamate (glutamine, asparagine, cysteine, homocysteic acid), in others not (valine, serine, homoserine, threonine, proline). All these amino acids with the exception of valine and homoserine prevented the transparency change caused normally by 0.5 mM glutamate. Most of them did not abolish the release of labeled glutamate caused by stimulation with the unlabeled amino acid. Only glutamine in 10 mM concentration suppressed both the transparency change and the release of the label.

Reference:

58. EFFECT OF AMINO ACIDS ON A “SCLERAL” PREPARATION OF THE CHICKEN RETINA

Investigators: A. Van Harreveld, Eva Fifkova

Support: National Institutes of Health, Public Health Service
National Science Foundation

The amino acids mentioned above were applied to a retinal preparation, consisting of the posterior half of the eye, which after removal of the vitreous body was immersed in a physiological salt solution. The retina remained attached to the choroidea and sclera and could conveniently be transferred from solution to solution. This preparation exhibits spontaneous spreading depression. The increased transparency of the tissue which characterizes this phenomenon in the retina appears as a dark sharply-outlined area which progresses slowly over the retina.

The result of the application of many of the amino acids in 10 mM concentration were in general agreement with the results obtained with the isolated retinas. The amino acids which had little effect on the latter preparation did not cause any transparency changes in the scleral preparations and did not prevent spontaneous spreading depression. The appearance of the retina did not change in these solutions, exhibiting a smooth membrane of moderate opacity.

The amino acids which affected the isolated retina caused a transparency change which sometimes appeared as an over-all increase in darkness of the preparation, sometimes as a spreading depression. Many of these amino acids changed the appearance of the retina profoundly. Starting 5 to 10 minutes after immersion in the amino acid solution the retina acquired a wrinkled appearance and became opaque, which seems indicative of damage to the tissue. These changes which were observed after immersion in serine, threonine, asparagine, cysteine and homocysteic acid solutions are so severe that they explain the absence of the reaction to glutamate stimulation. Other amino acids, such as glycine, alanine, proline and glutamine cause less severe changes, the retina after...
15 to 30 minutes still had a smooth or slightly wrinkled appearance, but did not exhibit spreading depression.

59. PROPAGATION OF SPREADING DEPRESSION

Investigators: A. Van Harreveld, Eva Fifkova

Support: National Institutes of Health, Public Health Service
National Science Foundation

Two compounds have been considered to be involved in the propagation of spreading depression. Grafstein (1956) proposed that the stimulus for this phenomenon would release K ions which would depolarize adjacent neuronal elements causing them to fire and to release additional K ions. The shifts in water and electrolytes which accompany spreading depression could not well be explained by this mechanism. It was proposed, therefore, that instead of potassium, glutamate which also causes depolarization of neuronal elements and can account for the fluid shifts (Van Harreveld and Ochs, 1956), would be involved in the propagation of spreading depression in the same way as postulated for potassium by Grafstein. This concept is supported by the observation of a transparency change in the retina typical for spreading depression after application of stimuli which cause this phenomenon in the cerebral cortex. Furthermore in retinas previously charged with labeled glutamate, stimulation with the unlabeled amino acid causes a release of $^{14}$C-glutamate. Both the application of glutamate and KCl to the cerebral cortex elicit spreading depression, although the necessary KCl concentration is considerably higher (100 mM) than that of glutamate (15 mM). The effect of these stimuli on the isolated retina were investigated in some detail. The threshold for glutamate stimulation is 0.2 mM which causes both the transparency change and the release of labeled glutamate. Stimulation of the retina with KCl causes a transparency change at a threshold of 5 mM above the normal K content of the perfusion fluid (2.5 mM). However, no glutamate is released by the tissue. This occurs only at concentrations of 100 mM and higher. These observations strongly support the involvement of glutamate in spreading depression of the cortex. KCl concentrations of about 100 mM are necessary to cause this phenomenon in the cortex. This is the minimum concentration which releases glutamate from the retina. Lower concentrations cause the same transparency change but do not elicit spreading depression in the cortex.

References:

60. IMPEDANCE MEASUREMENTS AND ELECTRON MICROSCOPY OF EDEMATOUS BRAIN

Investigators: Alexander J. Baethmann, A. Van Harreveld

Support: National Institutes of Health, Public Health Service
National Science Foundation
Deutsche Forschungsgemeinschaft

Studies of the fluid distribution in edematous brain tissue may provide information about the mechanisms which cause this condition. Measurement of the brain impedance and electron microscopy of freeze-substituted tissue were carried out on a metabolic type of brain edema. Part of the skull over one hemisphere was replaced by an epoxy resin plate in which two stainless steel electrodes had been embedded. The electrodes contacted the surface of the cerebral cortex. The epoxy plate was attached to the bone edges of a window in the skull with a fast-polymerizing dental cement. Insulated wires passing through the skin permitted impedance measurements in the unrestrained and conscious animal. The changes in specific tissue impedance during the development of brain edema could in this way be studied.

The edema was produced by administration of a nicotinamide homolog, 6-amino-nicotinamide (6.5 mg/kg body weight), which is exchanged against the natural nicotinamide in the pyridine nucleotides NAD and NADP (Zatman et al. 1954). Ensuing disturbances of the energy metabolism due to the failure of the resultant abnormal pyridine nucleotides, 6-amino-NAD and 6-amino-NADP, as hydrogen acceptors, may cause the development of the edema.

In control animals observed during a 48-hour
period after recovery from the surgery, a 9% decrease of the cortical conductivity was found, whereas in animals with developing brain edema the conductivity was reduced about 25% during the corresponding time period. This difference is statistically significant. Since the currents used in such measurements are mainly carried by extracellular electrolytes, the drop in conductivity suggests a reduction of the extracellular space. However, rendering the edematous cerebral cortex anoxic by circulatory arrest caused an additional drop in conductivity. This indicates that in edematous brain tissue an appreciable amount of electrolytes is still located in the extracellular space. This is taken up by the intracellular compartment under asphyxial conditions (Van Harreveld and Ochs, 1957).

In electron micrographs of freeze-substituted cerebellum from mice treated with 6-aminonicotinamide, a fair amount of extracellular space, especially in fields of nonmyelinated nerve fibers (parallel fibers) can still be demonstrated. In addition, glial elements were found to be swollen and electron-transparent. Micrographs of asphyxiated edematous tissue exhibited hardly any extracellular space. The features of edematous brain after glutaraldehyde fixation (perfusion via the abdominal aorta) are more difficult to assess due to interference of the fixation with the experimental effects.

References:

61. THE EFFECT OF ADRENAL INSUFFICIENCY ON THE WATER DISTRIBUTION IN THE BRAIN

Investigators: Alexander J. Baethmann, A. Van Harreveld

Support: National Institutes of Health, Public Health Service
National Science Foundation
Deutsche Forschungsgemeinschaft

Changes of the water and electrolyte composition in the brain which develop after the removal of the adrenal glands are similar to those observed in some types of brain edema, e.g. those produced by disturbances of energy metabolism (Biology 1972, No. 60). The mechanism of this endocrine disorder is not understood, nor is it known which of the adrenal cortical hormones prevents it. An attempt was made to characterize this type of cerebral fluid accumulation employing electron microscopy of freeze-substituted brain tissue and determination of the electrical resistance of the exposed cortex as a measure of the abundance of extracellular material. Male albino mice were adrenalectomized by paralumbar access; simultaneously a cranial window was made to prevent pressure increase due to the cerebral fluid uptake. After surgery the animals received a single dose of a potent glucocorticosteroid (prednisolone, 4 mg/kg body weight) and were maintained thereafter on a 1% NaCl solution as drinking water, and regular pellet food. Five to 6 days later the completeness of the removal of the adrenal tissue was checked by applying a water retention test. In addition, the water and electrolyte (Na, K) content of the brain were investigated in adrenalectomized mice, confirming earlier findings.

One day after the retention test the mouse cerebellum was subjected to freeze-substitution for electron microscopy. Compared to controls, which were craniotomized only, the extracellular space in the edematous animals appeared to be somewhat enlarged, occasionally showing local accumulation of extracellular material. The latter observation may need further confirmation. Contrary to the findings in a cerebral edema produced by a metabolic inhibitor (6-aminonicotinamide; see Biology 1972, No. 60) no swelling of cellular elements was visible.

The impedance studies were made in adrenalectomized rats, treated with the same procedure as the mice. The preasphyxial conductivity of the experimental animals was slightly higher (not statistically significant) than that of the controls, whereas no difference in the asphyxial conductivity drop was found. In adrenalectomized animals, the latency (time elapsing between circulatory arrest and the initial steep decline in conductivity) was significantly reduced, implying a reduced amount of metabolic energy reserves to maintain the normal intracellular electrolyte distribution.
THE IODIDE SPACE IN RABBIT BRAIN

Investigators: A. Van Harreveld, Alexander J. Baethmann

Support: National Institutes of Health, Public Health Service
National Science Foundation
Deutsche Forschungsgemeinschaft

Determination of extracellular space with extracellular markers depends on the assumption that the marker concentration in the extracellular material is equal to that of the plasma. Extracellular space determinations of the brain are hampered by the presence of the blood-brain barrier and of active mechanisms probably located in the choroid plexus which remove markers such as iodide, sulfate, etc. from the cerebrospinal fluid. Davson's concept of the "sink action" of the cerebrospinal fluid postulates that the marker diffuses from the extracellular space where it is present in a similar concentration as in the plasma into the cerebrospinal fluid which has a lower concentration and in this way is removed from the brain.

Attempts to inhibit the action of the choroid plexus by saturation of the active transport with a high concentration of the marker were only partly successful when iodide was used. The concentration of this ion in the cerebrospinal fluid did not exceed one-third of that of the plasma even when the plasma concentration was up to 50 mM. The possibility exists that also the extracellular fluid produced by the brain tissue has a lower iodide content than the plasma under these conditions, which would make iodide unsuitable as an extracellular marker for the brain.

Rabbits were injected with $^{131}$I and with unlabeled iodide to a plasma concentration of 35 mM. The distribution of the iodide was investigated in radioautograms of sections of the brain. In part of the animals the ventricles were perfused with an artificial cerebrospinal fluid containing the same concentration of labeled and unlabeled iodide as in the plasma. If the iodide concentration in the extracellular material is the same as in the plasma then the iodide concentration in the tissue adjacent to the ventricles should be lower in the nonperfused preparations due to sink action. If the iodide concentration is lower than in the plasma this region can be expected to contain a higher concentration of the marker in preparations in which the ventricles were perfused.

MECHANISM OF SYNAPTIC TRANSMISSION

Investigators: A. Van Harreveld, Janett Trubatch

Support: National Institutes of Health, Public Health Service
National Science Foundation

A fixation method was developed in this laboratory which provides a means of observing the short-lived process of synaptic transmission which is not accessible to conventional fixation techniques (Van Harreveld and Crowell, 1964). It consists of rapid freezing of the tissue by contact with a silver surface cooled to $-207^\circ$C with liquid nitrogen followed by substitution of the specimen in acetone containing 2% OsO$_4$.

An investigation was carried out with the isolated frog brain. It was shown that evoked potentials could be elicited in this preparation up to several hours after removal from the animal. The brain was placed on a carrier provided with a pair of stimulating electrodes and dropped onto the cold silver plate. A contact attached to the carrier hit the silver surface just before the brain and closed a circuit triggering a stimulator and the sweep of an oscilloscope. The output of the stimulator was applied through the stimulating electrodes to the brain. Contact of the brain with the silver surface changed the resistance in another circuit which was recorded as a voltage change on the oscilloscope. The time between stimulation and freezing could be controlled by adjusting the position of the contact attached to the holder.

Before freezing the brain was bathed in Ringer's solution containing an increased calcium content to enhance transmitter release. The opposite effect was achieved by an elevated magnesium content of the solution. An advantage of freeze-substitution is elimination of synaptic thickening which thus allows a clear view of the vesicles in the vicinity of the synaptic membrane. Preliminary results showed small vesicles in the immediate vicinity of the synaptic cleft. Union of the vesicles with the plasma membrane of the presynaptic terminal was observed.

Investigators: Janett Trubatch, Frank C. Verhulst, A. Van Harreveld

Support: National Institutes of Health, Public Health Service
National Science Foundation
Frank P. Hixon Fund

Although there is considerable evidence that glutamate is the transmitter at the neuromuscular junction of the crayfish claw (Van Harreveld and Mendelson, 1959; Takeuchi and Takeuchi, 1964) not much is known about the possible transmitters in the chicken retina. Recent findings in this laboratory (Van Harreveld and Fifkova, 1970) indicate that above a threshold concentration glutamate depolarizes retinal elements, and that low concentrations of this amino acid desensitize the retina to the effects of greater doses. This provides an additional criterion for the role of glutamate as a transmitter compound in either of these systems. If glutamate is the transmitter its presence in the tissue can be expected to block synaptic transmission by desensitization.

The adductor muscle of the crayfish claw is innervated by three nerve fibers, one of which is inhibitory; the other two are motor axons. One of the motor fibers causes a contraction when stimulated with single shocks and the second only when stimulated faradically. These “fast” and “slow” axons were isolated and prepared for stimulation. The effects of both electrical and chemical stimulation on the adductor muscle were recorded on a polygraph by a transducer attached to the pincer, and were also monitored intracellularly with glass micropipettes in single muscle fibers. The results of these experiments were that both the electrical and mechanical responses to stimulation of the fast and slow fibers as well as that caused by injection of glutamate were abolished when the claw was perfused with a physiological salt solution containing the amino acid. The glutamate contraction itself was most sensitive, and that caused by fast fiber stimulation was substantially more resistant to desensitization. Furthermore, no contractions could be elicited, except by direct stimulation of the muscle, as long as the glutamate remained in the tissue. Since the depolarization due to injection of glutamate was shown to last only a few seconds (Van Harreveld and Mendelson, 1959), this experiment is consistent with the proposition that glutamate is the transmitter on the neuromuscular junction of the crayfish claw.

The effect of glutamate on the reaction of the retina to light was investigated in chickens in which action potentials caused by light flashes were recorded from the optic tectum. To make the application of glutamate possible, the cornea, lens and corpus vitreum were removed from the opposite eye and the cavity thus formed was perfused with a physiological salt solution. Glutamate in concentrations of 0.2 to 1 mM promptly arrested the action potentials; however, after 1 or 2 minutes they returned to their original height despite continuous perfusion of the eye with the amino acid. Such concentrations of glutamate desensitize the isolated retina for the effects of glutamate in higher concentrations. The initial arrest of the action potentials can be ascribed to the depolarization of the retinal elements by glutamate, the return of tectal activity by the desensitization of the retina for this effect which, however, does not seem to include the postsynaptic membrane since synaptic conduction becomes possible. These observations suggest that glutamate is not the transmitter for these synapses.

References:

EFFECT OF ASPHYXIATION ON THE SPINAL REFLEX APPARATUS

Investigators: Janett Trubatch, A. Van Harreveld

Support: National Institutes of Health, Public Health Service

Asphyxiation of the spinal cord for certain periods of time (25 to 35 minutes) can cause a
profound change in the reflex activity. There is a depression of flexion reflexes and an exaggeration of the stretch reflex, especially in extensor muscles. Reflex inhibition is depressed although not completely absent. This abnormal activity tends to be present during three periods after the asphyxiation. A transient return of a weak stretch reflex may occur 10 to 15 minutes after the end of the asphyxial period (initial tone). After a few hours to 1 day a pronounced stretch reflex develops (secondary tone). Also this activity is transient and may disappear completely 2 to 3 days later. After a week the stretch reflex may again reappear (late tone) which remains for the life of the animal. Since asphyxiation seems to cause more extensive damage to interneurons than to the motoneurons it was postulated that an imbalance of the excitatory and inhibitory activities in the spinal cord would be responsible for these changes (Van Harreveld and Marmont, 1939).

An investigation is in progress of the reflex activity during the secondary and late tone, especially of changes in the normal reciprocal innervation pattern. The extensor contraction can be enhanced not only by stretch of the muscle itself (stretch reflex) but by many other muscles in the homo- and heterolateral leg. Nocuous stimuli which normally inhibit the homolateral extensor reflexes may enhance the extensor contraction. These patterns are investigated during the secondary and late tone. It seems likely that the damage to the interneurons is about the same during these periods of tone. However, plastic changes in the spinal cord may have occurred during the development of the late tone. The comparison of the reflex activity during these periods of activity may give an indication of the development of such plastic change.

Reference:

PUBLICATIONS


The research effort was concentrated this year on the study of the memory effect shown by crustacean horizontal optokinetic fibers, which were found previously in the rock lobster. For the purpose of these studies an automatic drive for the drum, in whose center the animal is supported, was devised so that either one or two dark periods from less than a second to minutes, separated by similarly variable light periods, could be obtained. It should be appreciated that for various reasons, but especially through the level of the state of excitement of the animal, rather wide variations in the results are present. For comparative reasons, 3 species have been used, the crayfish, Procambarus clarki, the rock lobster, Panulirus interruptus and the crab, Pachygrapsus crassipes, whose “visuality,” e.g. the use they make of visual clues, appears to ascend in this order. The over-all results check well with what is known about the resulting eye movements under similar circumstances as studied by Horridge (1966) and by Shepheard (1966). The main importance of this study is to be seen in its quantitative approach to a rather short-term memory system, in which no learning as such is necessary. So far we have studied a number of aspects, but by no means all, as will be evident from the more detailed reports.

Rearing of young crayfish in complete darkness from before hatching was found to cause no visible abnormalities in the newly-formed ommatidia, but affected the structure of the rhabdoms of the fully-developed ones. This indicates that light is necessary to maintain the light-absorbing apparatus intact after it has been formed, although exposure to too much light is also detrimental.

General References:

66. COMPARISON OF THE OPTOMOTOR FIBER PROPERTIES BETWEEN TWO CRABS

Investigators: C. A. G. Wiersma, P. Sanjeeva-Reddy
Support: National Science Foundation
National Institutes of Health, Public Health Service

As previously reported, the crab, Carcinus, has a relatively large number of various optomotor fibers, of which only some of the horizontal ones showed optokinetic reactions. This has been related to the fact that in the withdrawn position the eye turns by about 90° and that, therefore, in this position as in intermediate ones, conflicting messages would be sent out to the different eye muscles. It appears that in Pachygrapsus the same total motor fiber equipment is present, but here optokinetic responses can be obtained from others, such as “eye-up” fibers, though they are not as sensitive as the horizontal tonic ones. Even the very fast fibers, which in Carcinus react as strongly in darkness as in light, are here considerably less sensitive in total darkness, as shown by such experiments as reducing their small number of impulses by repeated dark turns, and then observing the increased number after illumination. The withdrawal reflex in Pachygrapsus proved to be considerably different from that in Carcinus for, instead of turning, the eye withdraws horizontally only, and even in the totally withdrawn position the enlarged anterior eye area remains exposed in its normal horizontal plane, as shown, e.g. by the optokinetic discharges obtainable when the eyes are fixed by gluing in this position. Therefore, in Pachygrapsus, no contradictory information with regard to eye position (which is presumably not read out by proprioceptive input) will result from light clues, and a better space read-out may result. It was also noted that the medium-sized fibers for horizontal optokinetic movements are here, as in Carcinus, responsive to both optokinetic input and horizontal turns in the dark, but the latter are less effective in Pachygrapsus, since the angular threshold for dark turning needed is considerably higher.

Reference:
67. COMPONENTS OF THE OPTIC TRACT OF THE CRAB, PACHYGRAPSUS CRASSIPES

Investigators: C. A. G. Wiersma, P. Sanjeeva-Reddy

Support: National Institutes of Health, Public Health Service
National Science Foundation

From the results on the Hawaiian swimming crab, Podophtalmus (Waterman, Wiersma and Bush, 1964; Wiersma, Bush and Waterman, 1964), it appears that the components of the optic nerve of highly visual crabs are as, if not more, diverse than in the rock lobster. In Carcinus, this could not be shown, since only larger units made a regular appearance. We now tried the same approach again in the local crab, but with about the same type of results. An interesting difference with other crustaceans is that, in the crabs, large spike-like responses are regularly obtained with one electrode behind the outer eyecup and the other in the body, when the first is not located in the optic nerve itself. In such leads various units can be sometimes identified with considerable certainty. The reason for the signals appears to be that the nerve runs in its central course through a narrow channel which serves as a fluid electrode. For the same reason electroretinograms clearly spike-like potentials in crabs can (Waterman, 1963). It is difficult to obtain electrode locations within the optic tract itself, but when successful the spikes become much brisker and often monophasic, and the lead is then more selective. With central leads no diphasic spikes from visual fibers are found, but one often obtains leads from primary sensory fibers to hairs located in the eye area, and finding the optic nerve is very difficult. Most often encountered were jittery and medium movement fibers, both with a representative in the space-constant class. Remarkable was the scarcity of sustaining and dimming fibers, confirming the difficulty of obtaining them in crabs in previous investigations.

References:

68. MEMORY IN THE CONTEXT OF CRUSTACEAN EYE MOVEMENTS


Support: National Science Foundation
National Institutes of Health, Public Health Service

Routinely the animals were placed in a striped drum, each stripe subtending 12°, and the drum was turned 3° during the start of 5 seconds of darkness. With the onset of darkness the frequency of the motor neuron activity decreased regularly in the lobster and usually in the crab, whereas the crayfish fibers often showed increased activity. With reillumination an increase or decrease relative to the original frequency is seen depending upon the direction in which the drum turned. From this sensitivity to change, memory can be inferred.

For angles larger than 3° the responses are similar. However, when a turn of 12° is given, which results in a black stripe occupying the position formerly occupied by a white one and vice versa, no significant frequency change occurs. From the animal's point of view the direction in which the drum turned is ambiguous under this condition. The size and duration of the response can often be increased by repeated turns in the same direction at short intervals. This reflects facilitatory processes in the effector system rather than memory facilitation since after such repetitions a response to a turn in the opposite direction is reduced in its inhibitory power. For this reason constant results are most readily obtained by alternating the direction of turning.

These preparations are particularly advantageous for the study of the physiological basis of memory. The system involved must consist of a relatively small number of cells. The operation of the memory is reflected in overt behavior, the eye movements, as governed by the optokinetic motor fibers. The stimulus is relatively well defined and its dimensions can be easily explored. This permits studies of encoding mechanisms. As discussed below the memory is of fairly long duration and...
its establishment can be readily controlled. Most importantly it allows a study of memory as information storage outside the context of learning, the acquisition of information.

69. INPUT TO THE OPTOMOTOR MEMORY IN CRUSTACEANS

Investigators: C. A. G. Wiersma, Richard L. Hirsh
Support: National Science Foundation
National Institutes of Health, Public Health Service

To be remembered a stimulus must consist of at least 3 stripes. The position of a single stripe is not remembered and 5 stripes are necessary for strong memory. To be remembered well by the lobster, each stripe must subtend more than 6°.

Both eyes contribute input to memory. A single eye of the crayfish or lobster was covered with a paper cap, or with plasticine in the crab. Covering the nonrecorded eye reduces memory responses slightly. Covering the recorded eye results in much reduced but still definite memory responses.

Input to an eye is summed in producing the memory response. The eyes of crabs were glued in place in the withdrawn position leaving only one-third of receptor surface exposed. The nonrecorded eye was covered with plasticine. As plasticine was applied in increasing amounts to the recorded eye, the number of exposed receptors decreases and so does the memory response, in agreement with the above mentioned necessity of the presence of several stripes in the visual field.

The acuity of the memory system was measured by progressively halving the size of the drum turn. The larger the turn the larger is the frequency change and the longer is the duration of the memory response. This is particularly true for small angles. Stripes of 12° and a period of darkness of 5 seconds were used. To control for response biasing and the effect of reillumination, turns in opposing directions were alternated. The criterion for memory was a detectable difference in the response to turns in opposite directions. The minimal turn to which memory is sensitive is about 0.1° for the crab, 0.3° for the crayfish, and 0.6° for the lobster.

As the size of the turn decreases the change of frequency in response to reillumination decreases, falling away sharply as the size of the turn approaches threshold. Interestingly, for small turns as the size of the turn decreases, the response is increasingly delayed.

Acuity measurements were repeated for crabs and crayfish whose eyes had been glued in place. Tentative results indicate that the acuity of the memory system is little if at all affected.

70. DURATION OF OPTOMOTOR MEMORY IN CRUSTACEANS

Investigators: C. A. G. Wiersma, Richard L. Hirsh
Support: National Institutes of Health, Public Health Service
National Science Foundation

The duration of optomotor memories in crayfish, crabs, and rock lobsters was measured. Preliminary investigations indicated values well in excess of 45 seconds. To prevent possibility of seeing the stripes due to ambient light after dark adaptation, testing was carried out within a light-proof tent for all longer-lasting tests. To control for the response to light onset the drum was alternatively turned in opposite directions. A discernible difference was used as a criterion of memory responses. Generally stripes of 12° were turned 3°. An interval of one minute separated trials.

Observed durations varied between 1 to 10 minutes. Considerable variation was noted between animals. During longer periods of testing, excited states, during which all optomotor fibers show increased frequencies and the eye presumably undergoes increased jitter, were often observed. However, memory survived these quite well. As the duration increases the size of the frequency change of the response decreases in an apparent exponential way.

The same experiment was repeated with crabs and crayfish whose eyes had been fixed by gluing. Tentative results indicate memory is not significantly affected.

In general the memory duration values so found are in agreement with those reported by Horridge (1966) and by Shepheard (1966) who observed eye movement. The long durations eliminate movement fibers from consideration as
the input to memory, because such fibers are only activated by changes in the position of an object occurring within much shorter time intervals.

References:


71. READ-IN TIME FOR THE OPTOMOTOR MEMORY IN CRUSTACEANS

Investigators: C. A. G. Wiersma, Richard L. Hirsh

Support: National Institutes of Health, Public Health Service
National Science Foundation

The time necessary for a new memory to become established was measured by turning the striped drum in one direction, illuminating the new position for a variable time period and then turning back in the opposite direction to the old position. The response to the first turn of a trial was used as an indicator of proper memory function. If the sign of the response to the second turn was different from that of the response to the first turn the intervening period of illumination was considered long enough to establish a memory for stripe position since it now determined the sign of the response.

Tests with positive initial and negative initial turns were alternatively done to control for response bias and the effects of light onset alone. Comparisons of the second responses in both sequences were particularly useful when the intervening light period was less than a second. The interval between the first onset of the light and the second onset was held constant. Generally 3° turns of stripes subtending 12° were used. The first period of darkness was generally 5 seconds and successive trials were separated by 1-minute intervals.

A new position will be sufficiently read into memory to determine the sign of the response after about 2 seconds or less.

However, the old position is still remembered. If the second turn of drum is less than the first, a longer intervening period of illumination, for reading in, is necessary if the new memory is to determine the sign of the response. In such a situation, the new memory becomes gradually more dominant, and the effect of the old memory declines proportionally, but at a more rapid rate than it fades in the dark.

Since the interval between the first and second onset of light was held constant, the read-in strength is solely a function of the time the new position is illuminated. Read-in strength is, therefore, not a function of time elapsed from initial illumination. Where the period of illumination was held constant while the succeeding period of darkness was increased, the memory response was never stronger than with shorter dark periods.

72. THE RELATIONSHIP BETWEEN OPTOMOTOR MEMORY AND OPTOKINETIC RESPONSES

Investigators: C. A. G. Wiersma, Richard L. Hirsh

Support: National Institutes of Health, Public Health Service
National Science Foundation

Optokinetic responses are eye movements which can be elicited by turning a striped drum or the animal in the light. Such responses are mediated by the same motor neuron fibers from which memory responses can be recorded.

We found that optokinetic responses appear unaffected by memory. The drum was turned at a speed of 3°/sec. Stripes of 6° were used. If memory affects optokinetic response then as the turn exceeds 6°, the stripes will move into the region in which the memory system gives responses of the opposite sign. A point of inflection in the frequency of fiber activity should, therefore, be observed. No effect was seen; instead the frequency increased progressively during the whole time.

Usually a fiber responsible for eye movement will continue to be active for a short time after the drum has stopped turning in the light. The frequency and duration of this activity is the same whether the drum stops in the positive regions of the stripe cycle for memory responses, or not. Thus memory does not affect eye movements occurring after a turn in the light.

Memory survives gluing of the eyes in place quite nicely. A new position is read in and read out after a turn in the dark quite well in treated crabs and crayfish. Tentative results indicate that both memory responses and afterdischarges on
turning in the light are greatly prolonged in eyes that have been fixed in place. This would indicate that negative feedback resulting from eye movement affects both responses.

73. MEMORY PHENOMENA IN OTHER OPTOMOTOR FIBERS


Support: National Institutes of Health, Public Health Service
National Science Foundation

Most of the experiments have been performed on the “tonic” horizontal motor fibers. At least in the lobster and the crab a more phasic type is also known to be present. These fibers do show some memory, but only when the turns are large, and they then give only a small number of impulses. This makes them less suitable for the present type of investigation. It may well be that the actual position of the eye with regard to the background raster plays so large a part here that it enters considerably into the discharge rate. Furthermore, such fibers will be much sooner influenced by the state of the animal. It has been frequently observed that memory loss is possible during an experiment, and in animals that did not survive for long afterwards, it was often noticed that memory was lost when the optokinetic reflex discharges were still quite strong. This indicates a larger dependence of the memory mechanism on adequate circulation, and such changes might well first affect the larger fibers. It has been found possible to show a memory effect for eye-up and eye-down fibers by putting the animal head down or up within the drum, but in this case the effect was always like that on the more phasic fibers for horizontal eye movements, and thus lower in frequency, less accurate and of shorter duration than for the tonic horizontal optokinetic fibers.

74. EFFECTS OF CONTINUAL DARKNESS ON THE VISUAL SYSTEM OF YOUNG CRAYFISH

Investigators: Joan L. Roach, C. A. G. Wiersma

Support: National Science Foundation
National Institutes of Health, Public Health Service

New retinal elements are continually arising and differentiating in the anterior area of the young crayfish eye (Parker, 1895). The structures considered necessary for the transduction of light into electrical events are the rhabdoms, which are composed of layers of parallel microvilli from 7 primary sensory retinula cells, each layer oriented at a right angle to the one just above or below it. Studies at the level of the light microscope indicated that development of the visual system occurred in continuous darkness; however, light was required for the maintenance of normal structure (Biology 1969, No. 109).

Eyes from animals subjected to continuous darkness from before hatching have now been examined with the electron microscope, since it was expected that the microvilli of the rhabdomeres, which are beyond the resolution of the light microscope, would be the most sensitive indicators of the effects of continued darkness (Eguchi and Waterman, 1966). All retinula cells examined from animals kept for more than 2 months after birth were considered to be quite active, on the basis of many free and clustered ribosomes, extensive rough and smooth endoplasmic reticulum, and Golgi bodies. Initial rhabdom formation in the growing zone appeared to be normal; however, microvilli of retinula cells some distance away from it no longer retained the $90^\circ$ orientation between layers nor their parallel course within a layer.

Since no animals have reached a large enough size for electrophysiological studies, we have been unable to test the responses in visual interneurons. However, a sudden bright beam of light failed to elicit an escape reflex in the one relatively large animal which was tested, indicating absence of vision.

Support for the assumption that junctions between the four crystalline cone cells are mainly structural (Biology 1971, No. 116) was provided by finding no differences in these junctions whether the animals were exposed to light or dark conditions.

References:


PUBLICATIONS


Neurons are basically conservative elements, from an evolutionary point of view. The separate mechanisms of conduction of an impulse along an axon and transmission at synaptic junctions between neurons are remarkably similar in all of the animal phyla that have been examined. The molecular components of membranes subserving these functions appear to have been conserved throughout evolution. Pharmacological agents such as tetrodotoxin and tetraethylammonium block the electrically excitable sodium and potassium channels, respectively, of neuronal and muscle membranes in all of the phyla so far examined including the highest vertebrates. Other pharmacological agents which block synaptic action by binding specific transmitter receptors (e.g., curare and bicuculline) also operate on these receptors (acetylcholine and GABA respectively) whether they occur in molluscs, crustaceans or mammals. These generalities and many others give us confidence that, when new principles of neuronal action are discovered in an animal simpler than a mammal, these principles probably also apply to the mammals including man.

Work on the physiology and biochemistry of neurons in the marine mollusc *Aplysia* has continued in this laboratory with emphasis on those special properties of neurons which are responsible for sleep-waking and reproductive behavior. Sleep-waking behavior in this animal appears to be controlled by special neurons with an endogenous 24-hour (circadian) cycle of activity. Strumwasser, Schlechte, Bower and Rempel have shown that the eyes of *Aplysia* are essential for entrainment of the sleep-waking cycle by external light-dark cycles as well as maintenance of this circadian rhythm itself.

The isolated eyes of *Aplysia* possess neurons with the ability to produce a circadian rhythm of optic nerve impulses in constant darkness.

This rhythm is probably the basis of synchronization of the sleep-waking cycle in *Aplysia*. The physiology of the circadian rhythm in the isolated eye is under continued investigation by Dr. Sener, Mr. Rothman and Mr. Audesirk in studies described in this section.

Specialized nerve cells which secrete a polypeptide controlling a large segment of reproductive behavior (egg-laying) have been discovered in *Aplysia*. Work on the biosynthesis, transport and release of this polypeptide has been published by Dr. Arch in the *Journal of General Physiology*. Mrs. Alvarez has been studying the fine structure of these specialized neurons (the “bag cells”) in order to determine the morphological basis of the synchronized discharge in these neurosecretory cells. It remains unclear whether these neurons are electrically or chemically coupled.

Arch and Berry have been working out biochemical fractionation procedures for whole ganglia in order to separate synaptic vesicles, neurosecretory granules and external membranes. It is hoped that these techniques can be scaled down in order to investigate these contents in some of the special large neurons in this organism.

Strumwasser and Arch have been interested in the pattern of proteins synthesized in the parabolic burster neuron, R15, which has an endogenous circadian cycle of impulse activity. Preliminary experiments have been directed at alterations in the pattern as a function of circadian time and the effects of membrane potential manipulation on the pattern. These experiments are a natural outgrowth of the techniques of single cell protein gel-electrophoresis pioneered by Dr. Wilson in our laboratory. Mr. Ram has continued his studies on alterations in the pattern of proteins synthesized in whole ganglia that have been depolarized by high external potassium.
75. SITE OF CIRCADIAN RHYTHM PRODUCTION IN APLYSIA EYE

Investigator: Robert Sener

Support: National Institutes of Health, Public Health Service

The isolated eye of the marine mollusc Aplysia californica discharges compound action potentials (CAPs) both in response to light and spontaneously in darkness, where their frequency follows a circadian rhythm (Jacklet, 1969). A necessary step toward understanding the controlling mechanism is to locate the site of rhythm production within the eye, so that further studies may concentrate on the physiology and structure of neurons within a specific known area. The basal region of the eye is selectively sensitive to localized passage of DC current at $1 \times 10^{-7}$ to $1 \times 10^{-6}$ amperes from an anodal extracellular micropipette. At the base such current blocks CAPs, while other parts of the eye and the optic nerve are relatively insensitive. Systematic reduction of tissue to the extreme base of the eye alters neither the occurrence of the circadian rhythm nor its period. Light responses are also unaffected. To quantify the relative amounts of tissue removed, its aqueous-soluble protein is measured by the technique of Lowry et al. (1951) and compared with values for whole control eyes, corrected to exclude protein contributed by the lens and its surrounding clear jelly. Electron-microscopical examination of isolated basal tissue shows no fine-structural damage and reveals that some primary photoreceptor cells are still present, explaining the normal light responses of tissue-reduced eyes.

These results diverge from the conclusions of Jacklet and Geronimo (1971) that (1) tissue reduction alters circadian periodicity and that (2) the circadian rhythm is produced by a large population of interacting noncircadian oscillators distributed throughout the retina. The present data require a model in which (1) sites of coupling among CAP-producing neurons are highly localized within the base of the eye, and (2) neuronal somata, processes and couplings in nonbasal regions are not immediately essential for normal light responses, spontaneous dark discharge and the production of circadian rhythmicity.

References:

76. THE INFLUENCE OF AFLATOXIN ON THE CIRCADIAN RHYTHM OF THE ISOLATED EYE OF APLYSIA CALIFORNICA

Investigator: Barry S. Rothman

Support: National Institutes of Health, Public Health Service

Last year it was reported that the pulse application of the RNA synthesis blocking agent aflatoxin B$_1$ (AFTX) inhibited the subsequent expression of circadian activity cycles of the Aplysia eye in vitro (Biology 1971, No. 121). This rhythm is characterized by changes in frequency of compound action potentials recorded from the optic nerve. The disappearance of rhythmicity occurred only when 3-hour AFTX pulses began 14 to 18 hours prior to projected peak activity in a control eye.

This study has now been extended to include more time points and the use of a different bathing medium. Data have been pooled from eyes with 23- to 28-hour rhythms by normalizing for period and phase differences from our clock time. A fraction is computed that shows how far along the interval between activity peaks of the control eye the drug was given.

The results show a "window" of sensitivity to AFTX from 0.5 to 0.8 cycles prior to peak control activity. A dose of AFTX given during this window will inhibit all subsequent rhythmic activity of the eye. A second smaller window of sensitivity may occur at 0.9 to 1.0 cycles prior to control peak. This remains to be more carefully studied.

The new results are consistent with the earlier report (Biology 1971, No. 121), and in fact help strengthen the case for a direct clock effect by showing that it is the relative time of AFTX administration and not the absolute time from projected peak activity that determines the subsequent effect on rhythmic expression.
77. PHASE SHIFTING A CIRCADIAN RHYTHM IN THE EYE OF APLYSIA BY HIGH POTASSIUM PULSES

**Investigator:** Arnold Eskin

**Support:**
- National Institutes of Health, Public Health Service
- National Aeronautics and Space Administration

The circadian rhythm of spontaneous optic nerve impulses from the isolated eye of *Aplysia californica* was studied. A general hypothesis was investigated: light-dark cycles, which can entrain *in vitro* the rhythm from the eye, couple to the intracellular clock mechanism through membrane depolarization. This hypothesis was tested by exposing isolated eyes for fixed durations of time to a depolarizing stimulus, namely, elevation of $K^+$ (hi-K pulses).

Depending on in which phase of the rhythm that the eyes were treated, hi-K pulses produced either advance or delay phase shifts in the rhythm. A phase response curve was thus generated for 107 mM $K^+$ pulses of 4-hour duration.

If the duration of the hi-K pulse was varied, keeping the phase at which the pulse started constant, the magnitude of the phase shift varied in a linear manner with the duration of the pulse.

To examine the involvement of transmitter and neurohormone release in the production of the phase shifts, eyes were exposed to hi-K pulses in the presence of a medium (high Mg$^{++}$, low Ca$^{++}$) which should inhibit such release. Since only slight differences were observed in the phase shifts produced by hi-K and by hi-K in the presence of high Mg$^{++}$, low Ca$^{++}$, transmitter and neurohormone release do not appear to be involved in phase shift production by hi-K. It is likely that membrane depolarization alone is responsible for the phase shifts.

Reference:

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78. SODIUM DEPENDENCE OF SPONTANEOUS ACTIVITY IN APLYSIA EYE

**Investigators:** Gerald J. Audesirk, Cheryl Laffer*

**Support:**
- National Science Foundation
- Sloan Fund for Basic Research

The isolated eye of *Aplysia* shows spontaneous impulse activity in the dark as well as a response to light consisting of two components, an initial phasic response and a later tonic response. The spontaneous dark discharge varies with a circadian rhythm. If some of the sodium in the bathing medium is replaced by tris, the dark discharge and tonic light response can be abolished, while the phasic light response is unaffected. The percent of normal sodium which must be replaced to completely suppress the dark discharge varies from eye to eye and also appears to change with time of day, ranging from about 30% to 80% replacement (highest % replacement needed near projected dawn). Further, pulses of reduced sodium solutions delay offset of impulse activity while not changing the time of the subsequent onset. The endogenous activity of the eye may be due, then, to a sodium oscillator cell similar to those in the abdominal ganglion (e.g., R15); also, the coupling between the pacemaker and its followers may be mediated by a chemical synapse which involves an increase in the sodium permeability of the postsynaptic membrane; or both of these may occur. The circadian effects imply the former, and these effects are presently being investigated.

Reference:

*Undergraduate, California Institute of Technology.

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79. OPTIC NERVE PROJECTION INTO THE CEREBRAL GANGLION

**Investigator:** Gerald J. Audesirk

**Support:** National Science Foundation

The eye of *Aplysia* is necessary in order for the locomotor activity of the intact animal to be entrained to a light-dark cycle. This could be due to direct neuronal connections from the eye into the cerebral ganglion or other ganglia, driving neurons there by synaptic input, or by neurosecretion released into the circulation at the eye or in the ganglia. Preliminary intracellular recordings of activities of cerebral ganglion cells have so far failed to find any neurons which receive synaptic
input from the eyes. However, extracellular recordings from the other nerves which join the cerebral ganglion have revealed, through signal averaging techniques using a computer of average transients, that the ipsilateral cerebropleural connective contains some axons whose activity appears to be initiated by the eye. Both pleurovisceral connectives also show eye-initiated activity. Other nerves have not yet been examined thoroughly. Further experiments, including histological study of the cerebral ganglion and procion yellow electrophoresis through the optic nerve, are in progress.

Reference:

80. PROTEIN SYNTHESIS IN THE EYE OF APlysIA CALIFORNICA

Investigator: Barry S. Rothman
Support: National Institutes of Health, Public Health Service

The eye of Aplysia californica was studied to see if protein synthesis in the eye could be influenced by the application of light. In a study by Burnel, Mahler and Moore (1970), performed on the primary visual cells of Limulus, the incorporation of $^3$H-leucine into protein was measured by autoradiography. They found that incorporation of label from a 24-hour dark incubation was greater than that in light. This effect could be amplified by nonradioactive preincubation in the light. Most of the increased protein synthesis was attributed to synthesis of the visual pigment rhodopsin.

If such an effect occurred in the Aplysia eye, then an increase in the level of visual pigment might be expected to appear relative to the levels of synthesis of other proteins. To make such a comparison a sodium dodecyl sulfate (SDS) polyacrylamide gel system was employed; the advantages being that 90% of the eye’s protein can be solubilized and that these proteins can be separated according to molecular weight.

Each of a pair of eyes from the same animal was dissected and the optic nerve sucked into a recording electrode immersed in 0.5 ml of filtered seawater at 15°C as a preincubation medium. Each recording apparatus was housed in a light-tight box that could be illuminated from within. The control eye was preincubated in the dark and later labeled for 2 hours in a defined medium containing $^3$H-leucine, also in the dark. The experimental eye was preincubated in the dark and labeled for 2 hours in the light using a $^{14}$C-leucine medium. After a brief rinse both eyes were quickly ground in a buffer and mixed together. The mixed grindate was then run on an SDS polyacrylamide gel; the gel was then fixed, sliced, and each slide counted for radioactivity. Such a double-label experiment allows a direct comparison of control and experimental proteins in each slice of the gel. The results showed no changes in the pattern of proteins synthesized nor in the gross amount of protein synthesized. A further experiment in which preincubation was done in light and incubation in dark still did not yield any change in the character of protein synthesis.

Three possible reasons for the above results follow: (1) protein synthesis is imperturbable by changes in levels of light; (2) a 2-hour label is too brief to see a change in synthesis; and (3) the assay method is too insensitive to measure the change in synthesis.

Reference:

81. EFFECTS OF HIGH POTASSIUM MEDIA ON RADIOACTIVE LEUCINE INCORPORATION INTO APlysIA NERVOUS TISSUE

Investigator: Jeffrey L. Ram
Support: National Institutes of Health, Public Health Service

To study possible coupling between membrane polarization and protein synthesis, elevated external K$^+$ levels (K$^*_o$) were used to depolarize the cell membranes in isolated Aplysia abdominal ganglia. The effect of this treatment on the incorporation of labeled leucine into proteins in the ganglia was analyzed on sodium dodecyl sulfate (SDS) polyacrylamide gels. Ganglia were preincubated 3 hours and incubated 4 hours in either control medium ($^{14}$C-leucine) or high K$^*_o$ ($^3$H-leucine). In high K$^*_o$ media, osmotic strength was controlled by making equimolar reductions in Na$^+$. A $^3$H- and a $^{14}$C-labeled ganglion were homogenized together in 1 mM phosphate buffer (pH 7.8) and centrifuged;
the supernatant was added to 2X SDS sample buffer and run on SDS gels, which were then stained, destained, traced, sliced, and counted.

There was a decrease in total leucine incorporation as $K^+$ was increased. At 50 mM $K^+$, the pattern of incorporation, as opposed to the amount of incorporation was essentially normal. At 90 to 110 mM $K^+$, a relative decrease in incorporation at higher molecular weights occurred, but a relative increase in incorporation appeared in a distinct peak at approximately 55,000 daltons (55 K). At 90 mM $K^+$, patterns for the right pleurovisceral connective nerve showed little relative increase at 55 K, suggesting that the 55 K peak might not have its origin in axons, glia, or connective tissue. At 90 mM $K^+$, the aqueous insoluble proteins of the ganglion did not show a significant relative increase at 55 K, showing that the 55 K effect did not occur with all 55 K proteins. The aqueous-insoluble proteins did, however, show a relative decrease at higher molecular weights and a decrease in total incorporation as $K^+$ was increased.

To check whether the high $K^+$ effect was a postsynthesis effect, ganglia were first labeled in normal media, rinsed, and then placed in 110 mM $K^+$. No increase occurred in the relative amount of previously labeled 55 K proteins. By using a second label in the high $K^+$ period and cutting the ganglion in half before homogenization, it was shown that the effect of high $K^+$ is the same on the left and right sides of the ganglion. If further experiments show that the effect on protein synthesis is due to the $K^+$-induced depolarization, this may provide a model system for studying the coupling of membrane polarization to protein synthesis.

### 82. THE EFFECT OF SYNAPTIC STIMULATION ON RNA AND PROTEIN METABOLISM IN THE R2 NEURON OF APLYSIA

**Investigators:** David L. Wilson, Robert W. Berry

**Support:** Helen Hay Whitney Foundation

**National Institutes of Health, Public Health Service**

Excitatory synaptic stimulation of the R2 neuron in the abdominal ganglion of *Aplysia californica* has been shown to cause an increase in its incorporation of $^3$H-uridine into RNA (Berry, 1969; Peterson and Kernell, 1970). This could have been a result of increased precursor specific activity rather than an increase in RNA synthesis. We have found that at low external uridine concentrations (1.5 µM) there is no increase in $^3$H-uridine incorporation correlated with synaptic stimulation. In addition, we could detect no change in total protein synthesis or in the pattern of newly synthesized proteins, resolved on sodium dodecyl sulfate (SDS) polyacrylamide gels. This neuron can thus be synaptically stimulated without detectable change in protein synthesis, and without a change in uridine incorporation under certain conditions. We therefore conclude that the increase in uridine incorporation observed in the previous studies at higher external uridine concentrations (100 µM) could be caused by an increased specific activity in a precursor pool rather than by an RNA synthesis change.

**References:**


### 83. SUBCELLULAR FRACTIONATION OF THE PARIETO-VISCERAL GANGLION

**Investigators:** Stephen Arch, Robert W. Berry, Renate Alvarez

**Support:**

**National Institutes of Health, Public Health Service**

**National Aeronautics and Space Administration**

The parieto-visceral ganglion of *Aplysia californica* contains approximately 2500 neurons. Since it is an integrative ganglion, there is ample opportunity to examine connectivity among various of the large neurons by means of electrophysiological techniques. Much effort has been devoted to analyzing and cataloging a wealth of diverse synaptic interactions within the ganglion (Tauc, 1967). Although it would be desirable to know the transmitter species participating in these interactions, technical difficulties have prevented extensive examination of this problem. Nonetheless, on the basis of enzyme assays, direct determinations, and iontophoretic applications, there is some evidence for three transmitter species: dopamine, serotonin, and acetylcholine (Koike, Eisenstadt and
In an attempt to circumvent some of the uncertainties of past attempts at transmitter identification we have begun a series of studies approaching the problem with the techniques of cell biology.

To purify synaptic vesicles we have undertaken a subcellular fractionation series using electron microscopy and gel electrophoresis to monitor our separation. When the cell somata and neuropil of the ganglion are homogenized in a 0.4 M sucrose solution (tris buffered and containing EDTA) it is possible, after sedimenting the nuclei and large membrane fragments, to achieve both synaptosomal and vesicle fractions on a sucrose step gradient. The synaptosome step also contains both large and small dense-cored vesicles. No dense-cored vesicles can be seen in the vesicle fraction. The partitioning of the dense-cored vesicles is supported by the observation that virtually all of the egg-laying hormone (contained in large dense-cored vesicles) is found in the synaptosomal fraction. When these fractions are sufficiently clean to permit the second phase of this study to begin, transmitter identification will be made by subjecting the ganglion to incubation in the appropriate radioactive precursors. Then, after vesicle purification, the vesicle contents will be assayed by means of thin-layer chromatography.

References:

84. THE EFFECTS OF SULFHYDRYL REAGENTS ON THE MEMBRANE PROPERTIES OF APLYSIA NEURONS

Investigators: Robert W. Berry, David A. Weisblat*

Support: National Institutes of Health, Public Health Service Rohm and Haas

Previous studies have shown that reagents which bind to sulfhydryl groups can block nerve impulse conduction (Huneeus-Cox, Fernandez and Smith, 1966) and the endplate potential (Del Castillo, Escobar and Gijon, 1971). This suggests that such reagents may be useful in investigating the biochemical properties of membrane conductance mechanisms. We are studying the effects of SH reagents on the electrical activity of two neurons of the Aplysia abdominal ganglion which exhibit different electrical behavior: cell R15, which is a bursting pacemaker and is depolarized by ACh, and cell R2, which is normally silent and is slightly hyperpolarized by ACh. In both cells, parachloromercurophenylsulfonic acid (PCMPS) causes a rapid depolarization and loss of excitability at 1 to 5 x 10^-4 M. PCMPS forms an ionic bond with SH groups. N-ethyl maleimide (NEM), which forms a covalent bond with sulfhydryls, blocks spike activity in both cells at 3 to 10 mM, and only later causes a significant depolarization. Dithiothreitol, a reducing agent, can reverse the effect of PCMPS, but not that of NEM, and itself has no effect on membrane phenomena at 10 mM. Both PCMPS and NEM act in the presence of low Ca++-high Mg++ solutions, which block synaptic transmission. NEM reduces excitability by causing a drastic (>50%) decrease in membrane resistance. Yet the membrane potential remains constant, suggesting that NEM's action is to increase membrane conductance to an ion that is already at equilibrium across the membrane. Experiments are in progress to determine which ionic channels are being affected.

References:

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PUBLICATIONS


Research in psychobiology has encompassed a range of rather diverse projects including: (1) hemispheric specialization and functional organization in human commissurotomy, hemispherectomy and other neurosurgical patients; (2) mechanisms and pathways for interhemispheric transfer and recall of visual information in monkeys; (3) neural plasticity after neonate surgery in kittens; (4) cerebral structures mediating memory consolidation, learning and motivation in brain-operated, newly hatched chicks; (5) embryonic plasticity and left-right specificity in the developmental organization of visual and tactile systems in frogs and goldfish. These different approaches are all concerned with various aspects of cerebral organization: its developmental patterning, its modification by experience, or its operational design and mode of integration. Progress in the developmental studies has shown that in goldfish, but not in anurans, the cytochemical gradients in both retinal and tectal fields that regulate synaptic affinity remain plastic and are subject to reorganization after large surgical ablations into late functional, almost adult, stages. The chick brain is found in combined lesion-behavior studies to contain the same basic structural and functional homologs for learning, memory and motivation known in the mammalian brain and appears to offer a simpler and more promising model for analysis of these functions. Work with human subjects (a) continues to confirm the presence in left and right hemispheres of two distinct complementary modes of central processing, (b) discloses additional functions mediated by the anterior half of the corpus callosum in patients with partial commissurotomy, and (c) indicates the functional capacity and relations of the second visual system in man.

85. MEMORY FOLLOWING COMPLETE AND PARTIAL COMMISSUROTOMY

Investigators: Dahlia Zaidel, R. W. Sperry  
Support: National Institutes of Health, Public Health Service, Frank P. Hixon Fund

The Wechsler Memory Scale and Benton’s Revised Visual Retention Test were administered in normal free vision to 10 commissurotomy patients (8 complete and 2 partial with splenium spared) of P. J. Vogel and J. E. Bogen. Time intervals since the commissurotomy operation ranged from 3 to 9 years and the present ages were from 20 to 45. Performance on the memory tests was compared with scores on the Wechsler Adult Intelligence Scale (WAIS), with the normal population, and with published findings on groups of epileptics. Marked disparities between the patients’ IQs and their scores on the memory tests were found while the same difference was less marked in other epileptic patients. Patients’ scores were far below those of the standardized population. Evidence was obtained for a selective role of the neocortical commissures in mnemonic functions. Memories stored in the right hemisphere remain inaccessible to verbal recall and all memory dependent on hemispheric integration for recall is impaired. Whereas the two patients with intact
spleenium exhibit very few of the split-brain phenomena that characterize those with complete commissurotomy, the memory scores were as low as in those with a complete section of the commissures.

86. INTERFERENCE BETWEEN LIMBS DURING INTERDEPENDENT BILATERAL MOVEMENTS: POSSIBLE INFLUENCE OF IPSILATERAL MOTOR CONTROL

**Investigator:** Bruno Preilowski

**Support:** National Institutes of Health, Public Health Service

It has been repeatedly shown that limb movements are influenced negatively by simultaneous action of the contralateral extremity. The mechanism of this interaction is not known. One hypothesis holds that the interference takes place at the cortical level via callosal interhemispheric connections. Another hypothesis proposes that interference occurs when the limited capacity of a central processor for movement-generated feedback is exceeded. Thus simultaneous mirror image movements, involving homologous muscles and supposedly redundant feedback, could be handled more easily than parallel movements which involve nonhomologous muscles and generate a greater amount of feedback. Neither of these two hypotheses is supported by the present investigation of bilateral motor coordination in “split-brain” persons and controls with intact neocommissures.

Patients in whom the neocommissures were partially or completely sectioned to control for intractable epilepsy were found unable to perform smooth bilateral movements involving simultaneous activity of nonhomologous muscles. Controls with intact commissures performed such bilateral movements as fast as unilateral movements, except when equal bilateral output was required.

These results cannot be explained in terms of the above mentioned mechanisms, rather they support a third hypothesis, namely that interference arises in bilateral movements from the interaction of ipsilateral motor outflow from one cerebral hemisphere with the contralateral motor command from the opposite hemisphere.

87. FUNCTIONAL COMPENSATION AND REEDUCATION FOLLOWING COMMISSUROTOMY

**Investigators:** Dahlia Zaidel, R. W. Sperry

**Support:** National Institutes of Health, Public Health Service

Frank P. Hixon Fund

To further determine the extent and nature of the reeducation and compensatory readjustments that are possible following cerebral commissurotomy, comparisons were made between the performance at one year after surgery and that at eight years in one subject operated on at age 12. Some tests administered at one year were given again under conditions as similar as possible. Cross-integrations between the left and right hands and left and right visual half-fields that had been impossible at one year were performed readily in the eighth year repetition. The subject also gave verbal reports about left hand and left visual field stimuli that he had formerly been unable to carry out. The general picture at eight years is thus found to be substantially changed from that seen at one year. Additional and more complex behavioral tests involving four sense modalities have further delineated the areas in which changes have occurred. New capabilities were found to fit into distinct categories when specific modalities were used for input and output. Possible explanatory factors include some development in the speech and linguistic processing of the right hemisphere and potentiated use of the ipsilateral systems of the brain for both somesthesia and vision.
88. AN ATTEMPT TO TEST FOR SELF-RECOGNITION IN THE RIGHT HEMISPHERE OF SPLIT-BRAIN PATIENTS

Investigators: Bruno Prellowski, Gregory Gray*, R. W. Sperry

Support: National Institutes of Health, Public Health Service

Frank P. Hixon Fund

Generally, left and right hemisphere capacities of man are described in terms of dichotomies, attributing language-related, i.e. intellectual, "human" functions, to the left hemisphere, and conceding spatial and perceptual capacities, i.e. those assumed to be present in animals as well, to the right half-brain. This gave rise to the distinction of a dominant major and a minor hemisphere and the discussion as to whether the right, minor hemisphere possesses consciousness or not. While it may be impossible at present to give an exact definition of consciousness, certain agreed-upon attributes of consciousness can be investigated experimentally. One such attribute is self-recognition.

It has been shown that the galvanic skin response (GSR) is greater to a picture of one's own face than to that of another person, even a familiar one. The present study used the GSR to investigate self-recognition in split-brain persons and in normal controls when portraits of the subject and of persons, either known or unknown to the subject, were projected into the right and left visual half-fields. The stimuli were presented in two series, one containing the subject's own face, a familiar face and an unknown face; the other containing only unknown faces. During the first part of the experiment the series with the subject's face was projected only into the left visual half-field and the other series only into the right visual half-field. During the second part of the experiment the order was reversed. Skin resistance, heart rate and breathing were recorded on a polygraph and overt behavior on video tape.

Data from two normal controls and one split-brain person showed indeed that GSRs to the subject's own picture were on the average greater than responses to faces of other persons, even the familiar ones. Most importantly, however, this was also the case with right hemisphere presentations in the split-brain patient, thus indicating a capacity of self-recognition in that hemisphere. On the other hand the responses in the patient were highly inconsistent and appeared with considerable latencies after stimulus presentation. Also, with right hemisphere presentations there was no overt sign of self-recognition, only an occasional frown or raising of eyebrows, and the subject reported to have only seen flashes of light. With left hemisphere presentations the patient showed no stimulus-related GSRs and reported to have seen women's faces, none of whom she recognized. This inability of split-brain persons to recognize faces when seen in the left hemisphere is consistent with previously shown difficulties in facial recognition of that hemisphere (Levy, Trevarthen and Sperry, 1972). Due to the fact that in the split-brain patient only an average predominance of GSRs to right hemisphere presentations of the subject's own face was obtained, further experiments with the same and other patients are under way to investigate the factors influencing the reliability of this response.

Reference:
Brain 95: 61-78.

*Undergraduate, California Institute of Technology.

89. NEUROPSYCHOLOGICAL STUDY FOLLOWING RIGHT OCCIPITAL LOBECTOMY IN MAN

Investigators: Dahlia Zaidel, R. W. Sperry

Support: National Institutes of Health, Public Health Service

Frank P. Hixon Fund

A battery of standardized tasks was applied to a 57-year-old man operated on three years previously for a brain abscess. A right occipital lobectomy was performed including partial removal of the parietal lobe. In addition to blindness in the left half-field of vision the operation has also resulted in an inability to recognize people by their faces. Tests for perceptual and spatial abilities were administered to further determine the extent of right hemisphere impairments.

In six tests (Kohs Block Design, Standard Raven's Progressive Matrices, Hooper Visual Organization Test, Picture Absurdities, Picture Discrimination, and Sherman Mental Impairment Test) normal and above normal scores were obtained for perceptuo-cognitive capabilities. In two tests
(Closure Speed and Closure Flexibility) scores showed gross defects. However, speed was a major factor in scoring these latter two tests and one would expect a considerably reduced visual scan rate due to left half-field blindness. In addition to the standardized tests a set of magazine-type chimeras of the human body or of isolated parts was presented in free vision. He was able to point out the abnormalities 100% correctly.

**90. LANGUAGE FOLLOWING DOMINANT HEMISPHERECTOMY**

*Investigator:* Peggy S. Gott  
*Support:* National Institutes of Health, Public Health Service

A 12-year-old girl whose dominant left hemisphere had been removed at age 10 because of tumor was tested to determine the abilities of the remaining hemisphere. Both verbal and numerical processing were investigated. It was found that she could properly identify 90% of the pictures presented from the Aphasia Therapeutic and Rehabilitation Kit. However, only 45% of the words from the Aphasia Kit were named correctly. She could rapidly say her ABCs but could not write them. Her performance on picking the called-for letter of the alphabet from a choice of 4 letters was only 31% correct. The results appear to reflect a failure of communication between the recently acquired centers for speech, and the centers responsible for writing and visual perception.

Further indication of undeveloped language associations was evidenced in numerical manipulations. She could count correctly to 40 and wrote numbers to 10. If, however, she was then asked to say the number pointed to by the experimenter, the only way she could say it was to begin at 1 and count up to it. She correctly indicated with her fingers the number pointed to, but she had to count her fingers one by one before verbalizing the number she had signaled. In a further test which required response to the number of dots presented in free vision on a screen, she repeatedly wrote one number and then said another. The written number was 70% correct.

The results indicate that although some language can be acquired by the right hemisphere after left hemispherectomy, at age 10, the language mechanisms developed in the right hemisphere at this age lack appropriate functional relationship with the motor and visual mechanisms.

**91. RESPONSE DOMINANCE BY THE DISCONNECTED “MINOR” HEMISPHERE WITH BILATERAL COMPETITIVE TACTUAL STIMULATION IN A WORD RECOGNITION TASK**

*Investigators:* Ute-Angelika Preilowski, R. W. Sperry  
*Support:* National Institutes of Health, Public Health Service  
*Frank P. Hixon Fund*

Recently, Levy, Tревarthen and Sperry (1972) concluded from their investigations of commissurotomy patients on perception of visual bilateral chimeric figures that “the primary tendency is for all visual stimuli to be perceived in the minor hemisphere,” and that hemispheric dominance of response control is determined primarily by the “central processing requirement.” The left hemisphere became “dominant when some form of verbal and/or conceptual symbolic transformation was required,” whereas the right hemisphere dominated response control “when immediate recognition and memory of visual shape was the only requirement.”

The latter inference appears to require qualification in view of the results obtained in the present experiment from a series of word (nouns and verbs) recognition and free recall tests with unilateral and bilateral tactual stimulation, administered to one of the commissurotomy patients (N.G.) who served as a subject in the above study. Though no significant lateral differences were observed after unilateral stimulation, a profound “minor” hemisphere dominance of response control obtained in the bilateral stimulation tests with immediate recognition of shape and also persisted when recognition required conceptual transformation (matching capital letter words to words written in script), regardless of whether the response had to be indicated by pointing with the left, right, or both hand(s). The “minor” hemisphere dominance was maintained even when the responses had to be vocalized while the response choice words were visually displayed. Dominance shifted to the left hemisphere only when verbal responses were required in absence of a visually
exposed response choice array.

The results seem to indicate that the primary role of the "minor" hemisphere in perception as noted in the visual modality, may also hold for the tactual modality. The data demonstrate that the disconnected "minor" hemisphere can clearly dominate response control under intellectually more demanding conditions than direct, immediate recognition of shape.

Reference:

92. HEMISPHERIC DOMINANCE FOR SINGLE LETTER AND GEOMETRIC FORM STIMULI WITH UNILATERAL AND BILATERAL TACTUAL PRESENTATION IN A COMMISSUROTOMY PATIENT

Investigator: Ute-Angelika Preilowski
Support: National Institutes of Health, Public Health Service

In a previous study (Biology 1972, No. 91) on hemispheric dominance a pronounced right hemisphere dominance was found in tactual, bilateral, simultaneous, competitive stimulation, whereas no lateral differences were observed after unilateral presentation. The highly accurate retrieval of the left-hand stimulus in this linguistic task was especially striking since it occurred together with a nearly total neglect of the right-hand stimulus. In an attempt to determine the possible influence of a memory factor (see White, 1969), two bilateral test series were run in the same subject using one-unit stimuli. Single letter stimulation and the complete set of response conditions employed in the bilateral word recognition study was used in one series; in the other, single geometric forms (employing only direct matching with manual pointing).

Unilateral tests (left and right hand pointing, direct matching) yielded identical scores for the left and right hemisphere performances, i.e. no lateral differences were present. Results with bilateral presentation demonstrate the same trend as the data of the word recognition experiment: right hemisphere dominance with nearly total neglect of the right hand stimulus. However, in two response conditions (both hands pointing simultaneously and vocal report with response choices), a large number of double responses occurred, and the dominance in terms of total number of correct stimulus retrievals actually shifted slightly to the left hemisphere in these two conditions when direct matching was requested in the letter and geometric form tests. In the more difficult task, requiring symbolic transformation (matching capital letters to script) the right hemisphere maintained a slight dominance.

The direction of stimulus response neglect cannot be explained directly by eye fixation (White, 1969) but a directional attention mechanism based on the same process could be postulated to explain part of the lateral dominance phenomenon in this tactual task. Though some influence of a memory factor is not excluded, the vocal response results, as well as the data from the tests with symbolic transformation, indicate that memory is not the main factor involved in determining this hemispheric dominance of response control.

Reference:

93. EXPRESSIVE LANGUAGE IN THE "MINOR" HEMISPHERE

Investigators: Ute-Angelika Preilowski, R. W. Sperry
Support: National Institutes of Health, Public Health Service
Frank P. Hixon Fund

Prior evidence for limited expressive language capacity in the disconnected minor hemisphere has come largely from two patients operated early in life in whom maturational plasticity is a complicating factor in interpreting results (Levy, Nebes and Sperry, 1971). The expressive language capacity of the "minor" hemisphere was explored further in the present study in a right-handed patient (N.G.) who had undergone commissurotomy at age 30, eight years previously.

The "minor" hemisphere was found highly efficient at cross-modal matching (visual to tactual or vice versa) of 3-letter nouns, verbs, adjectives and other words, (1) directly, i.e. capital letters to capital letters, or (2) by going from capital letter words to lower case or script versions. With the information restricted to the right hemisphere the patient could tactually arrange meaningful words
from 3 scrambled letters, and could successfully
discard 1 letter of 4 to form a specified verb,
instead of a familiar noun that could have been
made by discarding a different letter. The patient
was also able to print a word with her left hand
screened from view, after tactually perceiving it
prearranged, or after arranging it herself, but was
unable afterward to report the word verbally. Only
in a few instances was she able to write down the
name of a tactually-perceived object, possibly due
to major hemisphere interference effects, which
were prominent. However, the naming of visually-
perceived objects was consistently successful when
the patient could express herself by arranging 3
scrambled letters. Major hemisphere control tests
demonstrated that the correct responses were being
mediated by the minor hemisphere.

Reference:
Cortex 7: 49-58.

94. SPATIAL MEMORY IN DISCONNECTED HEMISPHERES OF MAN

Investigator: Santosh Kumar

Support: National Institutes of Health, Public Health Service

A new memory test was developed to explore
the spatial memory functions of the disconnected
right and left hemispheres of commissurotomized
patients. Four sets of stimuli were prepared as
follows: Set A contained 8 photo slides, each
showing a cross at different positions within a
square. Set B contained 8 slides, each showing a
cross at different positions within a square which
was bisected vertically or horizontally by a line, or
divided into 4 equal squares by 2 lines. Set C had
16 slides, each showing a cross and a circle at two
different positions within a square. Set D had 16
slides, each showing a cross and a circle within a
square which was divided as in Set B. A square
metal panel was used which could be bisected or
divided into 4 equal squares with removable
magnetized sticks. A wooden cross and a circle
were also prepared with magnets implanted so that
they could be held on the upright panel. The
response panel was on the far side of the screen
out of sight; the subject could see and handle the
cross and the circle.

The slides (stimuli) of each set were flashed on
the screen in front of the patient, in a prearranged
random order, to the right or left visual field. The
patient responded to the entire set with each hand
twice so that each stimulus could be projected to
the right or left visual field an equal number of
times during the use of a particular hand. A delay
of 60 seconds was used, after which the patient
tried to place the cross and/or circle in the correct
position in which it had been projected.

In the one patient (N.G.) tested to date the
right hemisphere shows better retention for the
spatial positions of the visual stimulus, whereas the
left hemisphere can retain spatial positions only
when the retention can be facilitated by such
verbal cues as right-left or up-down.

95. REACTION TIMES FOR THE IPSILATERAL AND CONTRALATERAL AUDITORY PATHWAYS

Investigator: Harold W. Gordon

Support: National Institutes of Health, Public Health Service

Contrasts between the ipsilateral and contra-
lateral auditory pathways were studied in patients
who had undergone complete section of the corpus
callosum for the alleviation of intractable epilepsy.
Previous work had shown that the major inter-
hemispheric pathway for cross-transfer of auditory
information had been severed along with the
callosum (Milner, Taylor and Sperry, 1968).
Therefore, it is assumed that one hemisphere
receives two auditory inputs via lower centers—one
from the ipsilateral ear and one from the
contralateral ear. A test was constructed that
primarily utilizes the speech centers in the left
hemisphere following stimulation to either the left
or right ear. Differences in performance between
the ears should reflect differences in the two
pathway systems to the left hemisphere.

The subjects were five patients who had
undergone complete forebrain commissurotomies
and one patient who has had a complete right
hemispherectomy for encephalitis. The test mate-
rial consisted of a list of familiar 1-syllable words
recorded on audio tape. The words were separated
by a silent interval ranging from 200 to 2000
msec. The tape was played to the patient through
stereo headphones with the words channeled to
the right or to the left ear in a predetermined
pseudorandom sequence. The task was to repeat
(shadow) each word as quickly as it sounded. The time between stimulus and response was measured by an electronic counter that was started by the stimulus word and stopped by the patient's response.

The results showed the patients able to repeat more quickly words heard in the right ear by a difference of 45 msec on the average. In control tests where the patients were required to say the word "Now," instead of repeating the actual word, there was no difference.

Data are still being collected but preliminary conclusions can be drawn. The control test indicates that the time for information to travel via the ipsilateral pathway is the same as that for the contralateral pathway. However, once the information reaches the left hemisphere an extra amount of time is needed before the message projects to the speech apparatus. This time (about 45 msec) must be related to cortical functioning of the left hemisphere and suggests the possibility that two cortical systems for word repetition exist within one hemisphere—one related to the contralateral pathway and the other to the ipsilateral pathway.

Reference:

96. AUDITORY TESTS IN TWO PATIENTS WITH A COMPLETE HEMISPHERECTOMY

Investigator: Harold W. Gordon
Support: National Institutes of Health, Public Health Service

Two young adolescent patients who each had undergone a complete hemispherectomy, one right, one left, were contrasted on tests involving dichotic listening of verbal material and auditory tests of musical abilities. The dichotic listening test consisted of an audio recording of three pairs of digits presented through stereo headphones so that one digit of a pair was heard in the left ear simultaneously with the other heard in the right ear. The patient's task was to repeat as many of the digits as he could remember. Results showed that the patient could report only those digits that were played to the ear contralateral to the hemisphere that had been removed; the ipsilateral digits were ignored. On the other hand, if either the right or the left ear, alone, were stimulated, the digits from each could be recalled. It is concluded that ipsilateral pathways are suppressed under conditions of simultaneous left-right stimulation but are functional in the absence of competing contralateral information. These findings for the right or left hemisphere support the conclusions found in patients with surgical division of the forebrain commissures (Milner, Taylor and Sperry, 1968).

The nonverbal tests consisted of different measures of musical abilities. The patient who had undergone a right hemispherectomy for encephalitis scored extremely poorly; his worst performances were in perception of pitch, rhythm, tonal memory, and timbre. In fact, it was found in later testing that this patient could not decide whether two tones, separated by as much as one musical step, were the same pitches or different. Singing was also poor, but melodies were recognizable. The patient's major problem in song production seemed to be his inability to sing correct pitch intervals—much like the problem faced by a person who is classically "tone deaf."

In sharp contrast, the patient who had undergone a left hemispherectomy for a recurring tumor could correctly sing pitch intervals that differed by less than one-quarter tone. Songs were also sung well including excellent pitch and rhythm production. However, in spite of good melody, the patient could not always remember the lyrics. In fact, when asked to speak the words without singing, she would usually become confused and would have to recite the song from the beginning; other times she would fail completely and refuse to continue after the first few words.

These observations strengthen evidence that the left hemisphere is poor in musical ability while the right hemisphere excels. This dichotomy is supportive of findings in another study where anesthetization of the right hemisphere markedly impaired singing while at the same time produced relatively milder speech disturbances (Bogen and Gordon, 1971). Additional studies, that will further contrast ipsilateral and contralateral auditory pathways as well as hemispheric asymmetries with respect to verbal and nonverbal stimuli, are in progress in these two hemispherectomy patients (see also Gott, Biology 1972, No. 98).

References:
97. INHIBITION IN THE MIDBRAIN VISUAL SYSTEM IN MAN

Investigator: Peggy S. Gott

Support: National Institutes of Health, Public Health Service

Evidence for a midbrain visual system in man which can serve to cross-integrate moving stimuli in the peripheral areas of the visual fields after commissurotomy is presented by Trevarthen (1970). The present study was an attempt to detect function in this midbrain visual system after partial or total removal of one cerebral cortex. Three patients were tested: a right hemispherectomy, a left hemispherectomy, and a right parietal-occipital lobectomy. When tested with stationary stimuli such as dots extending across the midline of the visual field, all subjects exhibited a homonymous hemianopia contralateral to the side of the lesion.

The ability to cross-integrate moving stimuli in the peripheral visual field was tested by presenting a moving dot which subtended 5° of the visual angle. The stimulus was presented pseudorandomly in the left or right visual field and moved along the horizontal meridian at a rate of 2 cm/sec and irregularly up and down to within 14° from the vertical meridian of the visual field. None of the subjects was able to detect the presence of the moving dot in the hemianopic visual field. However, all three subjects reported movement of the stimulus in the visual field projecting to the intact hemisphere. Function of the midbrain visual system after commissurotomy but not after cerebral lesions may be attributable to some kind of inhibition from the opposite superior colliculus as found by Sprague (1966) in the cat.

References:

98. COMPARISON OF THE HEMISPHERECTOMY AND THE SPLIT-BRAIN SUBJECT

Investigator: Peggy S. Gott

Support: National Institutes of Health, Public Health Service

Four split-brain subjects were given a series of 4 tests to determine which hemisphere was dominant, and the same tests were then administered to 3 hemispherectomy patients, 2 with the right hemisphere removed and one with the left hemisphere removed. All 4 tests required a “yes” or “no” response indicated by pushing 1 or the other of 2 buttons screened from view. Each test involved tachistoscopic presentation (100 msec) of visual stimuli pseudorandomly to right or left visual field with subject’s gaze fixated on a central point. In the first task (word-nonsense syllable) the subject had to determine whether 3 letters formed a word or just a nonsense syllable. The second task (hidden figure) consisted of determining whether a certain ambiguous figure previously shown in free vision was hidden in the flashed picture or not. In a third test (rhyming), the task was to decide if a word flashed to left or right visual field rhymed with a sample word exposed in free vision. The fourth test (matching) consisted of judging whether a flashed outline shape was the same as one presented centrally in free vision but rotated or flipped.

The results to date are shown in Table 1. The left hemisphere of the split-brain subjects performed significantly above chance (p > .05) on the word-nonsense syllable and rhyming tasks while the right hemisphere remained at chance level. In the matching test only the right hemisphere scored significantly above chance (p > .05). No definite hemisphere superiority was evident from the hidden figure test.

An adult right hemispherectomy patient (G.E.), performed as well as the splits on all tests, even on tests for which the right hemisphere was superior. An adolescent right hemispherectomy (D.W.), failed to score significantly above chance on the two verbal tasks but was as good as the splits on the nonverbal tests. A young left hemispherectomy patient (R.S.), performed as well as the splits except on the word-nonsense syllable task, and even exceeded the right hemisphere of the splits on the rhyming task.

Thus with the exception of the word-nonsense task in the two younger cases the hemispherectomies scored as well, if not better, than the individual hemispheres of the split-brain even on tasks for which the hemisphere specialized for that particular function had been removed.
Table 1. Hemispherectomies and Split Brains

<table>
<thead>
<tr>
<th>Patient condition</th>
<th>Word-nonsense</th>
<th>Hidden figure</th>
<th>Rhyme</th>
<th>Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.W. Right hand-right field</td>
<td>60</td>
<td>70</td>
<td>65</td>
<td>75</td>
</tr>
<tr>
<td>G.E. Right hand-right field</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>85</td>
</tr>
<tr>
<td>Splits* Left hand-left field</td>
<td>89</td>
<td>69</td>
<td>81</td>
<td>53</td>
</tr>
<tr>
<td>R.S. Left hand-left field</td>
<td>48</td>
<td>77</td>
<td>55</td>
<td>85</td>
</tr>
</tbody>
</table>

*SPLIT-BRAIN VALUES ARE THE AVERAGES FROM FOUR SUBJECTS.

99. A Technique for Presenting Continuous Lateralized Visual Information to Human Subjects

Investigator: Eran Zaidel*

Support: National Institutes of Health, Public Health Service

Past studies of cerebral laterality of function in commissurotomy patients as well as in normal subjects were confined largely to very brief visual inputs (on the order of 0.1 seconds) flashed tachistoscopically to the right- or to the left-half visual field while the subject fixated a central mark. The short duration is necessary to prevent eye movements. This precluded from direct investigation reading of phrases and sentences, the analysis of complex visual information and the temporal integration of visual scenes (e.g., in visual research) as they relate to hemispheric specialization.

The new technique consists of a variation on the contact lens mounted collimator method for stabilizing retinal images (Pritchard, 1961). Here instead of stabilizing an image, one stabilizes an opaque screen which occludes precisely one half visual field at a time. Using procedures followed by Dr. D. Fender (Fender, 1964) in experiments on visual perception, subjects are individually fitted with one custom-made flush-fitting (except for a small region around the limbus) 3-curvature scleral contact lens made of plexiglass. A small aluminum collimator (about 10 mm in length and 5 mm in diameter) is then mounted on the central region of the lens. The collimator contains a light (~50 mg), small (5 mm diameter) and powerful (~100 dioptres) double convex lens which fits against the contact lens. The point of regard is established with respect to the endpoint of the collimator, appropriate (approx.) half-circular, opaque rings are fitted on the end of the collimator to occlude either one of the two half visual fields. To obtain better contact lens fit, a 0.023" I.D. polyethylene tubing connects the contact lens to a bicarbonate of soda solution manometer to provide a suction of about 23 cm. The contact lens is also marked with a grid of 10 dots (spaced <0.003" apart and measuring <0.002" in diameter) for direct surveying of slippage using a Zeiss operating microscope used as a slit lamp at a magnification of X40. The total contact lens-collimator structure weighs less than 800 mg and slippage of less than 1° can be easily obtained with eye movements of up to 30° (see Riggs and Schick, 1968).

An optical relay system is then used to project a reduced optical image of the stimulus at a plane coincident with the endpoint of the collimator. The collimator lens then presents an enlarged virtual image at normal viewing distance and size. Thus free scanning but continuous lateralization of the image are possible. Optical relaying consisting of a camera lens, a dove prism, and either a mirror or a screen, permits the subject to view his own performance or projected pictures respectively.

The advantage of the technique is simplicity of design and hence versatility and easy modifiability. Its main drawbacks are expensive, limited comfortable continuous experimental time (about half an hour) and nontransferability. Two commissurotomy patients and one normal subject have so far been fitted with the device.

References:


*Division of Engineering and Applied Science, California Institute of Technology.
100. EFFECTS OF CONTINUOUS LATERALIZED VISUAL PRESENTATION ON COMMISSUROTOMY PATIENTS

Investigator: Eran Zaidel*
Support: National Institutes of Health, Public Health Service

A striking observation to come from use of the CLV technique (Biology 1972, No. 99) is that whereas left hemisphere performance is similar to that with free vision, performance with the right hemisphere seems to be subject to a very brief attention span. In spite of clear understanding of the task (demonstrated in correct responses) the subject will often “fall asleep” while using the right hemisphere irrespective of the nature of the stimulus, and has to be repeatedly “awakened” by the examiner at half-minute intervals or by continuous talking. With past testing methods this effect was avoided by providing continuous (if irrelevant) visual stimulation to the right visual field throughout testing. Thus it seems that active arousal in the left hemisphere is important to maintain a continuous level of activation in the right hemisphere. Furthermore, on exposing the right visual field to stimulation following extended unilateral left visual field testing, there occurs a brief “refractory period” of up to about a half-minute during which the subject seems still to be in a right hemisphere set, and is unable to verbalize the input.

Confirmation is seen for the existence of an ipsilateral (extrageniculo-striate?) visual projection system from the left visual field to the left cortex (Trevarthen, 1970), which mediates the transfer of crude shapes and contours (see D. Zaidel and R. W. Sperry, Biology 1972, No. 87). Thus a ball is perceived (verbalized) as a circle, a box or a house as a square, a spear as a long line, and prison bars are labeled “parallel lines.”

The new technique serves also as an important methodological control on the memory factor required by brief tachistoscopic presentations. Several experiments on spatial mental imagery suggest that memory components simply reinforce laterality dominance effects for certain types of information (e.g., verbal in the left hemisphere, visual-spatial in the right).

Reference:

*Division of Engineering and Applied Science, California Institute of Technology.

101. AUDITORY LANGUAGE COMPREHENSION IN THE MINOR HEMISPHERE

Investigator: Eran Zaidel*
Support: National Institutes of Health, Public Health Service

An array of 4 to 6 pictures is presented with the technique for continuously lateralized vision (CLV) (see Biology 1972, No. 99). A sentence is then spoken by the examiner and the subject has to point with his left hand to the picture best described by that sentence. The sentences are carefully controlled for redundancy and the pictures are selected and matched to make precisely the relevant syntactic (sentence structure) or semantic (meaning) distinctions. Since the association between the sentence and the matching picture can be of arbitrary “distance,” abstract as well as concrete linguistic information can be easily presented. The sentence-picture matching technique provides an approach to a systematic semantic analysis of right hemisphere comprehension that requires no reading and no speech.

A wide variety of syntactic structures have been demonstrated to be comprehended by the right hemisphere of L.B. These range from simple inflections of words (plural “s” on nouns, possessive “s” on nouns, etc.), to the use of personal pronouns and negative and passive sentential transformations. In order to test the capacity for nonredundant linguistic reference of phrases with multiple adjectives, the subject was administered a test in which he had to point to one of twelve objects (cylinder, cube, pyramid, which are either large or small and either light or dark) as described vocally by the examiner. This test resembles the so-called “token test” designed by De Renzi and Vignolo in order to detect mild receptive disturbances in aphasics (Boller and Vignolo, 1966). Again, lateralized performance by the right hemisphere of L.B. was near 100% correct. Inability to describe the test items verbally was taken as evidence of right hemisphere performance.

Semantic studies reveal a correlation between the right hemisphere’s ability to perform certain
skills and its ability to describe these linguistically. The right hemisphere seems to comprehend small numbers, adjectives of relative size and numerosity, etc. At the same time it proved superior to the left in a lateralized Piagetian seriation task (ordering a set of sticks by size) performing faster and more accurately than the left. In fact the right hemisphere seemed to use a characteristic strategy whereby the whole set of sticks is scanned at once for a smallest or largest element while the left hemisphere followed a more elaborate one-stick-at-a-time matching. Similarly right hemisphere comprehension of the semantics of spatial prepositions (in, under, behind, etc.) is matched by a corresponding apprehension of spatial relations. Conversely, on left-right discrimination tasks, when required to point to left or right limbs or sides of people or object arrangements in CLV pictures, the right hemisphere performed at chance level. Correspondingly, two versions of a test of topographic left-right discrimination (Money's Road-Map Test of Direction Sense) reveals consistent right hemispheric (as well as left hemispheric) deficiency. Furthermore, left hand deficiency was larger on a “descriptive” version which required the subject to characterize topographical moves executed by the examiner than on a specially designed “enactive” version where the subject was to perform the moves described by the examiner. Linguistically, the right hemisphere is more capable of acting on linguistic instructions than producing a linguistic description of a situation.

Various classifications of lexical information (the meaning of words) demonstrate minor hemisphere comprehension of such varied categories as actions and affective states (as expressed by facial expressions). Most unexpected perhaps was our failure to show right hemisphere deficit in the comprehension of abstract as opposed to concrete nouns. Pictures associated with words like “freedom” (prison), “justice” (a judge in court), and “medicine” (doctor), are promptly identified but not verbalized. Throughout the testing the transfer of visual information from the left visual field to the left hemisphere is checked and suspect trials are excluded from consideration.

References:
*Division of Engineering and Applied Science, California Institute of Technology.

102. LATERALITY EFFECTS IN SOME IMAGERY TASKS

Investigator: Eran Zaidel*

Support: National Institutes of Health, Public Health Service

In an adaptation of the old Hand Test of Thurstone applied with CLV (Biology 1972, No. 99), a picture of a left or a right hand—either a palm or a back—is flashed at 1/10 second. The subject is required to discriminate left from right hands by pressing a lever. Most normal subjects report that they respond by imagining their own preferred hand moved into the same position, and then check for a match or mismatch (Shepard, 1969). The hypothesis that the kinesthetic image of the left hand is better represented in the right hemisphere than the image of the right hand was not confirmed in tests on both split-brain patients and normal controls. Reaction time as well as percentage of correct responses to left hand image with left visual field input was not superior to that for right hand images. However, data from both population samples indicate that while both hemispheres process the information independently, the left hemisphere is superior.

A second study presented L.B. with lateralized pictures of pairs of computer-generated perspective line drawings from Shepard and Metzler (1971) of nonsymmetric 3-dimensional objects differing in spatial orientation. Half the pictures were of the same objects and half of different objects, and subjects had to determine equivalence of shape. While both spatial gestalt and analytic task components seem to be involved, the right hemisphere performed successfully beyond the 0.05 significance level.

The results qualify data from brain lesion studies which indicate a bilateral hemispheric involvement in imagery tasks (Humphrey and Zangwill, 1951). Just as Milner has shown for memory processes, it is the semantics of the imagery task that is crucial for hemispheric specialization. It seems that independent imagery mechanisms exist in the two hemispheres and that a spatial transformational component of imagery is specialized in the right hemisphere.

References:
103. INTERHEMISPHERIC INTERACTION DURING SIMULTANEOUS BILATERAL PRESENTATION OF DOTS IN COMMISSUROTOMIZED PATIENTS

Investigators: Evelyn Lee-Teng, R. W. Sperry

Support: National Institutes of Health, Public Health Service
Frank P. Hixon Fund

Dot-counting ability was compared between the left and the right hemisphere and between unilateral and bilateral presentations in 6 commissurotomized patients. At different trials in mixed order, a set of dots was presented for 0.1 second either in the left or in the right visual half-field alone, or two sets of dots were presented simultaneously for 0.1 second, one in each visual half-field. The size of the set varied from 1 to 5. The patient was asked to indicate the number of dots in each half-field with the ipsilateral hand. The results show that there was significant individual difference among the patients in general performance level and in relative hemispheric superiority, some performing better with the left hemisphere, some with the right. Over and above the individual differences, however, performance was generally better during unilateral dot presentation than during bilateral dot presentation. Analysis of type of errors does show consistent hemispheric differences in that the left hemisphere tends to undercount while the right hemisphere tends to overcount.

104. PARALLEL BUT SLOWED INFORMATION PROCESSING DURING SIMULTANEOUS BILATERAL TASK PRESENTATIONS IN A SPLIT-BrAIN PATIENT

Investigators: Evelyn Lee-Teng, R. W. Sperry

Support: National Institutes of Health, Public Health Service
Frank P. Hixon Fund

Whether two streams of thought can proceed simultaneously without mutual interference in the separated cerebral hemispheres was tested in a split-brain patient (L.B.) by presenting to him tasks that require fairly long durations of thinking. At different trials in mixed order, either a letter pair was presented for 0.1 second to the left or the right visual half-field alone, or two different pairs were presented simultaneously for 0.1 second, one to each visual half-field. The patient was asked to indicate with the ipsilateral hand the number of alphabetical gaps in between the letter pair; the correct answer ranged from 1 to 5. Comparison of the results for unilateral and bilateral task presentations showed an approximate 50% increase in reaction time for each hemisphere with bilateral presentation without any change in error rates.

105. INTERHEMISPHERIC INTERACTION DURING SIMULTANEOUS BILATERAL PRESENTATION OF LETTERS OR DIGITS IN COMMISSUROTOMIZED PATIENTS

Investigators: Evelyn Lee-Teng, R. W. Sperry

Support: National Institutes of Health, Public Health Service
Frank P. Hixon Fund

Interhemispheric interaction after forebrain commissurotomy was studied in 6 patients. Letters or digits were flashed for 0.1 second either in the right or in the left visual half-field alone, or in both fields simultaneously. Patients were asked to identify the stimuli either verbally or by hand. Results showed generally better performance for each hemisphere during unilateral than during bilateral stimulus presentation. For both verbal and manual responses, identification of right visual field stimuli was better than that of left visual field stimuli, the latter often being completely ignored during bilateral presentations. Some evidence was obtained for right hemisphere speech during unilateral presentation of left visual field stimuli.
**Does the Callosum Invert a Stimulus Left for Right?**

**Investigators:** Charles R. Hamilton, Suzannah Bliss Tieman, Betty Ann Brody*

**Support:** National Institutes of Health, Public Health Service

After a discrimination between left and right mirror images has been trained to one eye of chiasm-sectioned monkeys, a preference for the previously unrewarded stimulus is found when the untrained eye is tested. To interpret this paradoxical interocular transfer, Noble (1968) has postulated that the topographically-organized representation of a stimulus on the cortex is reversed left for right but not up for down when it is reprojected across the corpus callosum to the other hemisphere. The known anatomy of cortical topography and callosal connections is consistent with this suggestion, at least at a gross level.

We decided to test Noble’s “callosal inversion” hypothesis directly by requiring chiasm-sectioned monkeys to compare a sample stimulus, projected only to one hemisphere, with two possible matches, projected only to the other hemisphere. A response was correct when the monkey pushed the pattern that matched the sample. Noble must predict that with left-right (L-R) mirror images the monkey would pick the mirror-image pattern as a match since either the sample or the possible matches would be reversed as it was projected across the callosum. No such reversal should occur with up-down (U-D) mirror images.

Three chiasm-sectioned and four normal monkeys were tested on four problems each, 2 with U-D and 2 with L-R sets of mirror-image stimuli. All monkeys matched the U-D stimuli veridically as expected. The normal controls also matched the L-R stimuli veridically, but the average performance of the chiasm-sectioned monkeys was close to chance. Two of the three chiasm-sectioned monkeys performed significantly below chance on one of the two L-R problems, but all three performed significantly above chance on the other problem. Noble’s hypothesis is therefore contradicted since an anatomically-based model cannot account for this kind of variability in transfer of L-R discriminations by chiasm-sectioned monkeys.

We suggest that mirror-image difficulties are a function of the blind half visual field caused by the optic chiasm lesion. The monkey might be coding the stimuli in relation to the blind field, or the blind fields might mask part of the stimuli themselves (see Figure 1). To the extent that the monkey’s performance was affected by its blind fields the monkey should show mirror reversal. To the extent that the monkey depends on its callosal input—which we argue is veridical—it should show veridical transfer. Over-all, these two effects average out to chance level performance.

**Reference:**

*Department of Psychology, Stanford University, Stanford, California.

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**Figure 1.** Two possible ways by which blind visual half-fields might produce reversal of a monkey’s choice of a mirror-image stimulus when tested for interocular transfer of learning.
107. INTEROCULAR TRANSFER OF MIRROR-IMAGE DISCRIMINATIONS BY CHIASM-SECTIONED MONKEYS

Investigators: Charles R. Hamilton, Suzannah Bliss Tieman

Support: National Institutes of Health, Public Health Service

These experiments constitute four attempts to replicate Noble's (1968) finding of paradoxical interocular transfer of mirror-image discriminations (see Biology 1972, No. 106). Four chiasm-sectioned monkeys and four normal controls were tested for interocular transfer of both left-right (L-R) and up-down (U-D) mirror-image discriminations. The different variables manipulated included: (1) rewarding the positive stimulus versus double baiting during the transfer tests, (2) using our automated training apparatus versus a Wisconsin General Test Apparatus, (3) using simultaneous versus successive presentation of the two stimuli, (4) using 2-dimensional versus 3-dimensional stimuli, and (5) preventing vision through the unexposed eye with an opaque occluder versus blocking just the stimulus with polarizing filters.

The results were remarkably consistent. While the normals showed good transfer of both U-D and L-R problems, the chiasm-sectioned monkeys transferred only the U-D discriminations, not the L-R discriminations. No monkey ever showed a significant mirror-image preference on any problem. Thus we were never able to replicate Noble's results. This difference may be related to the type of eye occlusion. In our experiments eye occlusion occurred only when the monkey positioned its head behind the eyeholes of the apparatus. Noble used opaque contact occluders which were left in place for several days. We feel that this chronic occlusion causes the monkeys to rely more heavily on the blind fields while discriminating the mirror images, and thus show a mirror preference when tested for interocular transfer.

According to our hypotheses (see Biology 1972, No. 106), L-R mirror-image discriminations should be more readily learned by animals with optic chiasm section because the resultant blind fields should provide an important clue to the discrimination of L-R but not U-D mirror images. The results confirmed this prediction. While the normals took somewhat longer to learn the L-R mirror images than the U-D mirror images, the operated animals took significantly longer to learn the U-D mirror images than the L-R mirror images (p < .05).

We manipulated the fifth variable in an attempt to differentiate between the two forms of the blind field hypothesis (Biology 1972, No. 106). When polaroid filters are used to block the stimulus, both eyes are open and therefore everything in the visual field is visible to the monkey. As a result, the blind fields should be deemphasized as points of reference. In contrast, the masking of the stimulus by the eye that can see the stimulus should be unaffected. If the reference point version of the blind field hypothesis were correct then we would expect the chiasm-sectioned monkeys to show veridical transfer of the L-R mirror images and to take at least as long to learn the L-R mirror images as the U-D mirror images. However, the results of this experiment were essentially identical to those of the other three. We therefore conclude that the masking version of the blind field hypothesis is more likely correct. Experiments reported in the following abstract (Biology 1972, No. 108) support this conclusion.

Reference:
Noble, J. (1968) Brain Res. 10: 127-151.

108. OPTIC CHIASM SECTION AFFECTS DISCRIMINABILITY OF ASYMMETRIC PATTERNS BY MONKEYS

Investigators: Charles R. Hamilton, Suzannah Bliss Tieman, H. Leabah Winter

Support: National Institutes of Health, Public Health Service

Section of the optic chiasm causes the nasal half-retina of each eye to be blind. In the present study with 4 split-brain and 4 chiasm-sectioned monkeys we have shown that this blind area effectively masks half of a stimulus viewed by one eye. For example, when split-brain monkeys were trained on the discrimination × versus <, the left eye, which saw the right half of the patterns, averaged 1825 trials to a 90% criterion while the right eye averaged 105 trials. With the stimuli reversed, × versus >, the pattern of results reversed with the left eye averaging 122 trials and
the right eye 1330 trials. Therefore, we conclude that the two eyes saw opposite halves of the stimuli.

Such accurate fixation of the stimuli can cause several kinds of interpretive errors when testing split-brain or optic chiasm-sectioned monkeys with asymmetrical stimuli. (1) Apparent differences in learning rate by the two hemispheres could mimic effects of cerebral dominance. (2) Similarly, estimates of interocular transfer in chiasm-sectioned monkeys might be grossly over- or underestimated. (3) Reversal of discrimination of mirror images accompanying interocular transfer would be apparent rather than real since the monkeys would not have really learned to discriminate mirror images (see Biology 1972, Nos. 106 and 107). When not contraindicated, the use of bilaterally-symmetrical stimuli should be encouraged.

109. ESTABLISHMENT OF MEMORIES THROUGH THE ANTERIOR COMMISSURE

Investigators: Michael V. Sullivan*, Charles R. Hamilton
Support: National Institutes of Health, Public Health Service

It has been known for some time that pattern discriminations trained to one hemisphere of split-brain monkeys can be performed by the untrained hemisphere if only the anterior commissure is left intact. We have now shown that duplicate memories are established in the untrained hemisphere via the anterior commissure. Four rhesus monkeys with section of the optic chiasm, corpus callosum and hippocampal commissure were each trained two discriminations to one hemisphere and two to the other hemisphere. Following section of the anterior commissure, each hemisphere could perform all the problems, and hence each had a memory for the task. Thus the anterior commissure, which interconnects anterior inferotemporal cortex, can transmit information capable of laying down memories just as can the corpus callosum. Presumably all the requisite information is available to the inferotemporal cortex. The memories in the untrained hemisphere, however, were somewhat weaker than in the trained hemisphere, and performance with an untrained hemisphere was not as good as in other animals in which the anterior commissure was left intact during the critical tests.

110. SEPARATION OF VISUAL FUNCTIONS WITHIN THE CORPUS CALLOSUM OF MONKEYS

Investigators: Charles R. Hamilton, Betty Ann Brody*
Support: National Institutes of Health, Public Health Service

The posterior end (splenium) of the corpus callosum interconnects the occipital lobes and allows interhemispheric transfer of visual information. We have found that the posterior-most 5 mm of the splenium permits both the interocular transfer of pattern discriminations and the comparison of patterns separately projected to the two hemispheres. The penultimate 5 mm of the splenium, however, fails to support interocular transfer of simultaneous 2-choice discriminations although interhemispheric comparison of similar patterns is unimpaired. Clearly, then, this anterior region of the splenium is deficient in its ability to transfer information compared to the posterior splenium. Several interpretations are possible: (1) the anterior splenium might not transmit information specifying which stimulus is correct, which would prevent interocular transfer but not interhemispheric comparisons; (2) the unstimulated hemisphere might not remain alert and form memories during discrimination training, but would remain alert during comparison tasks since each hemisphere would be directly stimulated; or (3) in accordance with its connections to visual areas of the cortex not topographically organized, the anterior splenium may not transmit spatially relevant information, which is necessary for 2-choice but not comparison problems (see Biology 1972, No. 112).
111. FURTHER CHARACTERIZATION OF VISUAL ABILITIES OF THE ANTERIOR SPLENIUM


Support: National Institutes of Health, Public Health Service

The deficits, noted above, in the capability of the anterior splenium to transmit information pertinent to discriminations, have been further explored with additional monkeys in an attempt to specify more precisely the nature of perceptual processing occurring in the interconnected cortical areas (prestriate, posterior inferotemporal). We have now determined that the anterior splenium can do the following things. (1) Allow comparison of a sample pattern, presented to one hemisphere, with two possible matching patterns presented to the other hemisphere. This occurs regardless of whether or not each hemisphere has first learned the matching-to-sample task on its own. (2) Allow transfer of matching-to-sample trained with the left eye viewing the sample to tests performed with the right eye viewing the sample, or the reverse. It does not allow: (1) interocular transfer of matching-to-sample problems trained entirely through one eye; (2) interocular transfer of a simultaneous or successive 2-choice discrimination; or (3) interocular transfer of “learning sets” (the progressive increase in rate of learning on successive problems) for the matching-to-sample strategy.

These results argue against the third interpretation of the previous abstract (Biology 1972, No. 110) since spatial information is not critical for a successive 2-choice discrimination (see Biology 1972, No. 112). Furthermore, the second, attentional hypothesis is favored since transfer only seems to occur when both hemispheres are directly activated by visual input.

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**Calbiochem Summer Trainee, 1972.
†NSF Summer Trainee, 1972.

112. DOES THE ANTERIOR SPLENIUM TRANSMIT SPATIALLY-DEGENERATE INFORMATION?

Investigators: Suzannah Bliss Tieman, Charles R. Hamilton, Susan M. Schwartz*

Support: National Institutes of Health, Public Health Service

Hamilton and Brody (Biology 1972, No. 110) found that the anterior and posterior 5 mm of the splenium are functionally different in their ability to transfer visually-related information and suggested that this reflects a difference in the capabilities of the areas interconnected by these two regions. Recent anatomical evidence suggests that the posterior splenium interconnects the vertical midlines of the first three cortical representations of the visual field while the anterior splenium interconnects more anterior visual cortical areas. The first three representations are topographic but because of repeated convergence the spatial map degenerates after that. The following experiments test our hypothesis that the anterior splenium transmits spatially-degenerate information.

(1) Interocular transfer of go, no-go discriminations. Hamilton and Brody suggested that loss of spatial information in the anterior splenium would prevent interocular transfer of simultaneous 2-choice discriminations while still permitting interhemispheric comparisons. During monocular learning of a simultaneous 2-choice discrimination, the untrained side of the anterior splenium-intact monkey would see both the positive and negative stimuli but would not know their positions on the panel. Even knowing that its hand pushed the top screen, it wouldn’t know which stimulus was on that screen, and would therefore be unable to make its own associations between the stimuli, the response, and the rewards. By contrast, during the learning of a go, no-go discrimination (push one stimulus, don’t push the other), only one stimulus is present at a time and therefore its position in space is not relevant. Further, since only one of the two possible stimuli is paired with food the second side should be able to make its own association.

Four monkeys with midline section of the optic chiasm, anterior commissure, hippocampal commissure, and all of the corpus callosum except for the anterior 5 mm of the splenium were trained to discriminate eight pairs of patterns in a go, no-go situation with one eye and were tested for transfer of these discriminations to the other eye. The second eye showed no advantage. Two of these four monkeys have demonstrated the ability to perform the interhemispheric matching-to-sample task. The other two have yet to be tested. Because these monkeys fail to transfer discriminations for which spatial information should be
unnecessary, the spatial degeneracy hypothesis cannot account for the failure of monkeys with only the anterior splenium intact to demonstrate interocular transfer of simultaneous 2-choice discriminations.

(2) Spatial matching-to-sample. Monkeys with only the anterior splenium intact are able to successfully compare a sample pattern presented to one hemisphere with two possible matches presented to the other hemisphere. If the spatial degeneracy hypothesis is correct, and if the matching is made to depend on spatial information rather than pattern differences, then these monkeys should no longer be able to perform the task. This experiment is just starting.

*NSF Summer Trainee, 1972.

113. COMPARISON OF STIMULI PRESENTED TO SEPARATE VISUAL FIELDS OF NORMAL AND PARTIALLY SPLIT-BRAIN MONKIES

Investigator: Charles R. Hamilton
Support: National Institutes of Health, Public Health Service

Electrophysiological data indicate that the splenium of the corpus callosum contains neural fibers interconnecting only cells with receptive fields near the vertical midline of the visual field. How then can information concerning patterns seen in peripheral parts of the visual field be transferred between the hemispheres of monkeys? Or can it? For the usual tests of interocular transfer, information crossing from near the vertical midline may be sufficient for good performance since monkeys normally look at objects of interest with central vision. To test for transfer of peripherally-presented visual information we make the monkeys fixate a central position and then we flash two different stimuli to each peripheral visual field. Above-chance performance depends on comparing these two peripheral stimuli. Tests are currently under way with normal monkeys and with partially split-brain monkeys with either their anterior or posterior splenium intact. Normal monkeys, and those with anterior splenium intact, may perform well but not those monkeys with only the posterior splenium intact. This would show that representations of stimuli in the periphery are transferred through the anterior splenium which interconnects cortical areas that process data from large areas of the visual fields. Alternatively, none of the monkeys may be able to perform this task in accordance with the presently known anatomy and physiology. Since human subjects can perform this task, it might then be inferred that verbal labeling of the stimuli mediated the transfer in man.

114. CORTICAL AREAS INTERCONNECTED BY THE SPLENIUM OF THE CORPUS CALLOSUM

Investigators: Myrna Miller*, H. Leabah Winter**, Charles R. Hamilton
Support: National Institutes of Health, Public Health Service

Several of the above experiments test for differential behavioral functions of the anterior and posterior splenium. However, only relatively gross data exist on the anatomical terminations of fibers passing through these two callosal regions. Four monkeys have had lesions placed in the splenium, 2 in the posterior 5 mm and 2 in the anterior 5 mm. After 7 days the animals were sacrificed and stained according to the Fink-Heimer technique. The areas to which degenerating fibers project are now being determined. This data should provide a firmer basis on which to interpret our behavioral findings that these two segments of the callosum transmit different types of information.

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115. CEREBRAL DOMINANCE FOR ORIENTATION IN MONKEYS

Investigators: Charles R. Hamilton, Suzannah Bliss Tieman, William S. Farrell*
Support: National Institutes of Health, Public Health Service

Preliminary findings have shown that the left hemisphere of split-brain monkeys learns to discriminate patterns that differ only in orientation significantly faster than does the right hemisphere (Hamilton and Lund, 1970). This was true for discriminating between different directions of movement of a field of dots. No such dominance was found for discriminations based on the shape
of patterns. We are currently testing for cerebral dominance effects in the discrimination of other oriented stimuli, both moving (Biology 1972, No. 117) and static. So far we have found a left hemisphere dominance for discrimination of the orientation of a field of stripes. Because the tendency of split-brain monkeys to fixate the center of patterns masks half the stimulus (see Biology 1972, No. 108), simple orientations of asymmetric patterns such as arrowheads cannot be used. We overcome this problem by using pairs of bilaterally-symmetric patterns, such as ○ versus X, for which the right half of one pattern is the same as the left half of the other. Central fixation of such patterns produces a true orientation discrimination for split-brain monkeys.

Reference:

116. CEREBRAL DOMINANCE FOR FACIAL DISCRIMINATION IN MONKEYS

Investigators: Charles R. Hamilton, Bruno Preilowski, Suzannah Bliss Tieman, Gregory E. Gray*

Support: National Institutes of Health, Public Health Service

The right and left hemispheres of human beings are clearly specialized for different cognitive functions. The right tends to dominate in tasks involving the perception of spatial arrangements, the perception of musical forms, and the recognition of faces. The left hemisphere specializes in language and other complex analytic functions. To date little evidence has been obtained to favor similar cerebral asymmetries in other primates (see Biology 1972, No. 115). We are presently testing each hemisphere of split-brain monkeys for the ability to discriminate faces or facial expressions of monkeys. Control tests will ensure that the discrimination was based on facial characteristics. So far only one monkey has been tested; the left hemisphere appeared favored.

If we regard cerebral asymmetry as an adaptive product of evolution then the recognition of faces and expressions, which are of paramount importance to social interaction and communication, would seem likely candidates for cerebral specialization. A positive finding would dispel a commonly-held opinion that cerebral dominance is a strictly human characteristic reflecting the development of formal language.

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117. INTERHEMISPHERIC TRANSFER OF VISUAL DISCRIMINATION OF MOVEMENT

Investigators: Suzannah Bliss Tieman, Charles R. Hamilton

Support: National Institutes of Health, Public Health Service

Single cells in the mammalian superior colliculus tend to be very responsive to various kinds of moving stimuli (McIlwain, 1972). These results plus those of behavioral studies have led a number of investigators to postulate an important role for the midbrain in spatially-oriented behavior (Ingle and Schneider, 1970). We are presently testing the notion that discrimination of movement takes place in the midbrain by looking for interocular transfer through the midbrain commissures of a learned movement discrimination. This study attempts to maximize the chances of interocular transfer by using large expanding and contracting stimuli, which (1) resemble preferred stimuli of tectal and pretectal units in the cat, and (2) are usually considered important in “spatial” vision (Gibson, 1966). Eight rhesus monkeys with midline section of the corpus callosum, the hippocampal commissure, the anterior commissure, and the optic chiasm but with midbrain commissures intact, are being trained with one eye open to discriminate a spiral rotating clockwise from the same spiral rotating counterclockwise. Each monkey will also be trained on a discrimination involving rates of movement of the spiral. If the midbrain is important for the discrimination of movement, then it would be reasonable to expect the second eye to show some advantage when it is trained on the same problem. Additional splitting of the midbrain commissures should then prevent interocular transfer. Failure to find some advantage for the second eye would suggest that learning a movement discrimination is a function dependent on the cortex. In this case leaving the forebrain commissures intact should promote interocular transfer.

References:
118. DISCRIMINATION LEARNING FOLLOWING SUBCORTICAL LESIONS

Investigator: John David Johnson
Support: National Institutes of Health, Public Health Service

Previous research has shown that a somesthetic habit can be isolated in one hemisphere by "splitting the brain" and this learning is unaffected by large-scale destruction of non-somesthetic cortex (Sperry, 1959). In an effort to further narrow down the neurological basis of this learning the present study is testing for deficits in the learning and performance of somesthetic habits following various subcortical lesions. Destruction of much of the temporal lobe including the amygdala and hippocampus in one hemisphere of split-brain monkeys has had no major effect on the ability of this hemisphere to learn a new discrimination. However, early results suggest that a unilateral hypothalamic lesion undermines the performance of food-reinforced habits by the lesioned hemisphere without affecting its sensory-motor abilities. Further tests are planned to determine whether this effect is present when the interhemispheric commissures are intact. This effect should not appear if the undamaged hypothalamus of one hemisphere can serve the damaged hemisphere via these commissures. The results of this study should give some indication of the role of these pathways in conveying motivational information and may provide a way to study the interaction between motivational structures and those implicated as the location of memory storage.

Reference:

119. THE LOCUS OF REINFORCEMENT OF UNILATERAL PLEASURE-CENTER STIMULATION IN SPLIT-BRAIN CATS

Investigator: James J. Wright
Support: National Institutes of Health, Public Health Service

Despite demonstration of the critical role played by the intercortical commissures in interhemispheric transfer, there has been little clarification of the part played by subcortical mechanisms in cross-integration. It has been shown in commissurotomized humans and monkeys that both the stimulus patterns and the reinforcing reward must be perceived in appropriate sequence, by the same hemisphere, if learning of a pattern discrimination task is to occur.

In the present experiment transection of the corpus callosum, anterior commissure, and optic chiasm was carried out in cats, followed by unilateral chronic implantation of bipolar stimulating electrodes at pleasure-center sites in the supraoptic cortex, lateral hypothalamus, and midbrain tegmentum. The cats were then trained to a pattern discrimination with free vision while receiving an intracranial stimulus as reinforcement. After learning was established the cats were fitted with a plastic occluder over one eye and tested for learning first in one hemisphere, then the other. Present data from 5 cats indicates that learning is reinforced in both hemispheres from all the above sites of unilateral pleasure-center stimulation. To confirm disconnection of the animals’ hemispheres for pattern perception, each animal was subsequently trained monocularly to opposite pattern preference in opposite hemispheres, again using unilateral intracranial stimulation as reinforcement.

It can be concluded that unilateral pleasure-center stimulation exerts a reinforcing effect on both hemispheres, independent of transfer of information by direct intercortical commissures.

References:

120. CHANGES IN HEART RATE IN CAUDATE-LESIONED RATS

Investigator: John David Johnson
Support: National Institutes of Health, Public Health Service

The caudate nucleus has traditionally been associated with the regulation of motor processes. However, in the last decade a variety of data has suggested that it is involved in sensory and motivational processes as well. For example, the electrical stimulation of the caudate results in an inhibition of neuron activity in the hypothalamus and in the cortical sensory areas (Feldman and
Dafney, 1967; Nestianu, Bonciocat and Daneliuc, 1967). To further analyze the role of the caudate nucleus in motivational processes, this structure was lesioned in rats whose heart rate was being monitored. Lesioned and normal animals were given mild electrical shocks to determine the minimal intensity necessary to cause an acceleration of heart rate. The results from a small number of animals suggest that the caudate-lesioned rats require less intensity for acceleration than normals. These results would be consistent with the view that at the onset of stimulation the caudate nucleus inhibits motivational processes to prevent excessive arousal. They would suggest that defective function of the caudate allows a larger emotional response to mild aversive stimulation.

References:

121. MEMORY FORMATION PROCESSES IN THE CHICK
Investigators: Larry I. Benowitz, J. Geoffrey Magnus
Support: National Institutes of Health, Public Health Service

Recent evidence has indicated that several physiologically distinct representations of memory occur following a training experience (see review, McGaugh and Dawson, 1971). Determining the nature and interrelationship of these different phases is of importance in understanding the mechanisms by which memory is stored. To study this, we analyzed in quantitative detail the retrograde amnesia gradient resulting from passage of subconvulsive transcranial current (SCC) at various intervals following chicks' one-trial aversive conditioning (Lee-Teng and Sherman, 1966). Our results indicate that following the training experience, a metastable memory trace is immediately established (Phase I) and is maintained at a constant intensity. Within 30 seconds, this phase in turn induces the exponential growth of a semi-permanent memory trace (Phase II), which, once formed, is not susceptible to SCC. These findings, together with other consolidation experiments (Cherkin, 1965; Lee-Teng et al., 1970) suggest that in addition to its role as an inducer of permanent memory, Phase I also serves as behaviorally manifest short-term memory in the first few hours after training. During this period, the behaviorally latent Phase II is undergoing consolidation into long-term memory. A likely characterization of Phase I memory would be a population of metastable elements each in an all-or-none state. The first-order exponential kinetics of Phase II growth seem to reflect some finite neural capacity becoming saturated as Phase II memory forms.

References:

122. THE INTEGRATION OF FRACTIONAL MEMORY TRACES FOR CHICKS' ONE-TRIAL LEARNING
Investigators: Larry I. Benowitz, J. Geoffrey Magnus
Support: National Institutes of Health, Public Health Service

In order to investigate mechanisms that allow an animal to integrate a succession of similar experiences, we examined the memory that results from a sequence of two aversive trainings in chicks, each followed by SCC shortly after training. If the growth of memory after two such trainings were controlled only by the mechanisms described above (Benowitz and Magnus, Biology 1972, No. 121), then we might expect the resultant of the two experiences to be simple to predict; that is, the second training should immediately reinitiate the metastable Phase I, which in turn would be expected to cause the exponential growth of Phase II memory (the semipermanent precursor to long-term memory) to continue from the point at which its growth had been halted by the first post-training electroshock. On the other hand, if memory from the first training had not been fully manifest but nevertheless served to establish a "set" to facilitate integration of the second experience (Quartermain, McEwen and Amitzia, 1970), then the second training would cause a disproportionate increase in memory. Alternatively, one might propose that the
two partial memory traces might not get fully integrated together perhaps due to the use of separate neural substrates for the registration of each experience. Our results indicate that, in fact, the resultant of the two fractional memory traces is that predicted by a linear summation of the two training-current intervals in the exponential expression found to describe the growth of Phase II memory (see previous report). This indicates that the two memory traces are in full register and become completely integrated together, even in the absence of active mental associations. Since the rate of the second memory formation seems to be determined only by the amount of the memory remaining to be formed, these findings support the hypothesis that the exponential consolidation kinetics we have observed represent the saturation of some elemental mnemonic mechanism.

Reference:

123. LEARNING AND MOTIVATIONAL DEFICITS FOLLOWING FOREBRAIN ABLATIONS IN CHICKS

Investigators: Larry I. Benowitz, Evelyn Lee-Teng, M. Rone*

Support: National Institutes of Health, Public Health Service
National Science Foundation

Striking similarities in the telencephalic structures of birds and mammals have recently been indicated (see Karten, 1969). The more simplified anatomy of the avian brain, however, would seem to offer strategic advantages for studying functional correlations. In an attempt to understand cerebral organization underlying behavior, the effects of several different forebrain ablations on chicks’ appetitive learning and motivation were examined. Ablations of the dorsomedial telencephalon were found not to affect chicks’ acquisition of a simple pattern discrimination but did impair reversal training. Posterior lateral ablations impaired early stages of acquisition but had no other effects on learning or reversal. Frontal ablations increased the length of time required to perform the discrimination but did not harm learning itself in terms of trials to criterion.

These findings are all similar to results reported following ablations of the homologous components of the mammalian limbic system (i.e., hippocampus, amygdala and septal region) (Douglas and Pribram, 1966; Thomas et al., 1959). Together with previous findings concerning the effects of the same limbic system ablations on chicks’ avoidance learning (Benowitz, in preparation), it appears that despite approximately 300 million years of divergent evolution, learning of both birds and mammals is structured upon similar neuro-anatomical interrelationships.

To better define the functional significance of the regions removed, chicks with the same ablations as described above were further tested in several formalized behavioral tasks. Incomplete results indicate that none of the three ablations diminishes the general level of chicks’ activity as measured in several situations. However, both the frontal and dorsomedial ablations cause severe motivational deficits independent of learning (these chicks will freely walk off the depth of a “visual cliff” apparatus), while the posterolateral ablations do not. Dorsomedial ablations also severely affect chicks’ ability to perform a one-trial appetitive learning task, while the other two ablations do not. We are hopeful that further investigations of this kind will help refine our ideas about behavioral subroutines mediated by various forebrain structures and perhaps help to clarify the interrelationship between motivation and learning.

References:

*NSF Summer Trainee, 1971.
124. PLASTICITY AFTER NEONATAL CALLOSAL SECTION IN CATS

Investigator: James R. Carl
Support: National Institutes of Health, Public Health Service

Studies on human commissurotomy patients have shown that surgical separation creates a syndrome in which much of the normal exchange of information between right and left hemispheres is terminated. If, however, the separation is due to a congenital absence of the corpus callosum, tests for interhemispheric integration show a remarkable lack of functional impairment (Sperry, 1968). This result is not surprising, as the developing brains of young animals are known to be quite plastic and can often compensate for severe brain damage suffered as infants.

An attempt to examine the nature of the plasticity and mechanisms of compensation has been started by surgically sectioning the corpus callosum in a series of neonate kittens in the first week after birth. When the kittens are 2 to 3 months of age the optic chiasm will be sectioned and they will be tested for symptoms of hemisphere deconnection.

Reference:

125. RIGID PLACE SPECIFICITY OF RETINOTECTAL CONNECTIONS IN HALF-TECTUM FROGS

Investigator: Ronald L. Meyer
Support: National Institutes of Health, Public Health Service

In goldfish, Gaze and Sharma (1970) and Yoon (1971) have shown that the topographic map of retina onto the surface of the midbrain (optic tectum) is eventually restored in its entirety to a tectum that has been substantially reduced in size by surgical ablation. Using similar methods I have repeated these experiments on tree frogs. To date I have found no evidence for this kind of compression. My electrophysiological maps indicate that the portion of tectum left after surgery (approximately the anterior half tectum) receives the same retinal fibers it did prior to surgery even if the optic nerve is cut and allowed to regenerate. Careful behavioral testing (not done for goldfish) also shows that these frogs have a large, apparently permanent, blind area corresponding to the initial extent and locus of the lesion.

This evidence indicates that the tectal field of the frog is not subject to the kind of reorganization found in the goldfish. The results conform with findings for full-tectum frogs, and are in accord with the chemospecificity hypothesis of Sperry (1963).

References:

126. ON THE PROBLEM OF LEFT-RIGHT SPECIFICITY OF NEURAL TISSUE

Investigator: Margaret Y. Scott
Support: National Institutes of Health, Public Health Service

Genetic models for cerebral dominance in man and the general pattern of decussating fiber tracts in the vertebrate brain strongly suggest that left and right halves of the central nervous system must be biochemically differentiated. Whereas many regeneration experiments have indicated rather equal interchangeability between right and left neural tissues, the experiments of Franzisket (1959) on competitive reinnervation of cutaneous areas in frog by nerves from right and left sides seemed to demonstrate the existence of right-left specificity. The present experiment was an attempt to confirm and extend findings of Franzisket.

Electrophysiological recordings from dorsal cutaneous nerves revealed a slight, but consistent overlapping of receptive fields across the midline in 5 normal frogs. Recordings 5 to 7 weeks after the second dorsal cutaneous nerves had been surgically crossed in 5 frogs showed successful regeneration in all 5 with slightly abnormal innervation of the skin in one case. The area into which the crossed nerve regenerated was of approximately normal size in 3 animals and slightly smaller and centered across the midline in 1 animal. In the fifth, the surgically-manipulated left nerve regenerated to an area confined to the region of the right lateral ridge. Regeneration of the crossed right nerve was apparently unsuccessful. There was extensive over-
lap of the receptive fields of the crossed nerve and homolateral nerves as determined by electrical recording, in all cases.

These results are in conflict with those reported by Franzisket who found that cutaneous reinnervation of the surgically-crossed nerves did not overlap with that of ipsilateral nerves. In further tests all of the right dorsal cutaneous nerves were severed in 5 animals. Electrophysiological mapping of the receptive fields some 6 to 8 weeks later showed that the left cutaneous nerves had expanded their terminal fields to cover the entire dorsal region between the lateral ridges. Although the results thus far fail to support the hypothesis of right-left specificity, they do not exclude its presence in some form too subtle to have been detected by the present methods.

Reference:

127. COMPRESSION, DECOMPRESSION AND RECOMPRESSION OF RETINOTECTAL PROJECTIONS IN GOLDFISH

Investigator: Myonggeun Yoon
Support: National Institutes of Health, Public Health Service

The goldfish visual system manifests versatile plasticities in readjusting the visual projection from the retina onto the primary visual center (the optic tectum) in the midbrain. It has been found that the rostral half-tectum may reacquire an orderly compressed visual projection from the whole retina after removal of the caudal half of the tectum (Gaze and Sharma, 1970). The compression of visual projection may also be induced even in the absence of the denervated caudal half-tectum following surgical split of the latter from the rostral half of the tectum, regardless of whether the contralateral optic fibers were left intact or completely sectioned and then allowed to regenerate (Yoon, 1971). The compression along the rostrocaudal axis of the rostral half-tectum began to occur gradually from medial side of the half-tectum about a month after ablation of the caudal half of the tectum, and it became complete in the lateral side of the rostral tectum about a half-month later (Yoon, 1972a). These results suggest that the compression of retinotectal projection is a gradual process, and that it proceeds faster in the medial side than in the lateral side of the half-tectum along its rostrocaudal axis.

Furthermore, the compression of retinotectal projection was found to be a reversible phenomenon, which can be induced and then reinstated in correct retinotopic order (Yoon, 1972b,c): a normal visual projection from the whole retina onto the whole tectum may be restored following a previous compression, initially induced by surgical insertion of a mechanical barrier between the rostral and caudal halves of the tectum. In a further study, the newly decompressed visual projection was also found to be recompressed by removal of the caudal half of the tectum following the reinstatement of a normal projection from a previous compression.

It is suggested (Yoon, 1971, 1972b) that the plastic and reversible phenomenon of retinotectal reorganization may be due to a regulative mechanism which respecifies the tectal neurons according to their new relative positions within the half-tectum in correct retinotopic order.

References:

128. TRANSPOSITION OF RETINOTECTAL PROJECTION AS THE COMBINED RESULT OF EXPANSION AND COMPRESSION IN GOLDFISH

Investigator: Myonggeun Yoon
Support: National Institutes of Health, Public Health Service

In a group of juvenile goldfish, the temporal hemiretina was destroyed by electrical cauterization. This retinal lesion resulted in a scotoma on the rostral zone of the optic tectum. The caudal part of the tectum that was originally innervated by the surviving nasal hemiretina was also ablated in the same fish. About 4 months after the complementary surgical operations on the retina
and the tectum, the visual projection from the nasal hemiretina was found to become transposed onto the entire extent of the foreign rostral half-tectum in correct retinotopic order. Furthermore, if the newly reestablished retinotectal projection was then interrupted by crushing the optic nerve, the transposed projection became restored by orderly regeneration of the optic fibers from the surviving nasal hemiretina onto the entire extent of the remaining rostral half-tectum [Yoon, M. (1972) *Exptl. Neurol.*, in press].

In a later series of experiments, either the temporal half or the nasal half of the retinal area was destroyed. About 3 months after the retinal lesion, the visual projection from the surviving nasal or temporal hemiretina was found to have expanded over the entire extent of the intact contralateral tectum, regardless of whether the optic fibers were left intact or completely sectioned and then allowed to regenerate (Yoon, 1972). Following the expansion of visual projection from the hemiretina onto the whole optic tectum, the caudal half of the tectum was also ablated. About 4 months after the tectal ablation in addition to the preceding retinal lesion, the visual projection either from the nasal hemiretina or from the temporal hemiretina was found to become either transposed or reinstated, respectively, onto the entire extent of the remaining rostral half-tectum in correct retinotopic order (Yoon, 1972). These experiments show that the transposition of retinotectal projection is a combined result of the expansion after the retinal lesion and of the compression after the tectal ablation.

The reorganization of retinotectal projection (either compression, expansion, or transposition) in goldfish suggests that the formation of neural connections between the retina and the optic tectum is controlled not by rigid, place-specific determinants but by plastic, regulative mechanisms that preserve the over-all topological order in the pattern of connections regardless of whether the connections are compressed, expanded or unchanged.

**Reference:**

**PUBLICATIONS**


The development of an organism may be thought of as an orderly unfolding of a sequence of gene control functions much like the execution of steps in a computer program. We are attempting to study development by inserting gene mutations which block, so to speak, the execution of steps in the organism's developmental program. In *Drosophila* the body segmentation plan is subject to profound modification by certain mutants, especially those belonging to the cluster of bithorax genes in the third chromosome. These mutants bring about highly specific alterations of the normal pattern of segmentation of the thoracic and first-abdominal segments. Whether a fly will develop 2 wings or 4 or will develop 4, 6 or 8 legs is directly dependent upon the bithorax genes. The picture that emerges is one in which the genes of the bithorax cluster determine how far along a developmental pathway a given segment will develop. The first-abdominal segment, for example, has been found to be modifiable by mutant genes either in the direction of the thorax or in the direction of the second-abdominal segment. The thoracic versus abdominal decision appears to be made by a specific gene of the bithorax cluster. If the fly contains only a recessive mutant of that gene (known as the "d" mutant) then a thoracic state is manifested in the first-abdominal segment. On a number of grounds, the thoracic state is thought to be a more primitive state of development than the abdominal one. Apparently, in the presence of the mutant d gene, development does not proceed beyond the thoracic state as far as the first-abdominal segment is concerned, whereas in the presence of the normal d gene the first-abdominal segment proceeds to its normal abdominal state of differentiation. The first-abdominal segment is capable however of becoming more abdominal in the sense that it may take on characteristics of the much more highly differentiated second-abdominal segment in the presence of a dominant mutant, G, also a member of the bithorax cluster. A change of the first-abdominal segment to the second is evidently some kind of gain of function, possibly a hyperactivity of some gene similar to but probably not identical with the normal d* gene. We have been trying to study this kind of super-normal developmental change by inducing additional mutants of the G type. With the aid of the chemical mutagen, ethyl methane sulfonate, we have induced during the past year such a new mutant. This mutant is able to carry out a partial transformation of the first to the second-abdominal segment. It is remarkable in that it occupies a separate locus at some distance from the bithorax cluster, indicating that the control of the switch from first-abdominal to second-abdominal states of differentiation requires the cooperation of at least two genes. By means of gene duplication we are now attempting to increase the degree of hyperactivity of the gene or genes responsible for increasing the degree of abdominal differentiation. By means of combinations of mutants it may be possible to infer whether the different states of segmental differentiation are achieved through sequential or alternative pathways.

One of the approaches we have been using in studying the effects of the bithorax mutants is to construct flies that are genetically mosaic for such mutants. This has been accomplished by introducing the bithorax genes into an X-chromosome and then producing gynandromorphs in which the male tissue lacks the bithorax genes but the female

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**129. CONTROL OF DEVELOPMENTAL PROCESSES IN DROSOPHILA**

**Investigator:** Edward B. Lewis

**Support:**
- National Science Foundation
- National Institutes of Health, Public Health Service
- U.S. Atomic Energy Commission
tissue still carries them. Such sex mosaics can be produced by using genes which increase the spontaneous frequency of loss of X-chromosomes in somatic cells. Although the external parts of a fly can be sexed by means of a specific body color mutant, most internal tissues cannot be readily sexed. We are exploring a new approach based upon the intense fluorescence which the Drosophila Y-chromosome exhibits by comparison with that of the X-chromosome (Vosa, 1970). The fluorescence appears after staining with the dye quinacrine and examining under a UV microscope. Moreover, resting nuclei of males are more strongly fluorescent than are the resting nuclei of females. In order to increase the contrast between male and female cells, we have synthesized during the past year an X-chromosome which has two strongly fluorescent Y-chromosomes attached to it. The new chromosome has one long arm of Y at the tip of the X-chromosome and another long arm of Y at the base of the X-chromosome. We are indebted to Dr. Dean Parker for providing one of the special chromosomes used in synthesizing this X-chromosome. Mr. Chia has carried out preliminary examinations of the adult brain cells of females with two normal X-chromosomes and of similar cells from females carrying this special, X-double-Y-chromosome. The contrast between the two types of cells is sufficiently striking that it should be possible to make use of it to sex the internal tissues of a special type of gynandromorph, namely, a gynandromorph in which the female tissue carries one normal X-chromosome and one X-double-Y-chromosome, while the male tissue carries a single X- and no Y-chromosome. We plan to construct such gynandromorphs.

References:

130. PROPERTIES OF MOSAICS PRODUCED BY THE MUTANT mit OF DROSOPHILA MELANOGASTER

Investigator: William M. Gelbart
Support: Gosney Fund

Mutants causing somatic chromosome loss are very useful in the study of developmental processes, since they allow us to generate individuals composed of two genetically different types of tissue. For this reason, the properties of one such mutant, mit (mitotic loss), are being studied. This X-chromosome mutant, mapping near the f (forked bristle) locus, acts maternally and is recessive. Thus, mosaics are observed only among the progeny of homozygous mit females. From such females, chromosomes derived from either the maternal or paternal chromosome complement are somatically eliminated, and with equal frequencies. This is in contrast to previously described mutants which cause somatic chromosome loss in Drosophila melanogaster (ca\textsuperscript{ad} and mei-W5). ca\textsuperscript{ad}, like mit, acts maternally but only engenders loss of maternal chromosomes. mei-W5 acts paternally and leads to somatic elimination of paternal chromosomes only.

A fly which begins as an XX-zygote but which loses an X-chromosome in some of its tissues is termed a gynandromorph. Gynandromorphs account for 0.05 of the initially XX-progeny of homozygous mit mothers. Several hundred gynandromorphs have been produced and analyzed for the distribution of male tissue. These gynandromorphs arose from zygotes in which one X-chromosome was marked with y (yellow body and bristle color) and the other with sn\textsuperscript{3} (singed bristles). Female progeny are genetically y/sn\textsuperscript{3} and phenotypically wild type. Gynandromorphs express the recessive yellow or singed phenotype in portions of their bodies. These mutants allow male tissue to be detected in most of the adult cuticle. Among all gynandromorphs recovered, the mean frequency for which a given structure on the adult cuticle (i.e., a specific bristle) was male was 0.10. In other words, the average gynandromorph produced by mit females is 90% female and 10% male, at least for surface structures. Using the data derived from these gynandromorphs, a morphogenetic fate map, describing the positions on the embryo surface of those cells giving rise to specific adult cuticular structures, was constructed. Overall, this fate map is very similar to those produced by Garcia-Bellido and Merriam (1969) and Hotta and Benzer (unpublished), using two different gynandromorph-generating systems.

Reference:
BIOPHYSICAL CHEMISTRY
CELL BIOLOGY
ELECTRON MICROSCOPY
MOLECULAR BIOLOGY

Church-Alles
(Second Basement)
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Professor: Jerome Vinograd

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131. REPLICATION OF MITOCHONDRIAL DNA IN CULTURED MOUSE CELLS

Investigators: Harumi Kasamatsu, Donald L. Robberson

Support: Helen Hay Whitney Foundation
National Institutes of Health, Public Health Service

The covalently closed mitochondrial DNA from exponentially growing mouse cells contains approximately equal amounts of two major species: a mature DNA and a closed DNA which we have called D-loop DNA, or displacement loop DNA. The latter DNA, formed by displacement synthesis, contains a short progeny strand, hydrogen-bonded at a unique site to the light parental strand.

The mitochondrial DNA isolated from the upper band of an EthBr-CsCl gradient contains two distinctive replicative circular structures.

1) Expanded D-loops with expansions which vary in size up to that of genome size. The displacing strands in these molecules are again noncovalently attached. The displaced strand in the larger (>0.6 genome size) D-loops is often hydrogen-bonded along a significant part of its length to a progeny complement with duplex formation. The displaced single strand is the heavy parental strand.

2) Gapped circular molecules which contain a full-length circular single strand, a portion of which is hydrogen-bonded to a complement. The gapped region varies from <10% to full length. The single strand region of the large gapped circle formed a duplex only when the gapped molecule was annealed with the light parental strand.

Based on the structure and strandedness of these circular replicative intermediates, the following scheme for the replication of mitochondrial DNA has been developed (see Figure 1).

The mitochondrial DNA replication begins in a covalently closed circular DNA (A). A short 450-nucleotide strand is synthesized by a displacement mechanism with a unique region of the light strand serving as a template in the first discrete step of replication (B). After a nicking system becomes operative, displacement synthesis continues (C), and the short heavy strand is extended. Light strand synthesis occurs on the displaced heavy parental strand with the formation of a duplex (D,E). This duplex synthesis begins at 0.6 or more genome units from the origin for displacement of replication. The completion of displacement synthesis and separation before duplex synthesis is finished, gives α daughters and large gapped β Gpc molecules (F,G). Further duplex synthesis on β gapped circles forms β daughter molecules (H).

An implicit assumption in this model is that displacement replication is unidirectional and that only one of the two forks moves. This has been proved in an examination of double-length mitochondrial DNA molecules which contain a small D-loop in addition to the expanded D-loop. In earlier work we demonstrated that dimeric molecules often contain two small D-loops 180° apart. One of the two D-loops in some dimer molecules has been found to be expanded. In such molecules, one of the distances between one fork in the expanded D-loop and the nonexpanding D-loop remains constant, independent of the extent of the expansion.

References:
132. ABSENCE OF REPLICATING FORMS OF MITOCHONDRIAL DNA IN EARLY SEA URCHIN EMBRYOS

**Investigator:** Lajos Pikó*

**Support:** National Institutes of Health, Public Health Service

Our earlier studies (Pikó, 1969) have shown that mitochondrial DNA does not replicate to any appreciable extent during the first three days of development of the sea urchin *Lytechinus pictus*. During this time, the fertilized egg differentiates into a free-swimming larva containing about a thousand cells, but there is no increase in the total cytoplasmic volume. The mechanism of blockage of mitochondrial DNA replication is not known. In this study, the structure of the mitochondrial DNA was examined by electron microscopy with particular reference to the possible occurrence of a stable replicating intermediate, D-loop molecules, described in tissue culture cells (Kasamatsu, Robberson and Vinograd, 1971).

Mitochondria of unfertilized eggs and early embryos were isolated by sucrose density gradient centrifugation, and contaminating nuclear DNA was removed by DNase treatment. In buoyant density centrifugation in an EthBr-CsCl gradient, most of the mitochondrial DNA banded in the position of covalently closed circular DNA. This DNA was prepared for electron microscopy by the formamide-basic protein technique. Less than 1% of the molecules appeared to have a D-loop; therefore, the blockage must occur before this stage of replication.

It was shown earlier (Smith, Jordan and Vinograd, 1971) that the mitochondrial DNA of cultured tissue cells grown in the presence of ethidium bromide is subject to nicking-closing cycles resulting in a decrease in the buoyant density of closed circular DNA in EthBr-CsCl gradients. However, the mitochondrial DNA of sea urchin embryos grown in the presence of ethidium bromide, sufficient to inhibit mitochondrial DNA synthesis, shows no change in buoyant density, indicating an absence of nicking-closing cycles. This observation suggests that the enzymes involved in mitochondrial DNA replication may be absent or inactive in early sea urchin embryos.

**References:**


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133. SYNTHESIS OF MITOCHONDRIAL DNA WITH A PARTIALLY PURIFIED DNA POLYMERASE FROM THE MITOCHONDRIA OF HeLa CELLS

**Investigators:** Clark J. B. Tibbetts, Jerome Vinograd

**Support:** National Institutes of Health, Public Health Service

_in vitro_ DNA synthesis has been studied with a partially purified DNA polymerase from the mitochondria of HeLa cells and HeLa cell mitochondrial DNA. Product DNA, covalently attached to template DNA, was analyzed with respect to the complements separated in alkaline
CsCl buoyant density gradients. The results indicated base composition-complement fidelity of the product of DNA synthesis as well as preferential synthesis of DNA corresponding to the denser complement (in alkaline CsCl solution) of mitochondrial DNA. This asymmetric synthesis of mitochondrial DNA appears to arise from a bias in the number of single strand scissions sustained by the complementary strands of mitochondrial DNA during incubation with the protein fraction containing the mitochondrial DNA polymerase.

134. ISOLATION OF DNA BINDING PROTEINS FROM MITOCHONDRIA OF ANIMAL CELLS USING DNA-CELLULOSE CHROMATOGRAPHY

Investigator: Karin O. Sundquist*

Support: National Institutes of Health, Public Health Service

To continue the investigation of the mitochondrial DNA replication system, DNA binding proteins will be isolated from mitochondria of animal cells using DNA-cellulose chromatography. These proteins may include an RNA polymerase, one or two DNA polymerases (including the polymerase previously studied in partially purified form by Clark Tibbetts), a DNA ligase, and a Wang-type DNA unwinding protein (Wang, 1971). The binding proteins obtained will be assayed for DNA polymerization activity on nicked, gapped, and single-stranded templates. The method of Champoux and D'Auverge (1972) will be used to test for DNA unwinding activity. Particular emphasis will be placed on distinguishing these activities from the corresponding nuclear activities.

References:

*Division of Chemistry and Chemical Engineering, California Institute of Technology.

135. RELATEDNESS AMONG PRIMATE MITOCHONDRIAL DNAs

Investigators: Wesley M. Brown, Jerome Vinograd

Support: National Science Foundation

Mitochondrial DNA (mtDNA) has been prepared from human, chimpanzee and green monkey cell lines. Contour-length measurements in the electron microscope were made on the native DNAs mixed with ΦX174 RF DNA as an internal standard. The three DNAs do not differ significantly, all having a molecular weight of 11 million by this method. The buoyant densities of human and chimpanzee mtDNA are indistinguishable, but the value for green monkey mtDNA is lower by 2.5 mg/ml. The buoyant densities of the separated strands in alkaline CsCl are the same for all three species. The thermal absorbance melting profiles of the three mtDNAs are indistinguishable. Base compositional analysis of both duplex and separated strands shows no difference among the three mtDNAs. The heavy strand is 2.3 times as rich in G and 1.25 times as rich in T as the light strand in all three cases.

Heteroduplex mtDNAs have been formed by mixing and annealing equimolar amounts of the separated complementary strands. Buoyant density analysis of heteroduplexes shows 96% reannealing between human and chimpanzee, but only 73% between human and green monkey and between chimpanzee and green monkey. Thermal denaturation analysis of heteroduplexes, based on Tm lowering, indicated 99+% homology between chimpanzee and human mtDNAs and 71% between human and green monkey. These results are in qualitative agreement with the known phylogenetic relationship of these species. Electron microscope analysis of the heteroduplexes is being carried out at present.

136. BOND SCISSIONS IN ANIMAL CELL MITOCHONDRIAL DNA INDUCED BY EXPOSURE TO ALKALI

Investigators: Lawrence I. Grossman, Robert Watson*

Support: Jane Coffin Childs Memorial Fund for Medical Research
National Institutes of Health, Public Health Service

Mitochondrial DNA from animal cells can be readily isolated in the form of closed circular duplexes. This DNA is well known (reviewed in Borst, 1972) to suffer at least one nick during...
exposure to alkaline pH. The nature of this alkali-sensitive site is currently under investigation.

Mitochondrial DNA from mouse L cells suffers an initial nick at pH 13.0 (20°, in CsCl, ρ = 1.55) with a half-time (t½) of 48 minutes. This represents a rate approximately 100 times faster than would be expected for its molecular weight when compared to closed circular viral DNAs. The t½ for SV40, PM2 and bacteriophage λ DNAs (in buoyant alkaline CsCl, pH 13.0, 20°) range over several days and are in proportion to their molecular weights. The nicking rate for mitochondrial DNA in the range pH 12.4 to 13.0 is proportional to pH. The rate between pH 12.0 and 12.4 has a more complicated pH dependence which appears to correlate with the extent of denaturation of closed circular DNA. The nicking rate of DNA exposed briefly to pH 12.8 and assayed at pH 12.2 is proportional to pH.

A mouse line with mitochondrial DNA in the form of dimers of the monomer circles, and having two initiation sites for mitochondrial DNA replication, was examined. The nicking rate of this DNA is twice as fast as for the monomer DNA.

The presence of ribonucleotides in mitochondrial DNA, possibly arising as unexcised products of initiation of replication, is under investigation. Hydrolysis rates for RNA, presently being determined, tentatively allow the presence of as many as 8 ribonucleotides in mitochondrial DNA. Analysis of the products of hydrolysis, and direct tests for the presence of ribonucleotides, are currently in progress.

Reference:
*Division of Chemistry and Chemical Engineering, California Institute of Technology.

137. THE DEPENDENCE OF THE ALKALINE SEDIMENTATION VELOCITY OF CLOSED CIRCULAR DNA ON THE DEGREE OF STRAND INTERWINDING: THE ABSOLUTE SENSE OF SUPERCOILS

Investigators: Maurice Schmir*, Bernard M. J. Révet, Jerome Vinograd

Support: National Science Foundation
Centre National de la Recherche Scientifique

The sedimentation coefficients of a series of DNAs ranging in degree of supercoiling from zero to 10% have been measured in alkaline CsCl, in which the DNAs are fully titrated. The DNAs ranged in molecular weight from 3 x 10⁶ to 10 x 10⁶ daltons. The sedimentation coefficients, normalized by the appropriate S of the nonsupercoiled circular species, form a linear relation in a plot against the degree of interwinding, over the range 100 to 90%, with a slope of -0.5/10% decrease in degree of interwinding. These results are colinear with the relative sedimentation coefficients computed from the results of Sebring et al. (1971) which cover a range of 80 to 15% interwinding, and with the value calculated for singly interwound single-stranded circles.

An assumption made above, that naturally occurring, closed circular DNAs are negatively supercoiled, has been tested in a viscometric study of closed replicative mitochondrial DNA which contains a small unwound duplex region in the form of a D-loop. Such molecules require a lower concentration of ethidium bromide for relaxation than derivatives obtained upon the removal of the displacing strand and subsequent local renaturation. This result demonstrates that EthBr unwinds the duplex and that the supercoils in naturally occurring DNAs are negative.

Reference:
*Division of Chemistry and Chemical Engineering, California Institute of Technology.

PUBLICATIONS


In the past year, we have pursued our investigations on the expression of the mitochondrial genome and on mitochondrial development in HeLa cells. Studies on the mechanism and products of transcription of mitochondrial DNA have been centered around the problem of the extent of symmetric transcription and that of location of genes in mitochondrial DNA. The relative positions of the 4S RNA genes and of the ribosomal RNA genes in mitochondrial DNA have been determined by electron microscopy.

The amino acid composition of the proteins synthesized in mitochondria and the relationship to the cell cycle of mitochondrial protein synthesis have been investigated.

An analysis of the effects of inhibition of mitochondrial protein synthesis by chloramphenicol on mitochondrial development has shown that HeLa cell mitochondria continue to grow and replicate at a normal rate, and an apparently normal mitochondrial protein synthesizing apparatus continues to be formed, for at least five days of drug treatment. A cytochemical investigation at the electron microscopic level of the distribution of cytochrome oxidase activity in mitochondria of chloramphenicol-treated cells has provided evidence for an interstitial mode of growth of the inner mitochondrial membrane.

An analysis of the capacity of a thymidine kinase-deficient derivative of L cells to incorporate thymidine and 5-BrdU into mitochondrial DNA has unexpectedly revealed the persistence in these cells of a mitochondrial thymidine kinase activity.

Along a different line of investigation, the half-life of cytoplasmic messenger RNA in HeLa cells has been measured in the absence of inhibitors of RNA synthesis and shown to be much longer than previously assumed.

138. THE RELATIVE POSITIONS OF THE 4S RNA GENES AND OF THE RIBOSOMAL RNA GENES IN HeLa CELL MITOCHONDRIAL DNA

Investigators: Madeline Wu*, Norman Davidson*, Giuseppe Attardi, Yosef Aloni

Support: National Institutes of Health, Public Health Service
Dernham Fellowship of the American Cancer Society

In a previous electron microscope study (Biology 1971, No. 171), the relative position of the 16S and 12S rRNA genes in HeLa cell mitochondrial DNA was investigated by examining mitochondrial rRNA-DNA hybrids spread onto a basic protein film by the “aqueous” technique (Davis, Simon and Davidson, 1971). Under these conditions, duplex regions appear as gently curved filaments, while single-stranded DNA segments are collapsed into “bushes.” It was found that the genes for 16S and 12S rRNA are situated adjacent or very close to each other on mitochondrial DNA; the length of the DNA segment possibly separating the two genes was estimated to correspond to less than 500 nucleotide pairs. It was further concluded, from the absence in aqueous spreadings of any observed duplex regions in hybrids with 4S RNA, that not all of the approximately eight 4S RNA genes of the heavy (H) strand are clustered.

In the present experiments, we have analyzed the structure of hybrids of 12S plus 16S mitochondrial rRNA with the H strand and the location of the 4S RNA sites on the H and light (L) strands by the formamide modification of the basic protein film technique: by this procedure, both RNA-DNA duplexes and single-stranded DNA appear as extended filaments with a measurable contour length, and are distinguishable from each
other. In order to identify the individual 4S RNA sites on mitochondrial DNA, we have utilized for hybridization with this DNA mitochondrial 4S RNA covalently coupled to the electron opaque label, ferritin, which is visible in the electron microscope.

Mixtures of HeLa mitochondrial 12S rRNA, 16S rRNA, and/or the 4S RNA-ferritin conjugate were hybridized to separated H and L strands of HeLa mitochondrial DNA, or to a mixture of H and L strands. After spreading the DNA strands by the formamide procedure, the relative positions of the duplex regions corresponding to the 12S and 16S rRNA-DNA hybrids and of the ferritin-labeled 4S RNAs were mapped in the electron microscope.

The observed 12S and 16S duplex regions have lengths of $0.26 \pm 0.04 \mu m$ and $0.46 \pm 0.07 \mu m$, respectively. They are separated by a single-strand region of length $0.047 \pm 0.017 \mu m$, corresponding to $160 \pm 60$ nucleotides. There are nine reproducible binding sites for 4S RNA on the H strand. One such site lies within the spacer region between the 12S and 16S coding sequences; one site is immediately adjacent to the other side of the 12S sequence and one is adjacent to the other side of the 16S sequence. The other 4S sites are rather evenly spaced along the DNA strand of total length 15,600 nucleotides, except that two of them are clustered with a spacing of $120 \pm 30$ nucleotides between them. There are three 4S RNA coding sequences on the L strand, separated from one another by 2280 and 3900 nucleotides.

References:

**139. ISOLATION OF MITOCHONDRIAL RIBOSOMAL RNA GENES FROM HeLa CELLS**

**Investigators:** Giuseppe Attardi, Catherina Tu

**Support:** National Institutes of Health, Public Health Service

As described elsewhere in this report (Wu, Davidson, Attardi, and Aloni, *Biology* 1972, No. 138), the relative positions on the light (L) strand of HeLa mitochondrial DNA of the three 4S RNA genes coded by this strand (Aloni and Attardi, 1971) have been mapped in the electron microscope. This study, however, has not given any information on the distribution of the 4S RNA genes on the L strand relative to the ribosomal RNA and 4S RNA genes on the heavy (H) strand.

In order to provide markers hybridizable with the L strand to map the position of the L strand 4S RNA sites relative to the H strand sites, experiments are being carried out to isolate the H strand sequences coding for the 16S and 12S ribosomal RNA species. The approach followed involves hybridization, under saturation conditions, of purified 16S and 12S RNA with the separated H strand, followed by digestion of nonhybridized DNA with S1 nuclease from *Aspergillus oryzae*, specific for single-stranded DNA, and separation of RNA-DNA hybrids in a CsCl density gradient. The results obtained so far indicate a recovery of fully base-paired RNA-DNA hybrids in the amount expected from the known number of ribosomal RNA genes in mitochondrial DNA from HeLa cells (Aloni and Attardi, 1971).

References:

**140. EXTENT OF SYMMETRICAL TRANSCRIPTION OF MITOCHONDRIAL DNA IN HeLa CELLS**

**Investigators:** Barbara Attardi, Giuseppe Attardi

**Support:** National Institutes of Health, Public Health Service

Previous evidence (Biology 1971, No. 167), based upon the capacity of pulse-labeled mitochondrial RNA to hybridize with both the light (L) and the heavy (H) mitochondrial DNA strands and to form RNase-resistant double-stranded structures after self-annealing, combined with the earlier demonstration of complete transcription of the H strand, had indicated that mitochondrial DNA in HeLa cells is transcribed, at least in part, symmetrically. The observation that the initial rate of labeling with $5^3\text{H}$-uridine of the transcripts of the two mitochondrial DNA strands is about equal, and the sedimentation position of the L transcripts in sucrose gradients, suggested that this symmetric transcription involves a considerable portion of the length of mitochondrial DNA.

In order to determine the actual proportion of the L strand that is transcribed, hybridization
saturation experiments utilizing uniformly labeled L transcripts and isolated L mitochondrial DNA strands are being carried out. As a preliminary to these experiments, a procedure for separating the L transcripts from the bulk of the H transcripts has been developed.

References:

141. MITOCHONDRIAL DNA TRANSCRIPTION IN ISOLATED HeLa CELL MITOCHONDRIA

Investigators: Charles E. Novitski, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service

The observation that mitochondrial RNA (mit-RNA) synthesis is greatly accelerated in the S and especially the G2 phases of the cell cycle indicates the presence of a temporal control of the process (Pica-Mattoccia and Attardi, 1971). To approach the question of whether the transcription of mitochondrial DNA is normally subject to an extramitochondrial positive or negative control element, an assay based on RNA synthesis in isolated mitochondria is being developed.

When isolated HeLa cell mitochondria are incubated at 30°C in the presence of 3H-ATP, salts and sucrose, there is incorporation of radioactivity into RNA which exhibits the discrete species sedimenting at 4S, 12S, 16S and higher S values seen in mit-RNA labeled in growing HeLa cells. The labeled 4S RNA, due mostly to terminal labeling of tRNA is, however, more prominent in the profile of the RNA synthesized in isolated mitochondria than in the profile of mit-RNA 3H-uridine pulse-labeled in vivo.

Treatment of the isolated mitochondria with 1 µg of ethidium bromide/ml results in a 30 to 60% decrease in the labeling of high molecular weight RNA, with little effect on the labeling of 4S RNA. A 2-hour chase with unlabeled ATP following a 60-minute exposure to 3H-ATP results in a 2-fold decrease in total labeled RNA, with little decrease in the relative amount of labeled 4S material.

3H-UTP at the concentration of 0.1 mM is incorporated into labeled RNA with about half of the efficiency of 3H-ATP at the same concentration. The sedimentation profile of 3H-UTP-labeled RNA is very similar to that after 3H-ATP labeling, except for the much smaller amount of labeled 4S RNA with the 3H-UTP. When the 3H-UTP-labeled RNA was annealed (Aloni and Attardi, 1971a) with purified mitochondrial DNA, denatured and immobilized on filters, more than 50% of the RNA hybridized to the DNA. This is a minimum estimate of the proportion of labeled RNA which is transcribed from mitochondrial DNA, since there is very probably formation of mitochondrial RNA-RNA hybrids during incubation, due to symmetric transcription of mitochondrial DNA (Aloni and Attardi, 1971b).

References:

142. STUDIES ON THE CAPACITY OF HeLa CELL MITOCHONDRIA TO INCORPORATE DIFFERENT AMINO ACIDS

Investigators: Paolo E. Costantino, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service
Ente Nazionale Idrocarburi, Italy

Previous RNA-DNA hybridization experiments utilizing purified HeLa mitochondrial 4S RNA and separated heavy and light mitochondrial DNA strands from HeLa cells had indicated the presence of about 8 4S RNA genes in the heavy strand and about 3 genes in the light strand (Aloni and Attardi, 1971). More recently, an electron microscopic investigation of hybrids between mitochondrial DNA strands and complexes of 4S RNA with the electron opaque ferritin have substantially confirmed the above results, indicating the presence of 12 4S RNA sites in the HeLa mitochondrial genome (see Biology 1972, No. 138). These findings pose an upper limit for the number of tRNA species coded for by HeLa mitochondrial DNA, and raise the question of whether the HeLa cell mitochondrial protein synthesizing machinery utilizes only tRNA coded for by mitochondrial DNA, or whether, on the contrary, there is import of tRNA from the
in cytoplasm. In order to obtain an answer to this question, an investigation has been started on the incorporation capacity for the various amino acids of HeLa cell mitochondria. For this purpose, an in vitro mitochondrial protein synthesizing system (Lederman and Attardi, 1970) is being utilized.

**References:**

### 143. THE DEVELOPMENT OF A RECONSTRUCTED PROTEIN SYNTHESIZING SYSTEM UTILIZING THE MITOCHONDRIAL RIBOSOMES OF HeLa CELLS

**Investigators:** Karl E. Espelie, Giuseppe Attardi  
**Support:** Damon Runyon Memorial Fund for Cancer Research  
National Institutes of Health, Public Health Service

In an effort to learn more about the biogenesis of mitochondria, attempts are being made to develop a reconstructed protein-synthesizing system from the mitochondria of HeLa cells. Preliminary work has involved refining the procedure of Attardi and Ojala for the isolation of 60S mitochondrial ribosomes. These ribosomes are currently being used to establish a poly U-directed protein synthesizing system.

Future experiments will involve the use of mitochondrial mRNA from HeLa cells as the message in this in vitro system. Comparison of the protein products obtained in this way, with those produced in vivo (Lederman and Attardi, 1971), should yield information concerning the source of the genetic information for mitochondrial protein synthesis.

**References:**

### 144. MITOCHONDRIAL PROTEIN SYNTHESIS DURING THE CELL CYCLE

**Investigators:** James M. England, Giuseppe Attardi  
**Support:** Dernham Fellowship of the American Cancer Society  
National Institutes of Health, Public Health Service

HeLa cells, synchronized by the mitotic selection technique (Terasima and Tolmach, 1963; Robbins and Marcus, 1964), were pulse labeled with $^3$H-leucine in the presence of emetine [a potent inhibitor of extramitochondrial protein synthesis (Perlman and Penman, 1970)] at various stages of the cell cycle. In parallel control cultures, the $^3$H-leucine pulses were carried out in the presence of chloramphenicol (a selective inhibitor of mitochondrial protein synthesis in eukaryotic cells) in addition to emetine.

The rate of synthesis per cell was found to increase about 2-fold during the cell cycle. This increase did not appear to be linear, with a hint of a more marked acceleration at the beginning of the S-phase. Due to the increase in cellular protein content, the rate of synthesis per unit of protein was fairly constant throughout the cell cycle.

**References:**

### 145. THE VISUALIZATION OF MITOCHONDRIA BY HISTOCHEMICAL REACTIONS

**Investigators:** James W. Posakony*, James M. England, Giuseppe Attardi  
**Support:** National Institutes of Health, Public Health Service  
Dernham Fellowship of the American Cancer Society

In preparation for studies aimed at analyzing mitochondrial nucleic acid and protein synthesis at the cellular level, various histochemical reactions were tested for demonstrating mitochondria in the light and electron microscope. The diamino benzidine (DAB) reaction for cytochrome oxidase activity (Seligman et al., 1968) was found to be useful and was adapted to our cell system and modified to optimize its reproducibility and specificity. Results show that in the light microscope the entire complement of mitochondria in HeLa cells
can be recognized and measured: in the electron microscope every morphologically identifiable mitochondrion reacts with the DAB method.

146. EFFECT OF SELECTIVE INHIBITION OF CYTOPLASMIC OR MITOCHONDRIAL PROTEIN SYNTHESIS ON MITOCHONDRIAL NUCLEIC ACID SYNTHESIS

Investigators: Brian Storrie, Giuseppe Attardi
Support: National Science Foundation
National Institutes of Health, Public Health Service

In both bacteria and eukaryotic cells, nucleic acid synthesis is subject to regulatory mechanisms linked to protein synthesis. In mitochondria, because of the dependence of mitochondrial assembly upon both the mitochondrial and cytoplasmic protein synthesizing systems, this link may be either to mitochondrial or cytoplasmic protein synthesis. In HeLa cells, the observations of Pica-Mattoccia and Attardi (1971, 1972) indicating a restriction of maximal mitochondrial nucleic acid synthesis to a portion of the cell cycle, point to the existence of labile extramitochondrial signals, and suggest a possible close dependence of HeLa cell mitochondrial nucleic acid synthesis upon cytoplasmic protein synthesis.

The effect of selective inhibition of mitochondrial protein synthesis by chloramphenicol, or of cytoplasmic protein synthesis by cycloheximide, upon mitochondrial and, for comparison, total cell nucleic acid synthesis, has been investigated.

Pretreatment of HeLa cells with chloramphenicol for up to 3 days (2.5 generations of cell growth) has little effect on the rate of mitochondrial or total cell nucleic acid synthesis, while synthesis of mitochondrial DNA and RNA is progressively inhibited in cells exposed to cycloheximide, up to 55 and 80% respectively, after 4-hour treatment. Total cell DNA synthesis is stopped almost completely by 15-minute cycloheximide treatment, and total cell RNA synthesis is inhibited by about 50% after 4-hour cycloheximide treatment.

Mitochondrial malate dehydrogenase activity accumulates normally in cells treated with chloramphenicol, whereas cytochrome c oxidase activity is diluted proportionally to cell growth.

References:

147. EFFECT OF SELECTIVE INHIBITION OF MITOCHONDRIAL PROTEIN SYNTHESIS ON HeLa CELL MITOCHONDRIAL GROWTH AND REPLICATION

Investigators: Brian Storrie, Giuseppe Attardi
Support: National Science Foundation
National Institutes of Health, Public Health Service

The results reported above (Biology 1972, No. 146) of continued mitochondrial RNA and DNA synthesis, normal accumulation of mitochondrial DNA and mitochondrial malate dehydrogenase activity in the absence of mitochondrial protein synthesis, imply that either HeLa cell mitochondria are dividing at a normal rate in the absence of mitochondrial protein synthesis, or the amount per mitochondrion of DNA, malate dehydrogenase and presumably other mitochondrial enzymes synthesized in the cytoplasm, and possibly RNA, is increasing, with a probable increase in mitochondrial size.

No irreversible loss of mitochondrial function- ality is found upon growth of HeLa cells for periods up to 4 cell generations in the presence of sufficient concentrations of chloramphenicol to inhibit essentially completely over-all mitochondrial protein synthesis. Cells which have slowed their growth or just entered the stationary phase as a result of the exposure to chloramphenicol, resume growth at a normal rate immediately, or with a short lag, upon removal of the drug. The rate of mitochondrial protein synthesis, measured 1 hour after removal of chloramphenicol, is normal for periods up to 6 to 7 days of drug treatment at either 40 µg/ml or 200 µg/ml. Presumably normal amounts of the protein synthetic machinery are formed.

The average number of mitochondrial profiles per cell, the average size of mitochondria, and the relative amount of cristae are not altered by
prolonged cell multiplication in the absence of mitochondrial protein synthesis. In about 3% of the mitochondrial profiles, after chloramphenicol treatment, the inner mitochondrial membrane is organized into circular or whorled cristae. No circular or whorled cristae are seen in non-drug-treated cultures.

Preexisting cytochrome c oxidase activity, as assayed histochemically by the diaminobenzidine reaction, appears to be diluted uniformly along the inner mitochondrial membrane and the cristae, as the mitochondria proliferate in the absence of mitochondrial protein synthesis. This suggests an interstitial type of growth of the inner mitochondrial membrane.

148. STRUCTURES ACTIVE IN MITOCHONDRIAL DNA, RNA, AND PROTEIN SYNTHESIS

Investigators: Brian Storrie, Giuseppe Attardi

Support: National Science Foundation
National Institutes of Health, Public Health Service

As previously reported (Biology 1971, No. 176; Attardi et al., 1970), the distribution in a dextran-sucrose gradient of mitochondria active in DNA, RNA, and protein synthesis, and containing the bulk of mitochondrial DNA, is displaced to the heavy side of mitochondria as identified by the enzyme marker cytochrome c oxidase. A qualitatively similar distribution can be seen in a sucrose gradient. The distribution of the cytochrome c oxidase-positive structures is unaltered if the enzyme is assayed following digitonin disruption of the mitochondria instead of freeze-thawing disruption. The distribution of mitochondria as identified by a second enzyme, malate dehydrogenase, is intermediate between that of the synthetically-active mitochondria and that of the cytochrome oxidase-positive structures.

All HeLa cells grown in suspension culture appear to be positive for cytochrome c oxidase activity, as assayed histochemically by the diaminobenzidine reaction. The above described results cannot, therefore, be accounted for by cell heterogeneity. Furthermore, the histochemical results suggest that all or most mitochondria are positive for cytochrome c oxidase.

A morphological characterization of structures from portions of the dextran-sucrose gradient is being carried out to determine whether the distributions in such a gradient of mitochondrial synthetic activities, cytochrome oxidase, and malate dehydrogenase are the result of quantitative differences between mitochondria.

Reference:

149. STUDIES ON RNA SYNTHESIS BY RAT BRAIN SYNAPTOSOMES

Investigators: James M. England, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service
Dernham Fellowship of the American Cancer Society

As previously reported, all or almost all RNA synthesized by a rat brain synaptosome fraction in vitro is mitochondrial RNA (mit-RNA) (Biology 1971, No. 174). Electron microscopic studies have revealed that, in addition to synaptosomes, nonsynaptosomal cellular fragments containing mitochondria and a few free mitochondria contaminate this fraction. In order to study mit-RNA synthesis in synaptosomes, it was therefore essential to assess the distribution of in vitro synthesized RNA in these structures. Buoyant density fractionation and electron microscope autoradiography have been used to determine the distribution of radioactive RNA.

Fractionation studies have indicated that free mitochondria did not incorporate $^3$H-uridine into mit-RNA under the incubation conditions used. Due to incomplete fractionation, the distribution of label between synaptosomes and nonsynaptosomal cellular fragments was, on the contrary, equivocal.

Electron microscope autoradiography studies were complicated by the observation of a high background of adventitiously-bound label in material prepared by conventional techniques, due to the high input of labeled precursor and the low level of incorporation. An extensive chase and washing procedure was developed to reduce the background and to permit the localization of stable RNA species synthesized in vitro. Analysis of this material is in progress.
150. PERSISTENCE OF THYMIDINE KINASE ACTIVITY IN MITOCHONDRIA OF A THYMIDINE KINASE-DEFICIENT DERIVATIVE OF MOUSE L CELLS

Investigators: Barbara Attardi, Giuseppe Attardi
Support: National Institutes of Health, Public Health Service

A subline of L mouse fibroblasts LM(TK−) resistant to 5-bromodeoxyuridine (BrdU) was isolated by Kit et al. (1963) by continuous propagation of these cells in medium containing 25 µg/ml BrdU. The cells resistant to BrdU were characterized as deficient in the enzyme thymidine kinase which catalyzes the first step in the utilization of thymidine or BrdU. In the course of previous investigations by the present authors utilizing a clonal derivative (Cl 1D) of LM(TK−) cells, it was observed that mitochondrial DNA (mit-DNA) from this line (which was grown routinely in the presence of 30 µg/ml BrdU to prevent reappearance of thymidine kinase-positive cells) had an unusual sedimentation and density behavior in CsCl gradients, suggesting that BrdU incorporation and, possibly, photochemical nicking had occurred. The interest of these observations prompted a systematic and quantitative investigation of the capacity of Cl 1D cells to incorporate BrdU and 3H-thymidine into mitochondrial and nuclear DNAs.

Mit-DNA isolated from Cl 1D cells grown in the presence of BrdU, and centrifuged in a CsCl density gradient, was found to be heavier than normal mouse mit-DNA and heterogeneous in density, pointing to various degrees of substitution of thymidine residues by BrdU, while nuclear DNA from the same cells formed a relatively narrow band in CsCl in the position expected for mouse DNA unsubstituted with BrdU. The rate of 3H-thymidine incorporation into Cl 1D mit-DNA in different experiments was at least 30 to 60% of the rate of incorporation of this precursor into mit-DNA of A9 cells, an L cell derivative with no thymidine kinase deficiency; under the same conditions, incorporation of 3H-thymidine into nuclear DNA of Cl 1D was less than 1% of that into nuclear DNA of A9 cells. Ethidium bromide at 1 µg/ml inhibited by ~99% the incorporation of 3H-thymidine into the mit-DNA of Cl 1D, in agreement with published results on the effect of ethidium bromide on the incorporation of 3H-thymidine into mit-DNA of HeLa and SV3T3 cells.

References:

151. THE ISOLATION OF CHLORAMPHENICOL AND ERYTHROMYCIN-RESISTANT VARIANTS FROM HUMAN CELL LINES

Investigators: Carolyn H. Mitchell, Giuseppe Attardi
Support: National Institutes of Health, Public Health Service

Mitochondrial protein synthesis in mammalian cells is inhibited by low concentrations of the antibacterial antibiotics chloramphenicol and erythromycin. Variants of the human clone VA2-B (derived from the line WI-18-VA2 transformed by Simian virus 40) resistant to 40 µg/ml chloramphenicol have been isolated from cells treated with the mutagens ethylmethane sulfonate or N-methyl-N'-nitro-N-nitrosoguanidine. Resistance to chloramphenicol in these variants may be due to changes in cell permeability, inactivation of the antibiotic within the cell, or alterations in the mitochondria. In order to determine which variants are resistant to chloramphenicol as a result of altered mitochondrial protein synthesis or mitochondrial membrane permeability changes, the variants are being screened by measuring in vitro protein synthesis in isolated mitochondria by a method developed previously in this laboratory (Lederman and Attardi, 1970). Variants of the human cell line D98/AH2 which are resistant to higher levels of chloramphenicol (200 µg/ml) or to erythromycin (100 µg/ml and 500 µg/ml) are also being isolated, and will be tested for mitochondrial alterations by the above procedure.

Reference:
152. THE MITOCHONDRIAL OLIGOMYCN-SENSITIVE ATPase OF HUMAN CELLS

Investigators: Robert C. Anderson, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service

It has recently been shown in the yeast Saccharomyces cerevisiae that the mitochondrial oligomycin-sensitive ATPase complex consists of approximately 9 distinct polypeptides, 5 of which are synthesized on cytoplasmic ribosomes, the remaining 4 being synthesized on mitochondrial ribosomes (Tzagoloff and Meagher, 1972). (The oligomycin-sensitive ATPase complex is thought to be the enzyme system which catalyzes the last step in the respiratory chain-linked phosphorylation of ADP in mitochondria under physiological conditions.) This discovery has stimulated efforts to study the properties of the ATPase complex in the human cell line D98/AH2, with particular emphasis on the site of synthesis of its components, and on the possible segregation between the nuclear and mitochondrial genomes of genes for these components.

Reference:

153. THE STABILITY OF MESSENGER RNA IN HeLa CELLS

Investigators: William I. Murphy, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service

During the past year we have continued our investigation of the stability of the polysome-associated messenger RNA (mRNA) in HeLa cells. Previously, we have reported the development of a new pulse-chase technique which allows the accurate measurement of the rate of decay of mRNA under physiological conditions, thus avoiding the side effects accompanying the use of such drug inhibitors as actinomycin D (Biology 1970, No. 235). A prerequisite for these studies was the availability of a method for the isolation of mRNA free from other contaminating RNA species, especially ribosomal RNA. Testing a technique which was shown to be suitable for the isolation of mRNA from bacteria (Sedat, Lyon and Sinsheimer, 1969), we have found that the chromatography of RNA isolated from purified polysomes on benzoylated-DEAE cellulose separates the mRNA of HeLa cells from more than 95% of the ribosomal RNA. Using these techniques, the majority of the polysome-associated mRNA in HeLa cells was shown to possess a half-life greater than one cell generation (24 hours). HeLa cell mRNA appears, therefore, to be much more stable than previously reported by others on the basis of experiments involving the use of actinomycin D (Penman et al., 1963).

References:

PUBLICATIONS


Many different physiological processes are known to take place at the interface provided by the plasma membrane. Examination of cells and tissues by electron microscopy and other techniques reveals morphologically specialized regions of the membrane which one hopes to correlate with specific functional roles. Good examples of such morphological specialization are to be found in the cell coat, especially well developed in the apical region of epithelial cells, and at the level of cell junctions, whose role is to provide for attachment of cells to their neighbors, to contribute to the permeability barrier between the "milieu intérieur" and "milieu extérieur" of organisms, and to act as an important locus of cell-to-cell communications.

Most of these morphological specializations are "in the plane of the membrane," and occupy only a small portion of the total cell surface. In order to see whether other regions, which at first inspection are not specialized, are also found to be mosaic, special techniques must be employed. Freeze-cleaving, freeze-etching and shadowing which give one views of large areas of membranes, are therefore used in preference to sectioning. We are also trying to devise means to identify the specific chemical groupings which are expressed on the cell surface at different times. We would like to know whether or not one can also correlate the distribution of such groups with uptake of material by the cells, cell movement, contact inhibition, and other processes. In doing this we are attempting to reconstruct a dynamic image of the cell surface which can be related to cell behavior.

154. DISTRIBUTION OF JUNCTIONS IN VARIOUS TISSUES

Investigators: Jean-Paul Revel, Lily Chang, Phyllis Yip

Support: National Institutes of Health, Public Health Service

The roles of various specialized cell contacts are still far from being clearly understood in many cases. It is, therefore, important to try to establish whether there is a clear relationship between specific functional parameters, and the occurrence (or absence) of particular junctional specializations.

We have paid special attention to the possible correlation between the occurrence of "gap junctions" and the existence of electrotonic coupling between cells. Unpublished work by others on the epididymis had suggested that in the epithelial tubules of this organ such a correlation might not exist. There was physiologically demonstrable coupling, but an apparent absence of gap junctions. Using the powerful tool for cell membrane work which is offered by freeze-cleaving, we were however able to demonstrate the presence of numerous gap junctions.

It would, of course, be of particular interest to find tissues where gap junctions would be absent, but tight junctions present, since this would provide a basis for testing the hypothesis that only gap junctions can mediate electrotonic coupling. One such system could be MDCK kidney cells in culture in which up to the present only tight junctions have been found, whereas gap junctions have proved to be absent. Further experiments are planned to see whether such correlation can be thoroughly documented. One system which we had thought was of some promise in this respect was an analysis of chicken liver. Here, too, preliminary evidence suggested that only tight junctions were present and that gap junctions were absent. Further investigation, however, showed that this tissue did contain gap junctions, albeit only at very low concentrations.

References:
155. CELL JUNCTIONS IN EMBRYONIC TISSUES

Investigators: Lily Chang, Phyllis Yip, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

Circumstantial evidence seems to suggest that a possible morphological basis for electrotonic coupling is represented by gap junctions. The introduction and the increasingly widespread use of freeze-cleaving has shown that it is the method of choice for identification of these junctional contacts, and differentiation from other cell junctions such as tight junctions. For this reason we have decided to reinvestigate embryonic systems where “focal tight junctions” had been believed to mediate physiological coupling. We have now found evidence for the presence of extremely small gap junctions. We believe that these contacts could not be readily distinguished from focal tight junctions in sections, and that only through the use of a goniometer stage (Hyde et al., 1969) could one approach this problem in the past because of the small size of the contacts and the randomness of the plane of sectioning. It is hoped that an understanding of the nature of the junctional contacts which exist in early embryos will help us to understand how specific cell groupings are achieved.

References:


156. CONCANAVALIN A BINDING TO CELL SURFACES

Investigators: Susan B. Smith, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

Some proteins derived from plants have been shown to agglutinate transformed cells but not “normal” culture cells. Because the number of binding sites appears to be approximately the same in both cases it is likely that the distribution of the binding sites may be of great importance in agglutination. Of further interest is the fact that binding sites for the agglutinins seem to be specific carbohydrate groupings on cell surfaces. We have developed a technique for labeling carbohydrate residues on cell surfaces with concanavalin A, which in turn can be reacted with hemocyanin, a visual marker clearly recognizable in shadowcast replicas. Work has been slower than anticipated because we have encountered many difficulties, in particular with nonspecific binding to the glass substrate. Much time was expended in trying to solve this problem which has not yet been completely satisfactorily resolved.

A series of experiments has been carried out with polymorphonuclear leucocytes. Rabbit, but not guinea pig or rat cells, have an invariant distinct heterogeneous pattern at 37°C. In experiments carried out at 0°C the pattern is strikingly different, the label being homogeneously distributed over the cell surface. Cells first incubated at 37°C and then shifted to 0°C displayed the pattern characteristic of 37°C. Fixation with glutaraldehyde, osmium, or both, prevent the pattern change seen as the temperature is shifted. We have shown that BHK cells also have a temperature-dependent pattern, patchy at 37°C and homogeneous at low temperatures. In the presence of metabolic inhibitors (DNP, cyanide, azide) the homogeneous pattern characteristic of low temperature is observed. The effects do not seem to reverse if concanavalin A is added before the azide is removed.

An important stumbling block in interpreting the results is the possibility that the patterns observed are produced by the addition of concanavalin A, and therefore do not represent patterns which exist on the surface of untreated cells. Conflicting evidence is found in the literature but our data seem to favor the idea that concanavalin A may fix the membrane and demonstrate a real pattern reflecting cellular activity. Experiments now in progress in which monovalent concanavalin A is being used will hopefully be clear-cut enough to allow us to settle this point.

References:


157. DISTRIBUTION OF CHARGED GROUPS ON THE CELL SURFACE

Investigators: Pamela S. Hoch, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

Anionic groups play important roles on cell surfaces. They seem to vary in number in different pathological states and have been postulated to play an important role in cell contact and adhesion. We are now studying the distribution of such groups on cell surfaces, using colloidal thorium as a marker. We have found a great deal of variability in the distribution of negatively charged groups at pH 3.0. In the same cultures, some cells are highly reactive and others bind the marker only very poorly. We are presently investigating whether such variations may be correlated with position in the cell cycle. Three general patterns emerge: (1) the anionic groups are homogeneously distributed over the whole cell surface; (2) they are essentially absent from the whole cell surface; or (3) they are present only in some areas of the cell surface, particularly the ruffled membrane. In addition we have evidence for the presence of a microexudate in the form of long strands of material attached to the surface of the cells and also found in the vicinity of the cells on the substrate. Treatment of the preparations with pronase deeply affects the morphology of the cells, but does not seem to change the distribution of the colloidal thorium-binding material to a very great extent. The distribution is still reasonably homogeneous over the whole cell surface, with some indication of slightly increased patchiness. This is surprising because we expected a major portion of the anionic groups to be associated with glycoproteins, presumably attacked by pronase, rather than to glycolipids which would not be affected. Treatment with neuraminidase eliminates much of the binding but not all of it. Presumably it would be possible to differentiate between binding due to sialic acid and binding due to other negatively charged residues by such enzymatic treatment.

References:

158. CELL ATTACHMENT TO SUBSTRATE AND CELLULAR MOTILITY

Investigators: Pamela S. Hoch, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

Previous work has led to the development of techniques which allow one to examine the "underside" of cells, that part of a cell membrane which is facing its substrate (the petri dish). This and other techniques now allow us to examine the site of attachment of the cells to its substrate. We are presently examining cinematographically the behavior of moving cells. We can withdraw and add solutions while under cinematographic examination. In the first series of experiments we are following BHK cells and L cells, then trying to inhibit their movements by incubation in solutions containing dibutyryl cyclic AMP and theophylline, and finally washing out the inhibitors to allow a drop in cyclic AMP level so that the cells resume movement. We can thus examine cell contacts with the substrate in cases where the recent history and behavior of the cell is known, and hope to correlate this with cell movement. Similar experiments using transformed cells are also planned.

References:

159. DISTRIBUTION OF RHODOPSIN IN RETINAL ROD DISC MEMBRANES

Investigators: Lily Yeh Jan, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service
McCallum Fund

Rhodopsin is the major, if not the sole, protein molecule which is associated with the discs of retinal rods. We are investigating the appearance of rods in freeze-etch and other preparations in an attempt to gain information on the distribution of these molecules and whether or not their arrange-
ment changes in various physiological states. Freeze-etching, since it reveals the inside of the cell membrane, should be a particularly favorable tool for studying the distribution of such proteins inside biologically-active membranes.

**PUBLICATIONS**


BIOCHEMICAL EVOLUTION
BIOCHEMISTRY
BIOPHYSICS
DEVELOPMENTAL BIOLOGY
IMMUNOGENETICS

Kerckhoff-Church
(First Basement)
The efforts of our group are all directed toward an understanding of the control of gene expression in higher organisms. We have found that a fruitful way to study this matter is with isolated interphase chromatin which may be transcribed by RNA polymerase to yield transcripts whose nature may be investigated either by RNA-DNA hybridization or by formation of a couple to a ribosomal protein synthesis system.

We already know, and it is generally agreed, that DNA when complexed with histones is not available for transcription by RNA polymerase. Only a portion, 70 to 95% in general, of the DNA of the genome of any particular kind of specialized cell is complexed with histones. 5 to 30% is not thus complexed and is in contrast complexed with nonhistone chromosomal proteins. This has been shown particularly clearly by physical separation of the template-active from the template-inactive portions of the genome as is described in the following reports.

Histone chemistry continues to be an active field and we have collaborated during the past year with Professors Robert DeLange and Emil Smith in determination of the primary structure of arginine-rich histone III. Since our first announcement in 1968 of the primary structure of arginine-rich histone IV and of the high degree of conservation of that structure during evolution, the sequencing of histones has become a vast growth industry. Thus histones IV of pig and rat have been shown to be identical to that of calf. Histone III has been sequenced as noted above. The two principal slightly lysine-rich histones II have been sequenced by two groups. The histones I of the lysine-rich group alone remain to be completed. All histones share the peculiarity that the majority of their basic groups are clustered at one end of the molecule, the hydrophobic amino acids at the other. Clearly histones contain one end for DNA interaction, the other for protein interaction. The former is in part understood (ionic interaction, formation of DNA superhelix by histone-DNA interaction) but the latter is still terra incognita.

The fact that the nonhistone proteins are concentrated on the template-active portion of the genome has encouraged us to intensify our study of this group of chromosomal proteins. We have developed simple but rigorous methods for the isolation in native form of the entire nonhistone chromosomal protein group from respectively rat liver chromatin and pea plant chromatin. With the native proteins in hand we have been able to explore the question of the extent to which they include species which bind sequence-specifically to homologous DNA. We have found that although a majority of the liver nonhistone chromosomal proteins are DNA-binding ones, only a small fraction, approximately 2% of the total, bind sequence-specifically. We are determining the roles of these particular species of nonhistone chromosomal proteins in gene expression.

For study of the molecular events which occur during derepression of the higher organism's genome we use the regenerating rat liver system. After removal of two lobes of the rat liver, the cells of the remaining lobe exhibit renewed cell division and ultimately regenerate the lost liver. Although DNA synthesis starts about 15 hours after partial hepatectomy, many other biochemical alterations in the liver nuclei occur before this time. Thus between 1 and 6 hours after the operation the template activity of the regenerating rat liver chromatin for support of RNA synthesis increases by approximately 40%. This constitutes then a massive turning-on of genes previously repressed. Studies detailed below show that this increase in gene expression is accompanied by a corresponding decrease in histone-DNA ratio of the chromatin. The agent
which is responsible for removal of histones appears to be a chromosomally-bound histone protease which degrades histones to peptides which then fall off of the DNA. The next question is how does the protease know which histones to attack? Next year's report?

We and other groups have earlier reported the presence in chromatin of a small and characteristic class of RNA molecules, chromosomal RNA. Chromosomal RNA molecules are short, 40 to 80 nucleotides in length in different species, contain 8 to 10% dihydropyrimidine and hybridize to an unusually high proportion of the genome, 2 to 5% according to origin of the chromatin. Several groups have reported their inability to find chromosomal RNA in chromatin, due evidently to a variety of technical misadventures. We have therefore expanded and amplified our own procedures for preparation of chromosomal RNA and have published them. Hopefully these will prove helpful to others.

We have found that chromosomal RNA hybridizes only to repetitive segments of the genome and that it is derived from heterogeneous, rapidly-labeled, rapidly-turning-over nuclear RNA. We have previously ascribed chromosomal RNA a role in the sequence-specific interaction of chromosomal proteins and DNA. The relative importance of the parts played in this function by chromosomal RNA and by nonhistone chromosomal proteins is now being assessed.

160. ISOLATION AND CHARACTERIZATION OF THE ACTIVE REGION OF RAT ASCITES TUMOR CHROMATIN

Investigators: Ronald J. Billing, Lynda L. Uphouse

Support: National Institutes of Health, Public Health Service

Gosney Fund

When chromatin from various tissues is used as a template for *E. coli* DNA-dependent RNA polymerase, less than 20% of the chromatin is transcribed (Bonner et al., 1968). By physically separating this active region chromatin from the remaining inactive portion, the differences between these regions can be investigated. Characterization of the active region should provide information concerning regulation of gene expression. This report describes the isolation and partial analysis of the active region of rat ascites tumor chromatin.

Chromatin was incubated under mild conditions with DNase II (porcine spleen). Under these conditions, 60 to 80% of the chromatin is solubilized. Only 10 to 12% of the chromatin, however, remains soluble in 1.2 mM MgCl₂ in which histone-covered DNA is insoluble. That this is actually the chromatin transcribed *in vivo* is shown by labeling with radioactive precursors of RNA. 80 to 90% of the labeled nascent RNA remains in the supernatant with 10 to 12% of the chromatin.

Rat ascites chromatin was fractionated and analysis made of histone and nonhistone content. The histone to DNA content of the active region dropped 40 to 50% relative to the unfractonated chromatin. A similar increase in the histone to DNA ratio was found in the inactive region. This is to be expected if histones act as gene repressors.

A slight increase in the nonhistone to DNA ratio was observed in the active region with a corresponding decrease in the inactive region. Whether this nonhistone difference represents a functional distinction between the two regions or is an artifact depending on chromatin purification procedures is currently being investigated.

Reference:

161. DISTRIBUTION OF PROTEIN IN CHROMATIN

Investigator: Ronald J. Billing

Support: National Institutes of Health, Public Health Service

Gosney Fund

The main components of rat ascites cell chromatin are DNA, histone protein and nonhistone protein in the ratio by weight 1.0, 1.15, 0.70. The histone proteins are thought to play important roles in (1) maintaining the supercoiled structure of chromatin, and in (2) the repression of most of the genome. As the proportion of DNA transcribed is usually less than 20% it has been postulated that histones cover more than 80% of the chromatin DNA making it unavailable for transcription.
However, Clark and Felsenfeld (1971) have disputed this model claiming that 50% of the chromatin DNA is free from protein. Part of their experimental evidence came from the fact that over 50% of the chromatin DNA could be degraded by nucleases to acid-soluble products without any release of chromatin proteins. I have tried to show that it is not possible to determine the amount of the DNA which is free from protein using these conditions because the released protein aggregates and appears in the pellet fraction.

Rat Novikoff ascites cell chromatin was digested for various lengths of time with deoxyribonuclease II (DNase II). After centrifugation of the digest the degraded DNA appears in the supernatant. It was found that even when 95% of the DNA was degraded less than 6% of the protein appeared in the supernatant. In order to prevent aggregation of released protein the digest was centrifuged in the presence of 1.0 M urea, 0.2 M sodium phosphate pH 7.2. Under these conditions protein is released with degraded DNA into the supernatant. The release of histone is linear with that of DNA suggesting that histone is fairly evenly distributed along the chromatin DNA.

Reference:

162. FRACTIONATION OF RAT ASCITES CHROMATIN

Investigators: Ming-Ta Chong, Jung-Rung Wu

Support: McCallum Fund
National Institutes of Health, Public Health Service

We tried to separate the template-active chromatin from the inactive portion in rat ascites tumor cells. First, ribonucleoprotein-free ascites cell nuclei were isolated and sonicated in 0.25 M sucrose solution. Low speed centrifugation was used to get rid of unbroken nuclei and debris, and high speed centrifugation to pellet template-inactive chromatin (Ch 1), then NaCl solution was added to the remaining supernatant fraction to pellet template-active chromatin (Ch 2). Template activity was compared by using exogenous E. coli RNA polymerase. Ch 2 is 3-fold more active than Ch 1. The chromosomal protein was extracted with sodium dodecyl sulfate (SDS), and compared in SDS polyacrylamide gels. More histone was found in template-inactive than in template active chromatin (especially rich in histone IIa and IIb). Several nonhistone proteins not found in inactive chromatin are present in active chromatin. Chemical determination of histone and nonhistone confirmed results of the SDS gel pattern (NH/H ratios in template-inactive and active chromatin were 0.88 and 1.06 respectively). Further studies will attempt to isolate DNA from these two chromatins and compare their proportion of redundant and unique sequences.

We have asked the question do chromosomal proteins bind selectively to particular redundancy classes of DNA? Rat ascites DNA, sheared to an average length of 350 nucleotides, was denatured and fractionated into highly repetitive, middle repetitive, and unique DNA fractions. Each DNA fraction was then reconstituted to whole chromosomal protein. We find that the highly repetitive DNA binds more chromosomal protein by a factor 1.4 than the unique DNA, the middle repetitive DNA being intermediate.

163. STUDIES OF PEA SEEDLING HISTONES

Investigators: Jerry D. Johnson, Tom St. John*

Support: National Institutes of Health, Public Health Service
National Science Foundation

Chromatin has been isolated from seedlings of Pisum sativum by differential centrifugation. Extraction of this chromatin with 0.4 N H2SO4 solubilizes the histones plus a large amount of nonhistone protein. The separation of histones from nonhistone proteins and also a partial resolution of the various histone subfractions can be achieved by chromatography on the cation exchange resin Bio-Rex 70. The acid-soluble proteins are applied to columns of this resin in 0.1 M phosphate buffer (pH 6.8) containing 8% (w/v) guanidine hydrochloride (GtCl) and 0.1 M β-mercaptoethanol. The majority of the nonhistone proteins do not bind to the Bio-Rex 70 under these conditions and are collected as a runoff fraction. The proteins bound to the column are then eluted with a linear gradient of 8 to 13% GtCl in the phosphate buffer containing β-
mercaptoethanol. Three fractions of histone are partially resolved; the lysine-rich histones (I), slightly lysine-rich histones (IIb1 and IIb2), and the arginine-rich histones (III and IV). A fourth fraction containing principally nonhistone proteins plus a small amount of the arginine-rich histones can be eluted by washing the Bio-Rex 70 column with buffer containing 40% GuCl. This presents an improvement over previous uses of this system in that, in the presence of β-mercaptoethanol, the arginine-rich histones are eluted as a discrete fraction at about 12% GuCl. In the absence of mercaptoethanol these histones are not eluted from the resin until the GuCl concentration is increased to 40%. Under these conditions the arginine-rich histones are contaminated with a substantial amount of nonhistone protein which is also released from the column by the very high concentration of GuCl. When eluted at the lower GuCl concentration (in the presence of β-mercaptoethanol) the histone III and IV mixture contains no detectable contaminants.

The isolation of the individual histones was achieved by gel filtration of the fractions resolved by Bio-Rex 70 chromatography. A 5 x 150 cm column of Sephadex G-100 developed with 0.01 M HCl was found to be capable of resolving histone I from all other subfractions. The slightly lysine-rich histones, IIb1 and IIb2, eluted at the same position as histone III from these columns and hence could not be separated from this contaminant. The arginine-rich histones, III and IV, were cleanly separated from one another into the two respective fractions by gel filtration.

The various histones, prepared as described above, are now being studied in collaboration with several other laboratories. These investigations include: (1) a characterization of the subfractions present in the lysine-rich histones from pea seedlings and a partial study of the amino acid sequences of these proteins; (2) the further purification and complete amino acid sequence determination of the slightly lysine-rich histones, IIb1 and IIb2; (3) the complete amino acid sequence of histone III; and (4) X-ray crystallography studies of histone IV and histone IV-DNA complexes.

The knowledge of structural relationships involved in histone conformation and also in histone-DNA complexes which will be generated by these studies should help toward an understanding of the biological functions of these proteins.

*Undergraduate, California Institute of Technology.

164. PREPARATION OF PURE NONHISTONE CHROMOSOMAL PROTEINS IN NATIVE FORM

Investigators: Hendrikus W. J. van den Broek, Larry D. Noodén, J. Sanders Sevall

Support: The Netherlands Organization for the Advancement of Pure Research (Z.W.O.)
National Institutes of Health, Public Health Service
Damon Runyon Memorial Fund for Cancer Research

The nonhistone chromosomal proteins (NHCP) comprise a large percentage of the total protein of chromatin, but their biological roles are poorly understood. Much of this lack of knowledge stems from the difficulties encountered in preparing the NHCP in native condition and free of histones (Marushige, Brutlag and Bonner, 1968; Elgin and Bonner, 1970). We have developed a procedure for the preparation of histone-free NHCP from rat liver chromatin without the use of harsh pH conditions, denaturing agents (i.e., urea, guanidinium chloride) or detergents. We find that 3 M NaCl at pH 8 completely dissociates chromosomal proteins from DNA. These two components can be separated by filtration chromatography in a large pore gel (Bio-Gel A-50m). The chromosomal proteins are then concentrated by precipitation in 95% saturated (NH₄)₂SO₄. The histones can be removed by adsorption on a cation exchange resin (Bio-Rex 70). It is, however, necessary to run this step in 0.4 M NaCl in order to avoid adsorption of nonhistones to the Bio-Rex and to break up histone-nonhistone complexes which prevent adsorption of some of the histones. Considerable effort was made to use anion exchange resins (i.e., DEAE, QAE, AE cellulose) to purify and concentrate the NHCP but this turned out to be unsatisfactory because the adsorbed NHCP included histones and much of the NHCP did not stick. The chromosomal proteins were characterized at each stage of the procedure by polyacrylamide gel electrophoresis. The removal of the histones was monitored by electrophoresis of samples in acidic urea gels (pHs 4.3 and 3.2). The NHCP were examined by electrophoresis in pH 8.9 urea gels and sodium dodecyl sulfate (SDS)-tris gels. The SDS-tris gels in particular indicate a very high degree of heterogeneity and a wide range of
molecular weights.

NHCP prepared as described above are soluble in low ionic strength buffer. Previous reports of their insolubility may have been caused by the presence of contaminants as histone and/or DNA.

165. CHARACTERIZATION OF NONHISTONE CHROMOSOMAL PROTEINS BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED DNA

Investigators: J. Sanders Sevall, Hendrikus W. J. van den Broek, Larry D. Noodén

Support: Damon Runyon Memorial Fund for Cancer Research
The Netherlands Organization for the Advancement of Pure Research (Z.W.O.)
National Institutes of Health, Public Health Service

Nonhistone chromosomal proteins (NHCP) from rat liver chromatin have been isolated in the native state without harsh treatment by acid or base. Characterization by sodium dodecyl sulfate (SDS) gel electrophoresis indicates they are a highly heterogeneous group of proteins with a wide range of molecular weights. It has been frequently suggested that NHCP may be involved in the specificity of transcription and/or the structure of the chromosome. To investigate the role of NHCP, we have fractionated the species in our preparation according to their affinity for heterologous (E. coli) DNA or homologous (rat liver) DNA, both immobilized on a cellulose matrix.

Three fractions of NHCP are obtained when the total NHCP are passed over a tandem series of E. coli DNA and rat liver DNA cellulose. The unbound fraction, which has no affinity for either DNA, is highly heterogeneous and shows much similarity to the total protein. Another highly heterogeneous protein fraction which binds to E. coli DNA cellulose makes up 30% of the total NHCP. A specific DNA-binding fraction, which binds only to rat liver DNA, makes up 4% of the total NHCP and is characterized by a predominance of low molecular weight proteins by SDS gel electrophoresis.

The specificity of the homologous DNA-binding fraction was confirmed by two further methods. First, the protein was rechromatographed over heterologous and homologous DNA cellulose in which case 60% of the protein recovered was bound to the homologous DNA. Second, the membrane filtration technique of Riggs et al. (1968) (for isolation of labeled DNA-protein complexes) was used to test the affinity of the homologous protein-binding fraction for rat liver DNA. Competition studies with the latter technique indicate that the homologous binding fraction has a considerable preference for rat liver DNA over E. coli DNA.

References:

166. THE ISOLATION AND CHARACTERIZATION OF DNA-BINDING PROTEINS FROM EUKARYOTIC CELLS

Investigator: Jerry D. Johnson

Support: National Science Foundation
National Institutes of Health, Public Health Service

The existence of proteins which have the capacity to reversibly bind specific base sequences of a DNA polymer and to regulate the transcription of a specific portion of the genome have been well documented in prokaryotic organisms. The existence of similar proteins in eukaryotes has often been suggested but never unequivocally demonstrated. Such regulatory proteins are known to be present in vanishingly small amounts, 10 to 100 copies per cell, under normal conditions in prokaryotes. If the same is true of eukaryote cells, the problem of finding an assay of sufficient sensitivity to detect them is a formidable obstacle.

To identify and partially purify proteins which bind to DNA in a sequence-specific manner, the technique of DNA-cellulose chromatography has been chosen. Proteins from liver and Novikoff hepatoma ascites cells of rats were labeled in vivo with either 14C- or 3H-amino acids to afford a preliminary assay for the isolation of DNA-binding proteins. This approach has been refined to include a method for the isolation of labeled DNA-protein complexes. The characterization of these complexes is currently under way.

References:
proteins. The radioactive proteins prepared from these tissues were then passed over a series of columns containing first cellulose only, then E. coli DNA, calf thymus DNA, and finally rat DNA immobilized on cellulose. The cellulose column served to remove any insoluble material and also any proteins which would bind to the cellulose. The amount of such material in these preparations was always less than 0.2%. The proteins which bound to the various DNA-cellulose columns in low ionic strength buffers, 50 mM NaCl, were eluted by increasing the salt concentrations to 2.0 M. It was found that 20 to 22% of the soluble proteins isolated from rat liver nuclei were capable of binding to DNA while only about 2% of the soluble proteins from the cytoplasmic fraction of these cells were adsorbed on DNA cellulose. In ascites cells, approximately 8% of the total cellular protein was retained by the DNA-cellulose columns. The fraction of these proteins which passed through columns containing heterologous DNA but which subsequently bound to that from its tissue of origin should contain the proteins capable of sequence-specific binding. To test this hypothesis, these proteins were rechromatographed through a second series of DNA-cellulose columns to see if that fraction which bound to homologous DNA initially is enriched in sequence-specific binding proteins. This was also done with those proteins which bound to heterologous DNA to afford a comparison. The rechromatography showed that in all cases greater than 90% of the proteins which had bound to DNA in the first chromatography had retained their capacity for DNA binding. Comparing the ratios of proteins bound to heterologous and homologous DNA in the rechromatography showed that the fraction which initially bound to homologous DNA, after passing through a column of heterologous DNA, was, when compared to those proteins which first bound to the heterologous DNA, enriched 5- to 7-fold in proteins apparently capable of sequence-specific binding. Estimated from protein removal, the over-all purification of the initial unfractionated preparation is approximately 5000-fold. However, since only 20% of the final preparation was bound to homologous DNA the actual purification is probably closer to 1000-fold. Further characterization of the DNA-binding properties and heterogeneity of these proteins is in progress.

An additional fraction of the soluble proteins, about 1% of the total, binds to the DNA-cellulose columns and cannot be removed by buffer containing up to 4.0 M NaCl. However, these proteins can be eluted by treating the columns with DNase indicating that they are in fact bound very tightly to the DNA and are not either adsorbed to the cellulose or aggregated in the columns. Additional studies on this fraction are also being carried out.

167. ISOLATION AND CHARACTERIZATION OF CHROMOSOMAL PROTEINS FROM PEA TISSUES

Investigators: Paul P. Lin, Ralph F. Wilson
Support: National Institutes of Health, Public Health Service
The Goodyear Tire and Rubber Company
Union Carbide Corporation

Past biochemical and biophysical studies of isolated chromatin have shown that chromosomal proteins play a crucial role in the structure of chromatin and in the control of gene activity. Of these proteins, the histones have been most extensively studied and characterized while the study of nonhistone chromosomal proteins is still in an early stage. It has been suggested that nonhistone proteins may be candidates for all of the biological roles that histones seemingly have not fulfilled, especially the specific control of gene expression. It is our objective to isolate chromosomal proteins in their native state and to study their biological functions. In the present investigations, methods for the isolation and chemical and physical properties of chromosomal proteins have been developed.

After dissociation of chromatin in a buffer containing 3 M NaCl, 5 M urea, 1 mM NaHSO₃ and 10 mM tris, pH 8.0, the urea is removed by dialysis, and the chromosomal proteins completely separated from DNA by Bio-Gel A-50 column chromatography. Nonhistone proteins are then separated from histones on a Bio-Rex 70 (-COOH) ion exchange column in the presence of 0.35 M NaCl, 1 mM NaHSO₃, 5 M urea and 10 mM tris-acetate buffer, pH 7.2. The nonhistone proteins can then be concentrated on a DEAE-cellulose column after removal of salt and urea. By this procedure, all of the nonhistone proteins have been qualitatively but not quantitatively recovered as judged by SDS and urea gel electrophoresis.
As expected, nonhistone proteins co-precipitate with histones. Such complexes once formed can, however, be gradually solubilized by increasing the concentration of NaCl. The DNA-binding properties of the pea nonhistone proteins have been studied. Pea DNA has a finite number of binding sites for the nonhistone proteins of developing pea cotyledon. At saturation binding, the mass ratio of protein/DNA is about 0.17. An input mass ratio of protein/DNA of about 20 is required to give half-maximal binding. In vitro transcription by E. coli RNA polymerase is apparently not significantly affected by nonhistone proteins either with pea DNA or pea chromatin as template. The molecular weights of the major pea nonhistone proteins lie between 12,000 and 100,000. There are no qualitative differences in the major nonhistone proteins isolated from various pea tissues; bud, embryo and growing cotyledon. Amino acid analyses indicate that the ratios of \( \frac{(\text{Glu} + \text{Asp})}{(\text{Lys} + \text{Arg} + \text{His})} \) are 1.49, 1.59 and 1.76 for the nonhistone proteins isolated from pea embryo, pea bud and pea cotyledon, respectively. Finally, several enzyme activities have been found in the isolated nonhistone proteins preparation.

References:

168. GENE ACTIVATION IN REGENERATING LIVER

Investigator: William T. Garrard
Support: Damon Runyon Memorial Fund for Cancer Research
National Institutes of Health, Public Health Service

The process of liver regeneration offers a model system for the study of gene activation. Extirpation of two-thirds of the organ results in the triggering of the remaining hepatic tissue to undergo proliferation. The first wave of DNA synthesis occurs 18 to 20 hours after the operation, followed by mitosis at 24 to 28 hours. The proportion of liver chromosomal DNA available for transcription increases by ca. 40% within 12 hours after the operation. By studying the temporal changes in the chromatin of such cells we hope to discover more about how genes get turned on.

Chromatin samples from regenerating rat livers at different stages of development have the following chemical compositions:

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>No. of preparations</th>
<th>Histone/DNA</th>
<th>Nonhistone/DNA</th>
<th>RNA/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>1.13 ± 0.02</td>
<td>0.57 ± 0.12</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>sham</td>
<td>4</td>
<td>1.12 ± 0.05</td>
<td>0.62 ± 0.13</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>1.5</td>
<td>3</td>
<td>1.10 ± 0.05</td>
<td>0.57 ± 0.06</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0.96 ± 0.04</td>
<td>0.51 ± 0.13</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>0.97 ± 0.02</td>
<td>0.63 ± 0.16</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>0.97</td>
<td>0.48</td>
<td>0.04</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>0.89</td>
<td>0.53</td>
<td>0.03</td>
</tr>
<tr>
<td>44</td>
<td>2</td>
<td>1.02</td>
<td>0.68</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The most striking change observed is a rapid 14% decrease in the histone:DNA ratio within 3 hours after partial hepatectomy.

The histone and nonhistone chromosomal proteins from the above chromatin samples have been analyzed by urea and SDS polyacrylamide gel electrophoresis respectively. No significant change in the relative amounts of the five major histone classes occurs during development. Therefore, the 14% decrease in the histone:DNA ratio is not due to the preferential dissociation of a single histone species. However, a decrease in the amount of a minor histone component, histone A, occurs in 44-hour regenerating tissue.

Careful examination of the polypeptide chain profiles of the nonhistone chromosomal proteins displayed on SDS gels reveals about 55 bands, with molecular weights ranging from less than 15,000 to greater than 180,000 daltons. During development no new polypeptides were detected, although fluctuations in the relative amounts of individual components occur.
169. CHARACTERIZATION OF RNA POLYMERASES FROM NOVIKOFF HEPATOMA ASCITES TUMOR

Investigator: Stanley C. Froehner

Support: National Institutes of Health, Public Health Service

RNA polymerase from rat ascites tumor has been resolved into three activities (Ia, Ib, and II) by phosphocellulose and DEAE-cellulose chromatography. Ia and Ib are most active at low ionic strength with Mg++ on native DNA, while II prefers high ionic strength, Mn++ and denatured DNA. All three are sensitive to rifamycin SV, but only form II is inhibited by a-amanitin. These data, along with their elution positions on DEAE cellulose, indicate that forms Ia and Ib are localized in the nucleolus while II is nucleoplasmic (Roeder and Rutter, 1970). All three enzymes are stimulated by addition of the DEAE-cellulose runoff protein fractions, designated R01a-11 and R01b. Neither R01a-11 nor R01b contain polymerase activity and the stimulating factor is sensitive to heating, contrary to earlier reports (Biology 1971, No. 192). Ia and Ib are inactive at low enzyme concentrations, even in the presence of BSA, suggesting that the active complex dissociates at low concentrations. Addition of R01a-11 or R01b (each factor stimulates either enzyme equally well) eliminates the "cooperative" effect of enzyme concentration, suggesting that the factor may be a subunit of the enzyme.

Reference:

170. MOLECULAR STRUCTURE OF TWO NUCLEOLAR RNA POLYMERASES FROM NOVIKOFF HEPATOMA ASCITES TUMOR

Investigator: Stanley C. Froehner

Support: National Institutes of Health, Public Health Service

The nucleolar RNA polymerases (Ia and Ib) of rat ascites tumor have been purified by ammonium sulfate precipitation, phosphocellulose chromatography, DEAE-cellulose chromatography, and "high-salt" sucrose density gradient centrifugation. The sucrose gradient profiles show coincidence of the protein and polymerase activity peaks, suggesting that the enzymes may be homogeneous. Ia migrates as a single component on polyacrylamide gels under nondenaturing conditions. SDS polyacrylamide gels of Ia show a complex structure: polypeptides of 170,000, 125,000, 69,000, 49,000, 44,000 and 37,000 daltons are present in equimolar ratios except for the 69,000 and 37,000 dalton "subunits" which may be present twice per enzyme molecule. These molar ratios remain constant across the sucrose gradient activity peak as would be expected if the polypeptides sedimented as a single complex. The most likely structure for polymerase Ib is two polypeptides of 190,000 and 135,000 daltons, each present twice per enzyme complex, since the active enzyme has a molecular weight of about 600,000 (the same as Ia). Thus, these two form I RNA polymerases are physically distinct enzymes and have different molecular structures from form II (nucleoplasmic) polymerase from rat liver (Weaver, Blatti and Rutter, 1971).

Reference:

171. BIOPHYSICAL STUDIES ON TETRAHYMENA NUCLEAR DNA

Investigator: Jung-Rung Wu

Support: National Institutes of Health, Public Health Service

DNA was prepared from purified nuclear fraction of an amicronucleate strain of T. pyriformis W cells (generation time about 3 hours in the culture medium used). When total nuclear DNA was melted in 0.12 M phosphate buffer, the Tm was approximately 77.5°C and optical density increased 40% (hyperchromicity) from its native state. Its buoyant density (g/cm^3) determined by CsCl density gradient centrifugation was p = 1.688.

The DNA renaturation profile, measured by OD in a Gilford 2400, indicated that 85 to 90% of nuclear DNA was unique (single-copy) sequences
with a Cot 1/2 of 210. 8% of DNA was highly repetitive DNA with a Cot 1/2 less than 10^{-2} and redundancy of greater than 2 \times 10^{3} copies. If there was any middle repetitive DNA, it constituted no more than 2 to 4% of the nuclear DNA, with a Cot 1/2 of 10 and a redundancy of ca. 25.

The haploid nuclear genome size calculated from reannealing profile was about 2.4 \times 10^{13} g, twice that of Drosophila melanogaster.

Since Tetrahymena is a nondifferentiated eukaryote and lacks significant amounts of middle repetitive DNA in its genome (compared with 10 to 20% in Drosophila and rat ascites cells), it may indicate a correlation between cell differentiation and presence of middle repetitive DNA.

172. ORGANIZATION OF CHROMOSOMAL DNA IN EUKARYOTES

Investigators: Jung-Rung Wu, Ming-Ta Chong, James Bonner
Support: McCallum Fund
National Institutes of Health, Public Health Service

Repetitive DNA sequences constitute a sizable portion of the eukaryotic genome. They can be divided into highly repetitive DNA and middle repetitive DNA according to their different reannealing rates after denaturation. In most organisms, ca. 10% of total nuclear DNA reanneals very rapidly, is termed highly repetitive DNA and is of a redundancy ranging from tens of thousands to a million copies. Purified highly repetitive DNA, when denatured, reannealed and mounted for electron microscopy by standard aqueous method, yields a high proportion of circles. This repetitive region is therefore in tandem arrangement, that is, is serially repetitive, and it is known to be clustered in the centromeric area.

Middle repetitive DNA makes up to 10 to 20% of some eukaryotic genomes, such as Drosophila and rat ascites cells. Its redundancies range from as few as tens to thousands of copies. We have found that the middle repetitive DNA basic segments of Drosophila are short, perhaps around 150 to 200 base pairs in length. They are scattered throughout the genome which contains on the average one short, middle repetitive sequence per ca. 750 base pairs of single-copy DNA.

A different approach was also used by us to determine the size and distribution of middle repetitive DNA in rat ascites cells. A low molecular weight nuclear RNA which hybridizes with the middle repetitive DNA of the genome (see Biology 1971, No. 194) was prepared and coupled with ferritin. The RNA-ferritin complex was hybridized to ascites DNA and then prepared for electron microscopy. We measured the distances between the bound ferritin and found that they are located as close to one another as 100 to 300 nucleotides, and that there is another peak at from 500 to 1200 nucleotides. We conclude that in ascites, like Drosophila, middle repetitive DNA is also short and probably scattered throughout the genome. Two such short regions may sometimes be arranged in tandem and these are spaced by 500 to 1500 nucleotides of single-copy DNA. This is approximately the size of a structural gene.

From the information obtained in Xenopus DNA by Professor Eric Davidson’s group at Caltech, cyclization of eukaryotic DNA (Thomas et al., 1970), and our findings, we are in favor of the following model: the basic unit contains a middle repetitive DNA of 150 to 200 base pairs, and a unique (single-copy) DNA segment of structural gene size. They are distributed throughout the genome with the following several variations: (1) more than one piece (i.e., two or three) of middle repetitive DNA clustered together; (2) more than one piece of unique DNA clustered together; (3) many pieces of middle repetitive DNA; and (4) unique DNA only. As for highly repetitive DNA, located in the centromeric region, it is apparently serially repetitive.

References:

173. RENATURATION STUDIES ON DROSOPHILA DNA

Investigator: Stephen E. Harris
Support: National Institutes of Health, Public Health Service

Drosophila melanogaster DNA sheared to a fragment size of 300 nucleotides was used in the following study. The DNA was denatured and reannealed in 0.12 M PB (phosphate buffer), at
60°C to a Cot of 5. The DNA was then applied to hydroxyapatite column at 35°C and a thermal gradient was run to 60°C to remove single-stranded DNA. Duplex DNA was then removed from the column with 0.48 M PB. 28% of the DNA was in this bound fraction. This bound DNA (Cot 5 DNA) was then diluted to 0.12 M PB, denatured, and reannealed to a Cot of 0.003. The DNA was then applied to a hydroxyapatite column at 35°C and thermal gradient again run to 60°C. The highly repetitive DNA was then removed with 0.48 M PB. This constituted 35% of the input DNA; thus, 65% of the DNA (or 18% of the total) remained single-stranded. This single-stranded fraction is termed middle repetitive.

Single-copy DNA was prepared by incubating the unbound DNA from the first hydroxyapatite column to a Cot of 30 (60°C, 0.12 M PB) and collecting the unbound fraction from a hydroxyapatite column. Kinetic (by hydroxyapatite) and physical properties of these DNA fractions were then studied.

The single-copy DNA reannealed with second-order kinetics with a rate constant of 0.0097 (Cot 1/2 = 103) and when a bound fraction of this single-copy DNA was collected and melted, a rather sharp melting profile was found with a Tm of 83.5°C (native, main-band Tm = 85°C). However, the hyperchromicity was only 65% that of native DNA. CsCl density centrifugation of this single-copy-bound DNA had a p = 1.707 g/cc (native, p = 1.703; denatured, p = 1.716). The hyperchromicity and density analysis would indicate that these DNA structures have 60 to 70% duplex or double-stranded structure.

Similar analysis of the middle repetitive DNA indicate that 60% of this DNA renatures with single second-order kinetics with a rate constant of 3.28 (Cot 1/2 pure = 0.3). The melting profile of a bound fraction of this DNA gave a somewhat broader melting profile with a Tm of 80.5°C. The hyperchromicity and density analysis also indicate that these middle repetitive DNA structures have 60 to 70% duplex structure. Denatured middle repetitive DNA has a density the same as that of denatured main-band DNA and thus the 3°C lower 
Tm relative to single-copy DNA is probably due to its short base-paired regions and possibly to base-pair mismatch.

174. THE ORGANIZATION OF THE TRANSCRIPTIONAL UNIT

Investigator: David S. Holmes

Support: Lucy Mason Clark Fund
National Institutes of Health, Public Health Service

It has been shown that the middle-repetitive sequences of Drosophila DNA are about 150 base pairs in length and are distributed throughout the genome an average of one per unique segment. Preliminary evidence indicates that rat DNA might be similarly organized. If this is true then rat giant nuclear RNA (HnRNA), ca. 2 to 4 x 10^4 bases long, which is transcribed from such DNA will also contain unique sequences interspersed among repetitive sequences. Alternatively HnRNA might be transcribed from a small portion of the genome not organized in the above manner. For example, it might be transcribed from DNA containing exclusively repetitive or exclusively unique regions or from DNA containing both repetitive and unique regions but not ordered in the above way. The following study was designed to determine the classes of sequences present in HnRNA and to investigate their intramolecular organization within HnRNA molecules.

Purified HnRNA, prepared from rat ascites by a method involving phenol extraction of a nuclear lysate followed by a size separation of RNA using Sepharose 2-B and sucrose gradient sedimentation velocity has the following properties. It sediments heterogeneously with an s20,w > 45 and under denaturing conditions sediments slightly faster than the large ribosomal RNA (rRNA) of rat ascites. It has a compact structure as visualized in the electron microscope when spread from formamide. It has a broad melting profile with a hyperchromicity of 18% and a Tm of 56°C in 0.12 M phosphate buffer pH 6.8. It is concluded that HnRNA probably consists of molecules larger than rRNA and with considerable secondary structure possibly with intramolecular base pairing.

In an RNA-driven reaction, HnRNA hybridizes to more than 3% of denatured homologous DNA, and in a DNA-driven reaction about 40% of the recorded hybridization is to middle-repetitive DNA and the remainder to unique DNA. Therefore HnRNA contains both populations of sequences which are repetitive and which are unique. The question of whether repetitive and unique se-
quences are covalently linked in an HnRNA molecule and the distribution of such sequences along an HnRNA molecule is currently being studied by a combination of hybridization techniques and electron microscopy.

175. STUDIES ON POLYRIBOSOMES AND mRNA ISOLATED FROM RAT LIVER

Investigator: William R. Pearson

Support: National Institutes of Health, Public Health Service

Studies of heterogeneous rapidly-labeled nuclear RNA (HnRNA) and messenger RNA (mRNA) have indicated a precursor-product relationship, but the proportion of HnRNA which ends up in polyribosomes (polysomes) engaged in protein synthesis is quite small. Studies on isolated mRNA have been initiated to determine if there are structures in the message which may shed light on the selection of HnRNA molecules for use as message. Various theories of gene regulation have implications for the structure of mRNA. Georgiev et al. (1972) has proposed that there are regulatory sequences on the 5' and 3'-end of mRNA which the model of Britten and Davidson (1969) would allow for regulatory sequences on the 5'-end (if any such sequences are present). Tomkins et al. (1969) argues for a mechanism which protects presumptive mRNA from ribonuclease digestion after transcription. Present methods for mRNA isolation have been able to distinguish mRNA from ribosomal RNA both by sedimentation velocity and by the fact that polyadenylic acid sequences are present on some messages. The former technique allows for high purification only in the case of relatively homogeneous mRNA populations (e.g., hemoglobin message) while the latter assumes all message is bound to poly A.

A population of bound and free polysomes has been isolated from rat liver cells. Message has been labeled with a pulse of $^3$H-orotic acid (uridine precursor). The mRNA can be released from the polysomes by incubation with puromycin (Blobbel and Sabatini, 1971). CsCl preparative buoyant density analysis of rat liver polysomes separates the material into two bands of $\rho = 1.66$ and $\rho = 1.55$. When free polysomes are selected for in the isolation procedure only one band is found at $\rho = 1.66$. When pulse-labeled polysomes are incubated with puromycin all of the radioactivity associated with the heavier band is released and pelleted in the CsCl gradient. Radioactivity in the less dense band is not released. Thus mRNA can be released and separated from free polysomes by incubation with puromycin and banding in CsCl. This procedure, coupled with selection of polysomes in sucrose gradients before incubation and selection of low S-value material after dissociation should provide mRNA with low contamination from other RNAs.

References:

176. IS CHROMOSOMAL RNA REALLY TRANSFER RNA OR RIBOSOMAL RNA?

Investigator: David S. Holmes

Support: National Institutes of Health, Public Health Service

Lucy Mason Clark Fund

Previous reports from this laboratory have described the preparation and properties of a class of chromosomally-associated RNA molecules (cRNA) from a variety of tissues and organisms. The principal distinguishing features of cRNA are its small but variable size, high sequence diversity, high content of dihydrouracil and association with a protein.

Recently two reports in the literature have maintained that cRNA is an artifact of preparation resulting from contamination by transfer RNA (tRNA), ribosomal RNA (rRNA) or their breakdown products. It has been necessary, therefore, to review the current procedures used in this laboratory for the preparation of cRNA, and to repeat and extend the published work on the properties of cRNA.

Table 1 summarizes some of the properties of rat Novikoff ascites cRNA and compares these properties with those of tRNA and rRNA from the same tissue.

Clearly cRNA can be distinguished from
purified tRNA and rRNA. Furthermore, cRNA is unable to compete against either tRNA or rRNA in reciprocal hybridization competition experiments indicating that cRNA does not contain any hybridizable sequences found in either tRNA or rRNA. This makes it unlikely that cRNA is either degraded tRNA or rRNA.

The procedures for making cRNA start with chromatin (chromosomes). Thus the majority of the cellular rRNA and tRNA has already been removed. Even if labeled rRNA (in the form of ribosomal subunits) and tRNA are added to the chromatin, and the latter processed for cRNA, then less than 1% of the label is recovered with cRNA (the amount of the exogenously added rRNA and tRNA has been normalized to the quantity of these species normally found in ascites cells).

The present results, together with the data of previous workers, make it unlikely that cRNA from rat ascites cells is related to tRNA, rRNA or their degradation products.

**References:**


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**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>cRNA</th>
<th>tRNA</th>
<th>rRNA</th>
</tr>
</thead>
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<td>GC content (mole %)</td>
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<tr>
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<td>++</td>
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<tr>
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<tr>
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<tr>
<td>Chromatography on methylated albumin kieselguhr</td>
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</table>

- not determined
++ good resolution from cRNA
+ partial resolution from cRNA
0 not resolved from cRNA

---

**177. THE SEQUENCE DIVERSITY OF CHROMOSOMAL RNA FROM WHOLE RAT**

*Investigators:* Shane Allwright*, David S. Holmes

*Support:* Lucy Mason Clark Fund
National Institutes of Health, Public Health Service

The findings that new sequences of chromosomal RNA (cRNA) are produced during rat liver regeneration, and that different tissues of the same organism contain different sequences of cRNA, are consistent with the idea that the sequence diversity of cRNA is positively correlated with genetic activity.

The following experiment was designed to investigate further this correlation. It is probable that the pooled tissues of an organism contain a greater variety of active genes than any single tissue. Therefore, we isolated cRNA from whole newborn rats by the deoxycholate (DOC) method of Mayfield and Bonner (1971, 1972) and compared its sequence diversity with that of cRNA isolated from a variety of single rat tissues.

cRNA was labeled *in vitro* with $^{3}$H-dimethylsulfate and its sequence diversity tested by RNA-driven hybridization saturation to denatured rat DNA immobilized on nitrocellulose filters. cRNA from whole rat hybridized to 2.7% of the DNA, while cRNA from rat liver and kidney each hybridized to 2% and cRNA from Novikoff ascites hybridized to 5%.
The interpretation of this experiment is made difficult by two features: (1) we do not have a measure of the efficiency of the DOC method for extracting cRNA from whole rat; and (2) the conditions of hybridization used would not permit the detection of sequences of cRNA present at low concentration, yet possibly containing a significant amount of information.

References:
*NSF Summer Trainee, 1971.

178. POLY A SEQUENCES ON GIANT NUCLEAR RNA

Investigators: Gary A. Frankel*, Douglas W. Ridder*, David S. Holmes

Support: Lucy Mason Clark Fund
National Institutes of Health, Public Health Service

We have found that approximately 81% of the radioactivity associated with pulse-labeled giant nuclear RNA (HnRNA) is retained on nitrocellulose membrane filters under conditions in which the filters retain poly A. Control experiments show that the retention is due to poly A, and that poly A constitutes about 2% of the $^{3}$H-adenosine counts of HnRNA or about 0.4% of the $^{32}$P counts. Preliminary evidence indicates (1) that the poly A sequences are covalently linked to HnRNA molecules, (2) that HnRNA molecules are on average 2 to $4 \times 10^{4}$ nucleotides long, (3) that poly A sequences are on average 200 nucleotides long, and (4) that 0.5% of the mass of HnRNA is poly A. We estimate that there is on the average, about one poly A sequence per HnRNA molecule.

We are mapping the number and distribution of poly A sequences on HnRNA by hybridization with poly dT and visualization of the resulting poly dT-A duplex with the electron microscope.

*Undergraduate, California Institute of Technology.

179. THE RECONSTITUTION OF PEA EMBRYO CHROMATIN

Investigators: Paul P. Lin, Ralph F. Wilson

Support: National Institutes of Health, Public Health Service
The Goodyear Tire and Rubber Company
Union Carbide Corporation

It has been reported earlier that salt and urea-dissociated chromatin can be specifically reconstituted by gradient dialysis. This conclusion was based on the results obtained from hybridization competition in which only reiterated sequences were hybridized. To further examine the specificity of chromatin reconstitution, we ask: (1) what is the chemical composition and template activity of reconstituted chromatin compared to that of native chromatin? (2) can chromosomal proteins be reconstituted to DNA sequence specifically? and (3) is RNA transcribed from reconstituted chromatin similar to that transcribed from native chromatin of similar chemical composition and template activity?

Reconstitution was done according to the methods of Shih and Bonner (1970) and Spelsberg, Stiegles and O'Malley (1971), without the presence of 1 mM MgCl$_2$. It has been found that chromosomal proteins once dissociated cannot be quantitatively reconstituted to chromatin. Only 10 to 15% of the reconstituted chromatin is recovered in a form which is similar to native chromatin with respect to sedimentation behavior, chemical composition, and template activity. Using E. coli RNA polymerase for in vitro transcription (high salt), RNAs made from native pea embryo chromatin and pea embryo chromatin reconstituted simply by dissociation and reassociation of native chromatin are similar as judged by hybridization competition, while RNA made from pea embryo chromatin reconstituted from purified chromosomal components is not identical to that made from native pea embryo chromatin, as judged by hybridization competition. These were done at 37°C for 48 hours in 5X SSC and 50% formamide solution.

References:
180. STUDIES OF THE DISSOCIATION OF NATIVE AND RECONSTITUTED PEA EMBRYO CHROMATIN

Investigators: Paul P. Lin, James Bonner
Support: National Institutes of Health, Public Health Service
The Goodyear Tire and Rubber Company
Union Carbide Corporation

We ask the question: are proteins when they are dissociated from chromatin and then reassociated to DNA by gradient dialysis from salt and urea, as we have previously described, bound to DNA in the same manner as they are in native chromatin? To this end we have dissociated chromatin of pea embryos in varied concentrations of NaCl and in the presence of 1 mM NaHSO₃ and 10 mM tris buffer pH 8. We have separated the partially deproteinized chromatin from the dissociated proteins by differential centrifugation. The dissociated proteins were further separated into histone and nonhistone classes by chromatography on the weak cation exchanger Bio-Rex 70.

The over-all result is that chromatin dissociated in various NaCl concentrations, 0.2, 0.4, 0.6, 1.0 and 2.5 M, is different from reconstituted chromatin with respect to the salt concentration required to remove the two principal protein classes. About 50% of nonhistone proteins and 50% of histones of native chromatin can be extracted, respectively, with 1.2 M and 1.4 M NaCl. With reconstituted chromatin, however, 50% of nonhistone and histone proteins, respectively, are removed from DNA by 0.45 M and 0.70 M NaCl. The template activity of pea embryo chromatin for RNA synthesis as compared to pea DNA is the same for 0.4 M NaCl-dissociated and reconstituted chromatin as for that of 0.8 M NaCl-dissociated native chromatin, namely 50% of that of DNA. Thermoliquation profiles of native and reconstituted chromatin suggest also that the binding of chromosomal proteins to DNA is looser in reconstituted chromatin than in native chromatin.

It would appear therefore that our present methods of reconstitution of chromatin do not result in material exactly identical to native chromatin.

181. HYBRIDIZATION STUDIES OF RNA TRANSCRIBED FROM LIVER CHROMATIN

Investigators: Maurice Dupras, Andrew F. Cockburn*
Support: Medical Research Council of Canada
National Institutes of Health, Public Health Service

Rat liver chromatin is 5 to 10 times less efficient than pure DNA as a template for in vitro transcription by E. coli RNA polymerase. This is an indication that only a portion of the genome is expressed in chromatin. We have taken a closer look at the products of in vitro transcription of chromatin and of DNA by various hybridization techniques. Labeled RNAs were synthesized on each template, purified and hybridized to DNA under various conditions. When an excess of labeled RNA was added to a small amount of DNA trapped on filters, it was found that the RNA transcribed from DNA can occupy 10% of all available sites on DNA. The RNA transcribed from chromatin hybridized, in the same conditions, to only 5% of DNA. This suggests that a greater variety of RNA sequences are transcribed from DNA, although not as great as one would expect from the relative template activity of chromatin. It must be noted, however, that only repetitive RNA is analyzed by our technique. We then analyzed the chromatin and DNA transcripts by hybridization competition. A saturating amount of labeled RNA transcribed from DNA is challenged for hybridization by increasing amounts of unlabeled RNA transcribed from chromatin. Under these conditions RNAs made from these templates behave as if they contain identical sets of RNA. This seems in contradiction to template activity measures and to hybridization saturation results. A possible explanation for the discrepancy could be that the RNA transcribed from chromatin is richer in repetitive sequences than the DNA transcript. To test this hypothesis we used another hybridization technique, that of DNA excess hybridization. This technique permits direct measurement of the proportions of repetitive and unique RNA in a given mixture. Contrary to our expectations, the proportion of repetitive sequences is only 10% in chromatin transcripts, compared to 20% for RNA
Because of these unexpected results, the question of the specificity of the hybridization is raised. Our technique might allow so many mismatches that gross differences would not be detected. Indeed our RNA-DNA hybrids have broader melting curves and lower Tm's than would be predicted if matching were perfect. Our hybridization is not totally unspecific, however, because RNA transcribed from pea DNA competes very poorly with RNA transcribed from rat liver DNA.

We cannot discard entirely the possibility that the E. coli RNA polymerase cannot correctly read mammalian templates. As a matter of fact, we have examined the RNA transcribed from DNA and chromatin templates by the homologous rat liver RNA polymerase. We found that unlike the E. coli RNA polymerase situation, both chromatin and DNA transcripts contain about 20% of repetitive sequences. This is indirect evidence that the two enzymes do not function identically in vitro.

The present results obscure the interpretation of previous findings about chromatin reconstitution (see Biology 1971, No. 209).

*Undergraduate, California Institute of Technology.

**PUBLICATIONS**


Johnson, J. D. and Bonner, J. (1972) Histone structure and function. *Scienza Tecnica.* Accepted for publication.


We call our group “Immunogenetics” because our predominant concern is with the immune system—its nature, development, and uses—and because we look at things primarily from the vantage point of genetics, frequently using genetic tools in our research. During the past year we have worked with a variety of organisms—human, the rat, the mouse, the rabbit, the pigeon—and on occasion used a guinea pig, a sheep, a fish, a cow, or a worm; yeast or tumor viruses and marker-material from snails or horses. We have worked with specific markers on cell surfaces, especially one elaborated by cells as they go through the thymus, and during postnatal development of the brain; specificities on cattle and pigeon red cells, on peripheral lymphocytes, on cells from a variety of human and animal tumors and from human embryos, in the bursa of Fabricius and the thymus of pigeons. We have used pigeon eggs to study material they transmit from mother to developing squab, to protect the squab against infections; examined the effects of antibodies on the behavior of nematodes, and the composition of immunoglobulins from many different species of vertebrates. We have looked in the sera of people with particular diseases for types of antibodies that may be useful diagnostic agents; mapped a gene controlling a thymus specificity in the mouse and evaluated a possible relationship between this gene and leukemia incidence; hybridized pigeon lymphocytes with mouse cells.

The following abstracts, and the list of publications at the end, tell briefly about many of our explorations. It has been a good year.

182. A SEARCH FOR CYTOTOXIC ANTIBodies AGAINST ALLOGENEIC AND AUTOLOGOUS THYMOCyTES IN THE SERUM OF PATIENTS WITH MYASTHENIA GRAVIS (MG)


Support: Smith, Kline and French
U.S. Atomic Energy Commission
The California Chapter of the Myasthenia Gravis Foundation
National Institutes of Health, Public Health Service

In our continued effort to define a specific serological marker for human thymocytes we have investigated the possibility that cytotoxic antibodies against allogeneic and autologous thymocytes may occur in the serum of patients with Myasthenia Gravis (MG). The fact that antibodies to striated muscle, thyroid cells, nervous tissue and myoid epithelial cells of the thymus have been demonstrated in the serum of patients with MG led us to question whether or not antibodies to the alloantigens of thymic lymphocytes might also be present in association with this disease. The sera from 69 patients with MG have thus far been investigated. These sera were assayed for antibodies against thymocytes from 8 aborted fetuses, age 10 to 21 weeks, and two adults, age 23 and 52 years respectively. Twenty of the serum-donor patients had thymectomy before the study and of these 6 had thymomas. In addition, 7 of the patients underwent thymectomy during the course of this study and their thymocytes also were used in the testing. Two of these had thymomas. The patients ranged in age from 14 to 81 years. There were 50 females and 19 males. All ABO blood groups were represented. Of these sera only one demonstrated cytotoxic activity against 30 to 40% of the thymocytes from one of the thymic donors. This patient had previously received multiple transfusions which negates the significance of this finding. The reactivity most likely was against HLA specificities although this possibility was not tested.

An antiserum prepared in rabbits against human fetal thymocytes was shown to be cytotoxic for 80 to 90% of the thymic lymphocytes from 17 donors of varying ages. These findings are in keeping with the suggestion that MG may be due to antibodies directed toward
epithelioid rather than lymphoid thymic components.

Reference:

183. INVESTIGATIONS ON THE USEFULNESS OF A RABBIT ANTI-HUMAN THYMOCYTE SERUM (RAHTS) FOR SPECIFICALLY DETECTING THYMIC-DEPENDENT LYMPHOCYTES

Investigators: Ronald T. Acton, Maurice P. Barcos*, Robert Lukes*, John Parker*

Support: Smith, Kline and French
U.S. Atomic Energy Commission
National Institutes of Health, Public Health Service

It is now well established that there are at least two distinct populations of lymphocytes. One population is termed "thymic-dependent" lymphocytes (T-cells). Bone marrow precursor cells require the microenvironment of the thymus or thymic hormonal substances, in order to differentiate into these cells. This group of lymphocytes has to do with cellular immunity and has not been shown to secrete antibodies. The other population (B-cells) is derived from bone marrow precursor cells which receive their induction for maturation from an unknown source which presumably is analogous to the bursa of Fabricius found in birds. These cells are responsible for humoral immunity and either secrete antibody or have immunoglobulin molecules attached to their cell surface. In mice there are alloantigenic markers found on the cell surface which can be used to distinguish the two populations. These are termed the "theta antigen" for the T-cells and "plasma cell antigen" for B-cells.

Unfortunately, antisera against alloantigens which can be used as markers for the T- and B-cell populations in man have not been developed. However, we have raised an antiserum in rabbits against human fetal thymocytes (RAHTS). This antiserum is being tested as a possible reagent for detecting human T-cells. The results look promising in that the antiserum will kill 80 to 100% of the human thymocytes from all donors tested thus far in a cytotoxicity test. The antiserum will kill about 70% of human peripheral lymphocytes. Absorption with the thymocytes from any of the donors removes activity against all other thymocytes. The antiserum will not kill the cells from patients with chronic lymphocytic leukemia, all of which have immunoglobulin on their cell surface.

Although much more testing is needed, we are working on the hypothesis that this antiserum can be used to detect human T-cells. Another intriguing possibility is that RAHTS may be useful in determining the origin of various malignant cells.

184. STUDIES ON LEUKEMIA INCIDENCE AND THETA TYPE IN THE AKR/Cum AND AKR/Jax STRAINS OF MICE

Investigators: Ronald T. Acton, Tommy C. Douglas, Elizabeth P. Blankenhorn

Support: Smith, Kline and French
U.S. Atomic Energy Commission
National Defense Education Act
National Institutes of Health, Public Health Service

In the past the standard procedure for preparing anti-theta serum involved reciprocal immunization of strains AKR and C3H with thymocytes (Reif and Allen, 1964). Since these two strains of mice are matched at the major histocompatibility locus, relatively monospecific anti-theta serum could be obtained. In a report by Schlesinger and Hurvitz (1969), it was shown that AKR/Cum mice, which presumably had an origin common with AKR/Jax mice, expressed the C3H-theta antigen. Because AKR/Cum mice are homozygous for \( \theta^{C3H} \) while AKR/Jax are homozygous for \( \theta^{AKR} \), the two substrains should provide a better combination for producing theta antisera.

AKR/Cum mice were immunized with six weekly intraperitoneal injections of AKR/Jax normal thymus cells and bled for antiserum on the seventh week. The pooled antiserum was specific for \( \theta^{AKR} \) and had a high titer, comparable to C3H-anti-AKR serum. Other AKR/Cum mice were injected with thymus-derived leukemic cells from
the AKR/Jax strain following the same protocol. Notably, these AKR/Cum mice did not develop leukemia even though the transplanted AKR/Jax cells were viable and were actively secreting Gross leukemia virus. Three possibilities are offered for explaining this provocative observation: (1) AKR/Cum mice differ at histocompatibility loci and were rejecting the leukemic cells, (2) AKR/Cum mice are different at other genetic loci besides theta and this influences susceptibility to the leukemia virus, (3) Theta acts as a transplantation antigen. To test the first possibility we made arrangements with Dr. Edward A. Boyse at the Sloan-Kettering Institute for Cancer Research, New York, for his lab to type the thymocytes of the two strains of mice at additional loci coding for cell surface markers. As can be seen in Table 1, these two strains differ at the theta locus, but are identical with respect to a number of other cell surface antigens. Another interesting observation is that the AKR/Cum thymocytes also type positive for Gix and GCSA, two thymocyte cell surface markers indicative of Gross virus infection. We have not observed a high incidence of leukemia in the AKR/Cum strain, but observations over longer periods of time will be necessary before statistically sound data are available.

Further studies in collaboration with Dr. Jo Hilgers at the Netherlands Cancer Institute have concentrated on assaying the spleens of both strains for mouse leukemia virus group-specific (MULV-gs) antigen. This would give some insight as to the time at which the two strains begin to express the Gross virus on the thymocyte cell surface. So far, there appears to be no difference in the expression of MULV-gs antigen, both strains expressing the antigen throughout life.

We have recently found that the AKR/Cum and AKR/Jax sublines express different isozymic forms of the cytoplasmic malic enzyme. The gene coding for this enzyme is linked to theta. Genetic studies now in progress may give some insight into whether leukemia susceptibility is associated with the theta locus or genes closely linked to it.

References:

Table 1. Cell Surface and Biochemical Markers

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<th>Marker</th>
<th>AKR/Jax</th>
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<tr>
<td>θ-AKR</td>
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<td>-</td>
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<tr>
<td>θ-C3H</td>
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<td>+</td>
</tr>
<tr>
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<td>+</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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185. PRELIMINARY STUDIES ON SOLUBILIZATION OF THETA ANTIGEN FROM MOUSE BRAIN AND THYMUS

Investigators: Ronald T. Acton, Elizabeth P. Blankenhorn
Support: Smith, Kline and French
U.S. Atomic Energy Commission
National Institutes of Health, Public Health Service

The theta alloantigen of mice is a cell surface component found on thymocytes and in brain tissue. Quantitatively and qualitatively the theta antigen reflects the differentiation state of the respective cells. Although theta has recently been detected on epidermal cells, less is known about its relationship to differentiation in epithelium.

The fact that theta appeared on tissues as diverse as thymus and brain led us to investigate the chemical properties of this component. Several mouse leukemic cell lines have been analyzed for theta and shown to vary tremendously with respect to the cell surface expression. However, an ascites tumor termed L#2 which is passaged in A/He mice has been shown to contain as much theta per cell as appears on normal thymocytes. This tumor was obtained from Dr. Emma Shelton of the National Cancer Institute, where it has been carried since 1952.

Studies have therefore been initiated to learn the most suitable means of solubilizing theta from the L#2 tumor carried in A/He mice as well as the
brain from the same animals. Initially the hypertonic salt elution procedure was used and it was found to effectively elute the theta antigen from both tissue types. The eluted material was solubilized with sodium dodecyl sulfate and starch stearate. Although theta activity could be demonstrated in the eluted and solubilized material, the material was of high molecular weight; it was excluded through Sepharose 6-B. Also aggregates formed upon storage, preventing further studies. Another method of preparing the antigen was therefore sought. The 3 M KCl salt elution procedure was tried and found to be very useful in eluting soluble material from cell membranes which contained the theta antigen. The material eluted from both tissue types is still very large and is excluded through Sephadex G-200.

Inhibition studies have shown that the theta preparation from the two tissue types differs somewhat. The brain theta has a higher specific activity; lower concentrations of the theta extract from brain inhibit the cytotoxicity test more effectively than do extracts from the tumor.

Studies are now in progress to prepare large quantities of theta from brain and tumor for physical and chemical characterization.

References:

186. LOCATION OF THE GENE FOR THETA ANTIGEN

*Investigators:* Elizabeth P. Blankenhorn, Tommy C. Douglas

*Support:* U.S. Atomic Energy Commission

The theta antigen is a thymus- and brain-specific cell membrane constituent in mice. It has been recognized as a very useful marker for the elucidation of the differentiation states of thymus-dependent lymphocytes, and also for the physical separation of cells bearing this antigen by use of complement-mediated cytotoxic methods. The expression of theta is regulated by an autosomal gene with two known codominant alleles: $\theta^{AKR}$ and $\theta^{C3H}$. Reciprocal immunizations may be made with thymocytes between animals of either type to produce antibodies against either allelic product.

We are currently studying the genetic linkage of $\theta$ in the mouse. Previous studies have placed the gene on linkage group II, chromosome 9 (Itakura et al., 1971), at a distance of 16.8 ± 3.6 units from the dilute (d) coat color marker in the middle of the chromosome. To locate the $\theta$ gene more precisely, we are looking at recombination between the $\theta$, Trf, and Mod-1 loci in the second linkage group. The transferrin locus (Trf) has been shown to be at a position 13 units from d, Mod-1, the gene coding for a soluble, NADP-dependent malic enzyme (E.C. 1.1.1.40) which is found in the cytoplasm of mouse kidney cells, has been placed at a distance of 4 or 10 (Itakura et al., 1972) units from d. Our data show recombination frequencies of 11.3 ± 2.0% between Mod-1 and Trf, and of 22.9 ± 3.1% between Mod-1 and $\theta$. The recombination frequency between Trf and $\theta$ is 38.3 ± 3.1%. These results indicate that the gene order is as follows:

```
| Trf | 11.3 | Mod-1 | 22.9 | $\theta$ |
```

(d) (lu) (lu is a gene with skeletal effects included in the above map to identify the orientation of our markers with reference to other known loci of chromosome 9.)

In the three-point cross, the double recombinant class was absent, as is consistent with the above model. Another marker segregating in our cross is H-14, a red blood cell antigen coded for by an autosomal gene with two codominant alleles. H-14 has not previously been localized to any linkage group, and does not appear to be linked to any of the markers on linkage group II as determined by our data. Further studies on the linkage of $\theta$ in the mouse are being carried out.

References:
187. STUDIES ON RAT TISSUE ANTIGENS

Investigator: Tommy C. Douglas

Support: National Defense Education Act
         U.S. Atomic Energy Commission

The genetically defined $\theta$-antigen of mice provides a specific marker for those lymphocytes derived from the thymus. In addition to its presence on lymphocytes, the $\theta$-antigen is expressed in brain tissue. Some of the technical difficulties encountered in attempts to localize this antigen more precisely might be overcome if a similar antigenic system could be defined in another species. Such a finding might also be useful for structural and functional studies of the immune system. To these ends, thymus and brain antigens of the rat have been studied.

Thymus cells from rats of 8 different inbred strains were found to strongly absorb antibodies directed against the $11$-AKR, but not the $11$-C3H antigen. The former antisera were also toxic for rat thymocytes in the presence of complement whereas the latter were not.

Rat brain tissue specifically absorbs anti-$\theta$-AKR antibodies while other nonlymphoid tissues show much lower levels of such absorption. Little or none of the $\theta$-like antigen is present in neonatal rat brain. Between birth and age 3 weeks, an approximately logarithmic increase in antigen expression occurs. Following this stage, rat brain $\theta$-like antigenicity reaches a fairly stable level which persists throughout life.

The observed patterns of immunological cross-reactivity, organ distribution, and developmental appearance indicate that an antigenic system similar to the $\theta$-antigen of mice indeed exists in the rat.

188. BURSA AND THYMUS-SPECIFIC ANTIGENS OF THE PIGEON

Investigator: Susan L. Melvin

Support: National Institutes of Health, Public Health Service
         U.S. Atomic Energy Commission

The obvious differences between unlike organs must reflect different degrees and states of differentiation. In the case of the thymus and bursa of Fabricius, both are primary lymphoid organs but each plays a distinctly different role in the ontogeny and function of the avian immune system. Histologically, lymphocytes from either source are practically indistinguishable.

Antisera which might distinguish these organs were raised in rabbits. The rabbits were neonatally tolerated with other pigeon tissues before antigen challenge to reduce the antibody directed against common pigeon antigens. Unabsorbed sera still showed reactivity against pigeon serum, red blood cells, and saline extracts of many organs, as well as against suspensions of living bursacytes and thymocytes. Both soluble and membrane antigens were looked for. Test systems were chosen to take advantage of different properties of antibody populations including ability to precipitate or agglutinate the corresponding antigen and ability to fix complement.

One antiserum was chosen for more extensive study. Both this antibody and the antigen extracts were concentrated. Absorption of the antibody with pigeon serum left in reactivity against thymus, bursa and brain extracts. Tissue extracts were standardized for comparison to a constant OD$_{280}$ level. Immunodiffusion indicates a single thymus-specific antigen and a second antigen shared by bursa, thymus and brain, but not serum. The absence of the thymus antigen on brain suggests that this antigen is different from the mouse antigen, theta, which is present in high levels on mouse brain. Work is in progress to further define this serologic specificity, its organ and species distribution. Other work indicates that organ-specific antigens are similar and conserved in different species.

This thymus antigen appears to be a solubilized membrane antigen. It is soluble at 105,000 x g and is dialyzable. In immunoelectrophoresis it is found in the region of the well. It is relatively stable to freeze-thawing and does not stain for lipid. Different membrane solubilization techniques are being tested for antigen yield.

References:
189. SOMATIC CELL HYBRIDIZATION OF PIGEON LYMPHOCYTES

Investigator: Susan L. Melvin

Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

Cells of the thymus (T-cells) and bursa of Fabricius (B-cells) differ in their ability to secrete antibody. B-cells normally produce low levels of immunoglobulin (lg) in vitro; T-cells do not. In collaboration with Dr. Ray Teplitz at the City of Hope, attempts are being made to prepare B- and/or T-cell hybrids with a mouse thymidine-kinase-deficient cell line. B- and T-cells attach to the mouse cell line to a high degree after treatment with Sendai virus, 1-octadecyl-glycerophosphoryl-choline or trypsin-cold shock. The cells fail to undergo division or survive in selective medium. Medium has been collected in these initial stages of the hybridization procedure for determination of Ig levels. Heterokaryon formation and cell hybridization have been reported to activate antigen production by chick erythrocyte nuclei, whereas hybridization of mouse myeloma cells causes a severe reduction in Ig production by these cells. Hence the production of a particular cell product may be either enhanced or depressed after somatic cell hybridization. There are no reported data, either quantitative or qualitative, on the effect of hybridization on Ig production by normal cells.

References:

190. A DEVELOPMENTAL ANTIGEN ON SQUAB ERYTHROCYTES

Investigator: Susan L. Melvin

Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

An antigen is detectable on squab red blood cells (rbc) but not on adult rbc by direct agglutination with a properly absorbed rabbit antiserum. The antigen is present on the rbc of all tested embryos from 10 to 12 days of incubation until 3 days post-hatch. Adult rbc remain nonagglutinable after incubation with allantoic fluid, squab serum, saline extracts of egg white, egg yolk or crop milk. These results indicate that the antigen may be a cellular component rather than a secondarily attached specificity as in some other antigen systems. Loss of the antigen during development can occur either by failure to synthesize the antigen or by masking the antigen. Induced anemia reveals hidden specificity in some systems, but in this case rbc from anemic birds are negative. The only other type of pigeon cells tested, bursa and thymus cells from older squabs, show negligible reactivity to the antiserum in both agglutination and cytotoxic assays.

References:

191. CELL SURFACE TOPOLOGY OF BOVINE RED CELLS

Investigator: Suzanne O. Rosenberg

Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

Bovine red cells display over 50 serological specificities on their surface, grouped into 10 genetic complexes. One of these, the B complex, is responsible for over 30 specificities. The chemical basis for these specificities is not known. A mapping of these antigens on the cell surface may yield some information on the structure of these molecules, as well as define the topological relationships of various cell surface antigens.

Preliminary electron microscopic studies using conventional ferritin-labeled antibodies and bovine red cells (kindly supplied by Dr. C. Stormont of the University of California at Davis) which are identical in all the blood group complexes except the B complex, indicate that the B complex antigens are loosely clustered on the cell surface. It was felt that conventional ferritin labeling did not give a totally accurate picture of the cell surface, however, and therefore studies are presently being undertaken using the hybrid antibody method of
electron dense labels for localizing molecules on the cell surface using the electron microscope. Rabbit hybrid antibodies with specificities for bovine immunoglobulin and horse spleen ferritin, and bovine immunoglobulin and Busycon canicularis hemocyanin (from Dr. J.-P. Revel) have been prepared. Using these two electron microscopically distinguishable labels, I will be examining the topological display of B complex antigens vs. the antigens of a second complex, the C complex; the placement of the subspecificities within the B complex in relation to each other; and the cell surface distribution of bovine J substance which is a soluble blood group antigen that is present in the plasma and which adsors to the red blood cell surface.

Reference:

192. MAINTENANCE OF TRANSFERRIN POLYMORPHISM IN PIGEONS
Investigator: Jeffrey A. Frelinger
Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

Transferrin, a nonheme iron-binding protein, is polymorphic in most vertebrate species that have been examined. In pigeons it is controlled by an autosomal gene, with two known codominant alleles, TFA and TFβ. The two alleles are found in nearly equal frequencies and the three genotypes are at Hardy-Weinberg equilibrium in all populations studied. Ovotransferrins [both in crude egg white (Biology 1971) and purified protein] from heterozygous females inhibit microbial growth, using yeast as an assay organism, better than either of the homozygous types, or a mixture of homozygous types. Heterozygous females hatch a larger percentage of their eggs than homozygous females (Biology 1970). This difference is probably accounted for by the transferrin effect. The failure of the mixture of the homozygous types to act like the heterozygous type calls into question the currently accepted structure of transferrin as a monomeric protein. The greater fecundity of heterozygous females can account for the maintenance of transferrin polymorphism in pigeons.

193. THE MOLECULAR STRUCTURE OF PIGEON TRANSFERRIN
Investigator: Jeffrey A. Frelinger
Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

Transferrins from chicken and human have been reported to be ~80,000 MW single linear polypeptide chains. Pigeon egg white transferrin was examined by sodium dodecyl sulfate (SDS) gel electrophoresis. Native and reduced carboxymethylated (RCM) proteins give an apparent molecular weight of 80,000. When either native or RCM protein is heated for 30 minutes at 100° C in SDS, approximately 30% is converted into 40,000 MW. When either the native or RCM protein is extensively succinylated, most of the protein is converted to 40,000 MW. All preparations give a single amino-terminal amino acid, valine, by the dansyl chloride method. Cyanogen bromide cleavage results in four fragments when analyzed on SDS gels, although the proteins contain 8 methionines. This suggests that the protein consists of two similar or identical subunits.

194. BIOCHEMICAL CHARACTERIZATION OF ALLELIC DIFFERENCES BETWEEN PIGEON TRANSFERRINS
Investigators: Jeffrey A. Frelinger, M. Tomona*, William Niedermeier*
Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

A, B, and AB pigeon transferrins were purified by ion exchange and gel filtration chromatography and compared by tryptic peptide mapping. Only a single peptide difference was detected between A and B. Both peptides were present in AB transferrins. The peptides were eluted with water and their compositions compared by amino acid analysis. A single amino acid substitution, Ser (in A) → Asn (in B), was found. Differences in total carbohydrate composition between A and B
transferrins suggest that the amino acid change allows an additional carbohydrate prosthetic group per molecule. No differences were found in absorption spectra, gross amino acid composition, or NH₂ terminal amino acid analysis.

195. TRANSFERRIN POLYMORPHISM AND HARDY-WEINBERG RATIOS

Investigators: Jeffrey A. Frelinger, James F. Crow*

Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

It is known that strong selection can cause wide departures from Hardy-Weinberg ratios in a randomly mating population if the enumeration is made after selection. In pigeon transferrin polymorphism, selection is believed to act through embryonic mortality, so it might appear that enumeration of adults would show departures from Hardy-Weinberg ratios, especially in view of the large differences in egg hatch. But the selection is based on the maternal genotype, not that of the embryo. We have shown that in this case the equilibrium population is expected to be in Hardy-Weinberg proportions, as observed in real populations. However, if the population has not reached gene frequency equilibrium a slight excess of heterozygotes is expected.

196. INVESTIGATION OF NEMATODE ANTIGENS

Investigators: Elaine Kubota*, David B. Dusenbery, Elizabeth P. Blankenhorn

Support: National Science Foundation
National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

The characterization of various antigens by the use of antisera has proven to be of value in many different biological investigations. We were interested in determining the potential of these methods for characterizing behavioral mutants of the nematode Caenorhabditis elegans (see the reports of R. Russell's group).

The antisera were made by homogenizing 0.3 ml of packed C. elegans in 1 ml of saline, mixing with an equal volume of Freund's complete adjuvant, and injecting this into the toe pads of two rabbits. Three weeks later the rabbits were desensitized with a similar preparation injected intraperitoneally, and two days following this a similar intravenous injection was made. A week later the rabbits were bled and the sera stored at -20°C.

The antisera were characterized in several different ways. Passive hemagglutination tests (with ground-up nematodes as the sensitizing antigen) indicated that anti-nematode activity was present. However, surface antigens could not be demonstrated by either a modified immune adherence test or fluorescein conjugated antiserum, and soluble antigens could not be demonstrated by immunoelectrophoresis using any of several antigen extraction procedures. The antisera did appear to have a small but specific behavioral effect, causing the worms to writhe in shorter, larger-amplitude waves than normal, while normal rabbit serum had no such effect.

We conclude that making use of antisera to characterize mutants of C. elegans is not likely to be straightforward but will probably require the partial purification of specific antigens.

*NSF Summer Trainee, 1971.
197. THE CARBOHYDRATE COMPOSITION OF IMMUNOGLOBULINS FROM DIVERSE SPECIES OF VERTEBRATES


Support: Smith, Kline and French
U.S. Atomic Energy Commission
National Science Foundation
National Institutes of Health, Public Health Service

During the past several years the group of investigators listed has been involved in studying the phylogeny of immunoglobulins. A substantial number of species has been studied and their respective immunoglobulins physiochemically defined. With the report by Niedermeier of a sensitive procedure for analyzing neutral and amino sugars we elected to analyze the immunoglobulins of species more primitive than the mammals. With the Niedermeier technique carbohydrates are analyzed by gas chromatography utilizing the alditol acetate derivatives of the sugars released by acid hydrolysis. This allows one readily to detect 5 nanomoles of an alditol acetate.

There were 13 species of animals studied, representing all classes of vertebrates with the exception of the most primitive, the Agnatha. The high and low molecular weight immunoglobulins and their respective heavy (H) and light (L) polypeptide chains were analyzed for their carbohydrate compositions. Mannose, galactose, glucosamine and sialic acid were present in the immunoglobulins from all the species investigated. Fucose, however, was absent from the immunoglobulins of two species of animals studied. Although most of the carbohydrate was found associated with the H chains, significant quantities were also demonstrated in the L chains from many of the species. The carbohydrate composition was believed to be heterogeneous, because the quantities of fucose and sialic acid found often accounted for less than one residue per H chain. Whereas species representative of the mammals and birds had mannose to galactose ratios greater than one, the ratio of these sugars to each other in the immunoglobulins from animals below the birds was on the order of one.

The information collected in this study should prove useful in future attempts to elucidate the function of carbohydrates in immunoglobulins. It is anticipated that a complete understanding will probably require the study of glycopeptide structure and function from a phylogenetic standpoint.

Reference:

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PUBLICATIONS


Acton, R. T. and Shelton, E. (1972) Preliminary studies on the solubilization of the theta antigen from mouse brain and leukemic cells. Submitted for publication.


Professor: Max Delbrück

Visiting Associates: David C. Ailion, John W. Kauffman

Gosney Research Fellow: Tamotsu Ootaki

Research Fellows: Ruth L. Dusenbery, Edward D. Lipson, Stuart G. A. McLaughlin, Sheldon L. Trubatch

Graduate Students: Kostia Bergman, Moises G. Eisenberg, Kenneth W. Foster, Lily Jan, Y. N. Jan, Algirdas J. Jesaitis

Technical Assistant: Michael P. Walsh

Research Assistant: Jeanette Navest

PHYCOMYCES WORK: In July-August, 1971, a Phycomyces Genetics Workshop took place in Cold Spring Harbor, with 8 participants (3 from Caltech) and 6 visitors, under the direction of Cerda-Olmedo (Sevilla). Ootaki (Pittsburgh) applied his previous research on sporangiophore regeneration to the development of an elegant technique for obtaining heterokaryons and Hom (University of Pennsylvania) constructed a triple heterokaryon. Ootaki joined our group at Caltech in April, 1972, and has extended the heterokaryon work to a comprehensive series of allelism tests among our collection of mutants defective in carotene synthesis. Complementation between nonallelic albinos is a reliable check on heterokaryosis.

Genetic aspects were prominent also during the rest of the year. Bergman (Biology 1972, Nos. 198 and 199), Foster (Biology 1972, No. 201), and Ailion (Biology 1972, No. 202) characterized a variety of behavioral mutants so as to obtain a beginning of a network connecting the input of light, gravity, barriers and the output of sporangiophore initiation, growth responses, photocarotenogenesis. The semiautomatic tracking device described last year by Foster has proved very helpful in these studies (Biology 1972, Nos. 200 and 204), and will be especially so in the studies on bright-blind and bright-seeing mutants initiated by Lipson (Biology 1972, No. 204).

Jesaitis (Biology 1972, No. 205) has achieved the difficult goal of assessing the orientation of the photoreceptor pigment using polarized light stimulation and isolation of the proximal from the distal side by two independent methods. One of these involves a "super yellow" mutant which produces enough β-carotene to shield the distal side from light, leading to reversal of the phototropic effect.

Foster (Biology 1972, No. 203) has shown, by an ingenious analysis of old and new experiments, that the "spiral growth" of the sporangiophore (twisting around its long axis) is an essential element in the sustained phototropic response.

Jan has initiated a highly interesting new inroad toward the understanding of the responses of Phycomyces, by showing that chitin synthetase is amenable to a variety of studies (Biology 1972, No. 207). Jan also has contributed some new approaches to the understanding of the avoidance response (Biology 1972, No. 206).

MEMBRANE WORK: The control of membrane permeabilities by chemicals or light or electrical potentials is basic to the functioning of many cell types and the problem of understanding the mechanisms of these controls has become the central problem of membranology. Alamethicin is a cyclic oligopeptide conferring upon artificial black lipid films ion-permeabilities sharply controlled by applied potentials. Eisenberg and Hall (Biology 1972, No. 212) have undertaken a very detailed study of this phenomenon which permits one to make plausible guesses as to the molecular processes involved.

198. BLUE-LIGHT CONTROL OF SPORANGIOPHORE INITIATION IN PHYCOMYCES

Investigator: Kostia Bergman

Support: National Science Foundation
National Institutes of Health, Public Health Service

Many fungi produce spores or spore-bearing structures under the control of blue light. Phycomyces sporangiophores (spphs) are produced continuously along racing tube cultures grown in constant darkness or constant light. However, if a dark-grown culture is exposed to light for a short time on one day a narrow, dense band of spphs is observed the next day at that point of the tube occupied by the mycelial tips during the light pulse. A periodic program with "short days," (e.g.,
4-hour light/20-hour dark) leads to periodic bands of spphs spaced at intervals corresponding to one period length (in this case 24 hours) of mycelial growth. Spph initiation is inhibited by a light to dark transition and is stimulated by a dark to light transition. A partial action spectrum of the initiation response, covering the critical 480 nm to 540 nm region, strongly suggests that the same photoreceptor pigment is involved as in the phototropic response and light growth response of spphs. Mutants with altered light control of spph initiation have been found among those selected for altered phototropism. This joint elimination of these two responses to blue light by a single mutation is evidence for a common early transduction system.

199. CLASSIFICATION OF mad MUTANTS OF PHYCOMYCES

Investigator: Kostia Bergman
Support: National Science Foundation
National Institutes of Health, Public Health Service

Mutants of Phycomyces with altered responses of their sporangiophores (spphs) to light are called mad. Simple quantitative tests have been devised and used to separate the mad mutants into phenotypic classes based on three sensory responses: phototropism of spphs, avoidance of solid objects by spphs (or autochemotropism), and light control of spph initiation. Class 1 mutants have altered phototropism but normal avoidance. Some of these mutants show phototropism only at intensities 10^6 times higher than the wild-type threshold, but none are completely nonphototropic. Class 1 is divided into two subclasses. Class 1-1 mutants are also deficient in light control of spph initiation. Class 1-2 mutants show normal light control of spph initiation. Class 2 mutants have altered phototropism and altered avoidance but normal light control of spph initiation. A first outline of the organization of the sensory systems of Phycomyces is proposed.

200. CHARACTERIZATION OF THE LIGHT RESPONSE OF PHYCOMYCES

Investigators: Kenneth W. Foster, Edward D. Lipson
Support: Sloan Fund for Basic Research
McCallum Fund
National Institutes of Health, Public Health Service

Using this machine the latency and the shape of the response have been characterized as a function of temperature, stimulus size, level of adaptation and incident light wavelength. Latency is dependent on the logarithm of the absolute adaptation level. The response as a function of stimulus size is the same at wavelengths 380 and 455 nm. The shape of the response is remarkably independent of the adaptation level over a wide range of intensities. For large stimuli the shape of the response changes markedly, with the rising of an early peak in the growth velocity and inhibition of a later second peak.

These characteristics are quite similar to the visual stimulus photocurrent response systems of animals, as analyzed especially by Hagins' group (1972), Lipetz (1969), Glantz (1968), and others.

References:
201. CHARACTERIZATION OF MUTANTS OF THE STIMULUS RESPONSE PATHWAY

Investigator: Kenneth W. Foster
Support: National Science Foundation
McCallum Fund

Bergman (1972) has studied a series of mutants with abnormal responses to light. He hypothesized that at a specific point each mutant interrupts the pathway from the reception of a stimulus to the expression of a response. With different types of receptors and outputs these pathways form a branched tree. He was able to define three classes: class 1-1, in which light does not trigger sporangiophore (spph) initiation or blue-light tropism, but spphs do avoid; class 1-2, those that fail only to show light tropism; and class 2, those whose only response to light is spph initiation. Further observation of these mutants reveals that the growth response of each of these mutants is in some way altered.

Although the mutants do belong to clear classes and subclasses, each mutant also has individual response characteristics. In class 1-1 there is C47 with threshold raised 10,000-fold but otherwise normal response at moderate and high intensities and C21 with an abnormally large clockwise error in its bending direction sufficiently near its response threshold to direct specimens 180° from the correct direction of the source. For C21 this also results in abnormally fast hunting.

In class 1-2, one subclass, all of them albinos, S37, S14, S18 and S5 show abnormally long refractory periods, i.e. in a sequence of three stimuli separated by 45 minutes, the second stimulus gives a small response. This occurs with wild type only if the stimuli are separated by a much shorter time.

Mutants in class 2 can be separated into two subclasses, those that geotrope and those that do not (see Biology 1972, No. 202). Of the subclass that does geotrope C110, C63, C68 and C149 all show an abnormally long drawn-out growth response of small amplitude both to pulses of light and to step changes. All have poor phototropism in blue light and apparently higher thresholds than for wild type. They all trope to the horizontal away from 280 nm, with thresholds not more than 10 times above wild type. Their troping rate is, however, about one-thirtieth of wild type. The subclass of nongeotropers C106 and C150 show smaller than wild-type responses to light. Responses to step changes are drawn out, but those to pulse changes are of normal duration.

The mutants mentioned are reproducibly distinguishable from each other under certain conditions. It is hoped that the understanding of these and other mutants will help to illuminate the stimulus response pathway in Phycomyces.

Reference:

202. GEOTROPISM IN PHYCOMYCES

Investigators: David C. Ailion, Max Delbrück
Support: National Institutes of Health, Public Health Service
National Science Foundation

We are presently trying to determine the mechanism responsible for geotropism in Phycomyces. Two candidates for this mechanism are (1) the motion of cytoplasm relative to the vacuole and (2) the sedimentation of statoliths (possibly in the vacuole). In order to distinguish between these possibilities, we are in the process of screening mutants for rapid geotropism and for nongeotropism.

At present we have studied geotropism in wild type, in the albino mutant C5, and in seven class 2 mutants (i.e., mutants which have diminished avoidance and phototropic responses). We observed nongeotropism in two of the class 2 mutants, C106 and C150, indicating that these mutants are most probably altered in a gene common to all three properties. The fact that some of the class 2 mutants do not respond to gravity while others have normal geotropism indicates that the position of the entry of geotropism into the stimulus-response network (Bergman, 1972) is on the output side relative to the confluence of avoidance and light. Geotropic bending rates of 1°/minute, three times that of wild type, have been confirmed in C5. Complementation tests between heterokaryons of nongeotropic mutants will be used to determine the number of specific geotropism genes.
203. MECHANISM OF PHOTOTROPISM IN PHYCOMYCES

Investigator: Kenneth W. Foster
Support: Sloan Fund for Basic Research
         McCallum Fund

A model has been developed which indicates that an indispensable factor in phototropism is the cell’s natural twist (spiral growth, about 6°/min in the middle of the growing zone). Each region locally adapts to the local light intensity. As a result of the lens properties of the transparent cylindrical cell the incident light is focused into a band on the far side of the cell. Due to the twist of the cell, at the leading edge of the focused band there will be a continuous entry of dark-adapted area into the higher intensity band, thus giving rise to transient positive responses in a succession of surface elements. At the trailing edge of the focused band there will be a step down in intensity and a corresponding negative response; however, this negative response is known to be much smaller and drawn out. The relatively large positive response at the leading edge of the band is sufficient to cause Phycomyces to trope towards the light.

This model accounts for phototropism entirely on the basis of the transient growth response to light changes, the optics of the cell, local adaptation and the physical twist of the cell.

The effect of absorption of light within the cell has been measured and been found to have a very small effect in reducing phototropism. This would seem reasonable if adaptation is considered and if the stimulus is proportional to the contrast between the high intensity band and the adjacent dark-adapted area which are equally affected by absorption. Consequently it takes an absorption of 90% of the light across the cell before phototropism reverses from positive to negative. Scattering of light within the cell has a much more significant effect probably because it reduces this contrast.

This model has been successful in explaining a number of experiments from the literature involving asymmetric illumination and adaptation of Phycomyces.

Reference:

204. PHYSIOLOGY OF PHYCOMYCES AT HIGH LIGHT INTENSITIES

Investigators: Edward D. Lipson, Kenneth W. Foster
Support: National Institutes of Health, Public Health Service
         Sloan Fund for Basic Research
         National Science Foundation

When adapted to high light intensities, Phycomyces is less sensitive to still brighter signals. One presumes that receptor pigment regeneration is unable to keep pace with bleaching. Using the Phycomyces tracking machine we have observed a breakdown of the reciprocity relation for pulse stimuli, which states that the response normally is a function of the product of the intensity and the duration of the pulse. Assuming a first-order kinetics model for the pigment, we have shown how a careful examination of reciprocity breakdown can provide two important pigment parameters, namely the cross-section for bleaching and the regeneration rate constant. This information will provide another assay for the pigment in addition to the known action spectrum (Delbrück and Shropshire, 1960).

At high intensities we have also observed a dependence of the steady-state growth rate on the prevailing intensity; this is in contrast to the normal range where the steady-state growth rate is independent of the light intensity. This finding suggests an intimate connection between bleached pigment and the regulation of growth.

Reference:
205. DICHLROISM AND ORIENTATION OF PHYCOMYCES VISUAL PIGMENT

Investigator: Algirdas J. Jesaitis

Support: National Institutes of Health, Public Health Service

Phycomyces sporangiophores (spphs) contain large amounts (10 mg/ml) of gallic acid (Dennison, 1959). At this concentration ultraviolet light of wavelength 280 nm is nearly completely absorbed in traversing the cell. This means that collimated light, entering the cell perpendicularly, will influence the proximal photoreceptors of the cell much more than those in the distal region. This is demonstrated by the negative phototropism of the spph in this type of light.

Because of this fact, I have been able to measure the average photopigment orientation for 280 nm light. Light polarized at 50° to the long axis of the cell and incident perpendicularly is 30% more effective in eliciting a growth response than light polarized at 140° to the cell axis. This light is effective only on the proximal region of the cell. The difference in response is maximum at these angles.

A mutant (car-41) has been isolated which exhibits negative phototropism in the blue region of the spectrum. This behavior results in an overabundance of yellow pigment, presumably β-carotene, in the spphs of this strain. The optical density of the mature spph is greater than 1. Car-41 is currently being used to measure the average orientation of the 455 nm transition moment in the proximal region of the cell.

Reference:

206. AUTOCHEMOTROPISM (AVOIDANCE RESPONSE) OF PHYCOMYCES

Investigators: Y. N. Jan, Max Delbrück

Support: McCallum Fund
National Science Foundation

The sporangiophore (spph) of Phycomyces can sense the presence of any object near its growing zone and grows away from it. The most plausible explanation is the following: The sporangiophore emits some gas X. The growth of any region in the growing zone is regulated by the local concentration of X. The nearby object alters the distribution of X and hence causes the tropic response.

There can be two alternative hypotheses: (1) Positive control: X is a growth promoter. The nearby object serves as a reflecting barrier for X. (2) Negative control: X is a growth inhibitor. The nearby object adsorbs X.

To resolve this alternative, several experiments were designed. (1) If X is reflected by the barrier then the spph should not bend away from a nearby barrier which is made of material which adsorbs instead of reflecting X. But our experiments showed that spph avoids even a barrier made of activated charcoal, a very good adsorbant. (2) The spph avoids a thin wire (diameter ~50 µ) placed adjacent but at right angles to the growing zone. The effectiveness as a barrier is comparable with that of a big plate placed at the same distance from the spph. If the wire reflects, then the modification of the concentration of X due to the wire would be negligible. If the wire adsorbs, the wire is a "sink." In this case, a thin wire could cause a significant avoidance. (3) To detect X, Phycomyces must have some receptors for X on the cell surface of its spph. When the receptors on one side of the spph surface are blocked from receiving X, the spph should bend away from that side if X is a growth inhibitor and it should bend toward that side if X is a growth promoter. To block the receptors, a thin layer of protein solution was applied to one side of the cell surface. One of the proteins used was concanavalin A. By using fluorescence labeling techniques it has been shown to bind to the surface of spph's growing zone (presumably by recognizing the terminal mannside-like residues). The spph bent away from this side. The latency is comparable with that of the avoidance response.

All the above evidence is strongly in favor of hypothesis (2).

What is the molecular nature of X? To identify X, it is desirable to have X radioactively labeled. Using 14C as carbon source for the growth of Phycomyces would be impractical because too small a fraction of radioactivity would end up in gas X. In the case of insect pheromones, most of the molecules are simple compounds of relatively low molecular weight and many are derivatives of fatty acids or terpenes. X might be of similar nature. Therefore 14C-sodium acetate was fed to
Phycomyces. The Phycomyces does emit labeled gas. The nature of this gas is to be determined next.

207. CHITIN SYNTHETASE IN PHYCOMYCES

Investigator: Y. N. Jan
Support: McCallum Fund
National Science Foundation

The output of Phycomyces sensory processes involves variations of the rate of cell wall elongation. Since chitin is the major component of the cell wall of Phycomyces, we expect that regulation of chitin synthetase might play an important role in the output of these sensory transduction processes.

The first step of such a study is to find an assay method for chitin synthetase. The assay was done in the following manner (Ohta et al., 1970). (1) The biosynthetic preparation of the substrate of the enzyme, UDP-N-acetylglucosamine (¹⁴C), was done according to R. J. O'Brien's procedure (O’Brien, 1965). (2) The reaction mixture contains an aliquot of substrate and cellular material from various cell fractions. (3) After various periods of incubation the reaction was stopped and applied to a Whatman 3MM filter paper for descending chromatography. 18 hours later, the radioactivity remaining at the origin was scored as chitin.

We found that: (1) extracts from exponentially growing mycelia show chitin synthetase activity; (2) the enzyme is membrane bound; (3) polyoxin D, an inhibitor of this enzyme in other organisms (Ohta et al., 1970), is a very potent competitive inhibitor of the Phycomyces enzyme; (4) cyclic AMP, a suspected second messenger in the regulation of cell wall synthesis, shows a concentration-dependent inhibitory effect on chitin synthetase activity.

References:

208. STUDIES OF NUCLEOPROTEINS FROM PHYCOMYCES

Investigator: Ruth L. Dusenbery
Support: National Institutes of Health, Public Health Service
National Science Foundation

The development of a method for isolation and purification of chromatin (Biology 1971, No. 223) has continued. A purified nuclear fraction can now be obtained by initial homogenization in the presence of 25 mM ascorbic acid and 10 mg/ml bovine serum albumin to reduce cellular phenolic compounds and bind cellular lipoid material, respectively. Final purification of the nuclear material is achieved on a discontinuous sucrose gradient. This fraction is then treated briefly with a low concentration of nonionic detergent to strip away the nuclear membrane and attached ribosomes, producing a fraction free from non-nuclear proteinaceous material. Both light and electron microscopy as well as chemical tests are used to monitor the composition of material obtained from each step in the procedure. Work is now in progress to characterize the basic protein fraction from purified chromatin.

A part of this work was done in collaboration with Dr. G. Meissner and Dr. S. Fleischer, Vanderbilt University, Nashville, Tennessee.

209. MYCELIAL GROWTH AND CAROTENOID PRODUCTION OF PHYCOMYCES IN LIQUID MEDIA

Investigators: David C. Ailion, Wan-Jean Hsu*, Max Delbrück
Support: National Institutes of Health, Public Health Service
National Science Foundation
U.S. Department of Agriculture

Observations by Cerda-Olmedo and co-workers on carotenoid production in heterokaryons of Phycomyces have been explained by postulating the existence of enzyme aggregates necessary for the production of various carotenoids.

This hypothesis can be checked by forming heterokaryons between mutant strains which have defects at different steps in the biosynthetic chain leading to β-carotene. In the heterokaryon, each strain will supply enzymes which are either absent or defective in the other; accordingly, it will be possible for the heterokaryon to form enzyme...
aggregates containing nondefective enzymes in addition to those containing various patterns of defective enzymes. The relative number of each kind of aggregate, and thus the amount of the corresponding carotenoid produced, should then depend on the numbers of nuclei of each strain present in the heterokaryon (de La Guardia et al., 1971).

In order to test these concepts, we have performed experiments in which we observed the time course of both mycelial growth and of carotenoid production in agitated liquid media for a number of different strains and for the heterokaryon C2*C9. In particular we found that typically a mycelium would grow exponentially and would double every 5 or 6 hours until saturation was reached. For the wild-type strains, production of \( \beta \)-carotene, \( \beta \)-zeacarotene, and phytofluene was observed to parallel the mycelial growth.

In the heterokaryon C2*C9, we observed during the exponential growth period a large amount of lycopene accumulation which increased at a considerably slower rate than the \( \beta \)-carotene production. In other words, the lycopene percentage of our total carotenoids decreased with time while the \( \beta \)-carotene percentage increased. These observations of nonconstancy in time of the percentage fractions of individual carotenoids contradicts the behavior required by the enzyme aggregate hypothesis (de La Guardia et al., 1971) and suggests a more complicated pathway of carotenoid production in Phycomyces.

Observations on wild type grown in dark vs. wild type grown in white light have indicated a much lower rate of \( \beta \)-carotene production for the dark-grown samples, confirming the phenomenon of photoinduced carotenogenesis (Goodwin, 1952).

References:


*U.S. Department of Agriculture Research Station, Pasadena, California.

210. EFFECT OF \( \text{O}_2 \) AND \( \text{N}_2 \) GAS ON PHOTOTROPISM OF SPORANGIOPHORES AND ON MYCELIAL GROWTH IN PHYCOMYCES

Investigators: David C. Ailion, Max Delbrück

Support: National Institutes of Health, Public Health Service
National Science Foundation

It has been suggested (K. C. Poff, personal communication) that the presence of pure \( \text{O}_2 \) may cause deterioration in Phycomyces. We tested this question by studying phototropism to blue light of the wild-type strain in gaseous atmospheres containing \( \text{O}_2 \) and \( \text{N}_2 \) in which we varied the percentage of \( \text{O}_2 \). In particular we found that the phototropic bending rate was higher in pure \( \text{O}_2 \) (~5.6°/min). At lower \( \text{O}_2 \) concentrations, both the phototropic and growth rates of sporangiophores slowed down, with phototropism ceasing for \( \text{O}_2 \) concentrations of 10% or below and growth slowing to 2 \( \mu \)/min for \( \text{O}_2 \) concentrations of 5 to 10% (as compared to a normal growth rate of 30 to 50 \( \mu \)/min). In pure \( \text{N}_2 \), we observed no growth or phototropism.

To test for the possibilities of anaerobic mycelial growth in \( \text{N}_2 \), we studied mycelial growth in cultures of liquid glucose-asparagine-yeast medium in which \( \text{N}_2 \) gas was bubbled through the medium. In particular we performed our observation on the wild-type strain and on two mutants, C2 and C5. After leaving the sample exposed to \( \text{N}_2 \) for periods of from 1 day to 3 weeks, the mycelia were removed. Microscopic study revealed no protoplasmic streaming. Plating of the mycelia on growth medium resulted in no new mycelial growth. These observations imply that the absence of \( \text{O}_2 \) is lethal to the organism and that there is no anaerobic growth mode.

211. SHORT ZYGOTE DORMANCY TIME STRAINS OF PHYCOMYCES

Investigator: Sheldon L. Trubatch

Support: National Science Foundation
California State University, Long Beach

Zygotes formed during the sexual reproduction cycle of most strains of Phycomyces germinate only after a long dormant period of six months. This excessively long dormancy time prevents a practical genetic analysis of the many mutant strains of Phycomyces. Furthermore, no chemical or physical procedure which induces a speedier germination has been found.
However, wild-type strain UBC21 has a zygote dormancy time of only 45 days, but is suspected of having a different sensory physiology from the extensively studied NRRL 1555 strain because it produces short sporangiophores. Strain UBC21 was crossed with NRRL 1555 to try to produce a new strain with known sensory physiology and short zygote dormancy time. These crosses produced a low zygote yield (less than 1% of normal), and the few zygotes that were formed had a low viability. All of the spores from a given zygote were found to be of the same sex. As a result, it was suspected that crossing had not occurred.

A positive test for recombination was performed by crossing UBC21 with strain C2, a white mutant otherwise physiologically identical with NRRL 1555. Because UBC21 is sexually plus and C2 is sexually minus, the appearance of a sexually plus white offspring confirms the occurrence of crossing. Although crossing yield and zygote viability were again much lower than normally obtained, two positive white strains now known as C169 and C170 were found. These are now being crossed with NRRL 1555 to determine if the short zygote dormancy time can be inherited.

212. ION CONDUCTING CHANNELS PRODUCED IN MODEL MEMBRANES BY THEIR INTERACTION WITH ALAMETHICIN

Investigators: Moises G. Eisenberg, James E. Hall*

Support: Rockefeller Foundation

Artificial membranes made from chromatographically pure phosphatidyl ethanolamine in n-decane (2.5%, w/v), characterized by a very small conductance to small ions (about $10^{-10}$ mho/mm$^2$ for 0.1 M NaCl), have been modified by the addition of the cyclic polypeptide antibiotic alamethicin (MW 1667), whose primary structure is known. At fixed alamethicin and salt concentrations, there is a threshold in the voltage applied across the lipid membrane, below which the conductance is identical to that of the unmodified artificial membrane, but above which the current increases exponentially with the applied voltage. This increase is a consequence of the onset of conductance steps of approximately uniform size (about $10^{-9}$ mho/unit-step, in 1.0 M NaCl). The steps occur only when the side to which alamethicin has been added is positive relative to the other side. This system shows gateable conductance channels similar to nerve cell membranes. Emphasis has been placed on instrumentation suitable for observing the behavior of the unit channels, and for their statistical analysis. The unit channels have ohmic conductance and behave, when open, as if ions were flowing through a very small pore which connects the aqueous environments at both sides of the membrane. These unit channels seem to arrange themselves in patches of 4 to 5; within a patch of such pores, there is a binomial distribution of the number of unit channels simultaneously open, averaging between 2 and 3, with opening and closing rates in the millisecond range. The patch number also has a statistical distribution, but its mean is very voltage-dependent. It accounts for the voltage dependence of the over-all conductance of the modified membrane.

Our goal is the understanding of these pores in terms of molecular mechanisms. The system itself is well characterized in terms of its components and is by far the simplest one available to date which shows pronounced voltage gating.

References:

*Division of Engineering and Applied Science, California Institute of Technology.

213. THE EFFECTS OF A CYCLIC POLYETHER ON THE ELECTRICAL PROPERTIES OF PHOSPHOLIPID BILAYER MEMBRANES

Investigators: Stuart G. A. McLaughlin, G. Szabo*, S. Ciani*, G. Eisenman*

Support: Ruddock Fund
National Science Foundation

The cyclic polyether XXXII, a neutral, lipid-soluble molecule, produces large increases in the conductance of bilayer membranes formed from a variety of lipids. The conductance increases linearly with the concentration of alkali metal cation but with the square, and at higher
concentrations the cube, of the polyether concentration. This implies that two or three polyether molecules combine with a single cation to carry it across the membrane. In the presence of XXXII the bilayer is permeable solely to cations and the membrane potential is described by an equation of the Goldman-Hodgkin-Katz type. The permeability ratios determined from potential measurements are independent of salt concentration, decrease in the sequence Cs>Rb>K>NH₄>Na>Li (1.0, .25, .15, .075, .007, .0013) and are equal to the conductance ratios at low (e.g., 10⁻³ M) salt concentration. At higher salt concentrations, the permeability and conductance ratios are not equal and maxima in the conductance vs. salt concentration curves are observed. Both these phenomena are postulated to be due to the formation of relatively impermeant 1:1 polyether cation complexes in the aqueous phase. The 1:1 aqueous association constants deduced from bilayer measurements decrease in the sequence K>Rb>Na>NH₄>Cs>Li (120, 34, 26, 19, 12, 4 liter per mole) and agree quantitatively with the literature values for the more water-soluble polyether XXXI, which lacks only the t-butyl groups of XXXII.

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**PUBLICATIONS**


The focus of interest in this laboratory is the molecular basis of gene regulation in higher organisms. We consider the genomic regulatory system from a dual point of view, as our studies deal both with genomic control of early embryonic differentiation and with evolution of higher organism DNA. It is evident that understanding of the structural basis of regulation is also key to a more comprehensive view of evolutionary process, since metazoan evolution has progressed by way of important changes in the patterns of gene regulation. Our studies fall into four closely related areas: the organization of repetitive and nonrepetitive sequences in DNA; evolutionary comparisons between the DNAs of closely related organisms; the organization and kind of the DNA sequences transcribed in various differentiating systems; and the control of embryonic gene activity by elements stored in egg cytoplasm during oogenesis.

214. THE ORGANIZATION OF XENOPUS DNA

Investigators: Eric H. Davidson, Barbara R. Hough, Christopher S. Amenson, Roy J. Britten

Support: National Institutes of Health, Public Health Service
         National Science Foundation
         American Cancer Society
         Carnegie Institution of Washington
         Charles B. Corser Fund for Biological Research

In each of the species that have so far been studied (calf, sea urchin and Drosophila), measurements indicate that repeated sequences are scattered throughout the DNA, interspersed among stretches of nonrepeated sequences. New studies now demonstrate conclusively that an intimate and extensive interspersion exists in the DNA of Xenopus laevis. The principal method employed is to assay, with hydroxyapatite, the reassociation of trace quantities of labeled fragments of various sizes with an excess of 450-nucleotide-long fragments. Labeled Xenopus DNA was sheared at chosen speeds in a blender to produce five fragment sizes (200, 700, 870, 1500, and 3700 nucleotides as measured by alkaline sucrose density gradient centrifugation). Reassociation kinetics of these fragments were determined in the presence of 10,000-fold excess of 450-long fragments at the hydroxyapatite criterion of 0.12 M phosphate buffer at 60°C.

An interesting observation needs to be mentioned before discussing the implications of these measurements for interspersion of the principal repetitive DNA sequences. A fraction of Xenopus DNA was found which binds to hydroxyapatite in the single-stranded state, and the evidence indicates that the sequences responsible are themselves widely interspersed through the DNA. Similar observations have been made previously with reference to other DNAs. This DNA fraction binds almost completely when passed over hydroxyapatite a second time, while the original unbound fraction does not bind appreciably in such a retest. Sequence interspersion of this “zero time binding fraction” is indicated by the fact the amount of DNA in the zero time binding fraction rises with the fragment size. It is 3.8% of the total DNA for 450-long fragments and 25% for 3700-nucleotide fragments. Preparations of this 3700-long, low Cot binding fraction were sheared to 450 nucleotides and reassociation kinetics measured in the presence of excess unfractionated 450-long fragments. The kinetics indicate that this fraction contains the same ratio of repeated to single copy DNA as the total, with the expected augmented proportion of zero time binding DNA. No evidence exists as to
the kind of sequence responsible for the binding of this fraction of the DNA. Whatever the sequences are, they appear to be widely scattered throughout the DNA with a proportional expectation of being adjacent either to single copy or repeated sequences.

The amount of reassociation of the labeled fragments at a given Cot increases sharply with fragment length from 200 to 800 nucleotides, then rises more slowly to the 3700 length. At Cot 50, almost all of the repetitive DNA of the *Xenopus* genome has reassociated. This is about 45% of the DNA when measured with 450-nucleotide fragments, but almost 80% of 3700-nucleotide-long fragments are bound at Cot 50. Numerical analysis of these data indicates that there is a large class of DNA (50 to 55%) in which stretches of nonrepetitive DNA averaging 900 to 1100 nucleotides are interspersed with short repeated sequences. There is another large class of DNA (at least 25%) in which the single copy stretches are longer (4000 to 8000 nucleotides) and there may be a small class in which repeated sequences are rare or absent. At the other end of the spectrum of interspersion, there may be repetitive sequences which are present in clusters, with little or no nonrepetitive DNA present. However, experiments designed specifically to test for and isolate clustered repetitive sequences have failed to demonstrate a clustered component. These experiments show that if clustered repetitive sequences exist in the genome of *Xenopus*, they constitute less than 6 to 8% of the DNA.

Measurements of the hyperchromicity of repetitive DNA duplex support the conclusion that repetitive and nonrepetitive sequences are intimately interspersed. Samples of DNA of two fragment sizes (450 and 1800 nucleotides) were separately incubated so that only repeated sequences reassociated, bound to hydroxyapatite, eluted with 0.5 M phosphate buffer, diluted and melted in the spectrophotometer in 0.12 M phosphate buffer at 260 nm. The hyperchromicities (corrected for single-strand collapse) of the two preparations were 17% and 10% (expressed as fraction of denatured optical density). The reassociated longer fragments apparently contain extensive nonreassociated regions amounting to something like half of their lengths. When the reassociated 450-long DNA is digested with a single-strand-specific enzyme (S-1 from *Aspergillus*), about one-third of the DNA may be recovered on hydroxyapatite and this fraction shows a hyperchromicity of 25%. This large hyperchromicity is nearly that observed for native *Xenopus* DNA (27%) and thus the pairing of the enzyme-resistant regions is almost complete.

Information derived from both the reassociation kinetics and these hyperchromicity experiments indicate that the average length of the interspersed repeated sequences is 300 ± 100 nucleotides.

The biological significance of the finding that such a large fraction of the nonrepetitive DNA of *Xenopus* is interspersed with many short repetitive sequence elements is as yet unknown. It seems likely that this highly ordered arrangement carries out a role with selective value to the species. The lengths of the repeated and single copy interspersed regions observed for *Xenopus* are consistent with the predictions of the model of gene regulation proposed earlier (Britten and Davidson, 1969, 1971). However, they of course do not yet supply a crucial test of the model.

References:

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**215. LENGTH OF REPEATED SEQUENCE ELEMENTS**

**Investigators:** Dale E. Graham, Michael Henerey, Roy J. Britten

**Support:**
- Damon Runyon Memorial Fund for Cancer Research
- National Institutes of Health, Public Health Service
- National Science Foundation
- Charles B. Corser Fund for Biological Research
- Carnegie Institution of Washington

Knowledge of the length of individual stretches of repeated nucleotide sequences in higher organism DNA is important in attempting to establish the role or function of repeated sequences in the genome. The studies of the arrangement of DNA sequences that have been done so far imply not only that there is an intimate interspersion of repeated and nonrepeated sequences (Biology
1972, No. 214) but also that many of the repeated sequence elements are probably short. Some appear to have a length of a few hundred nucleotide pairs and a few may be less than a hundred long.

A series of measurements has been initiated in an attempt to determine the length and distribution of the repeated sequences in the genomes of the sea urchin *Strongylocentrotus purpuratus* and the amphibian *Xenopus laevis*. Several approaches are being pursued. The melting temperature and ultraviolet hyperchromicity of reassociated repeated DNA formed from various DNA fragment sizes are being measured. In addition, the lengths of the reassociated repeated DNA fragments are estimated after digestion with a single-strand-specific nuclease (S-1 from *Aspergillus*). From this combination of measurements it should be possible to deduce the length of even moderately imperfectly repeated sequence elements.

Very short fragments (25 to 100 nucleotides) are prepared by controlled DNase I digestion followed by Sephadex G-200 fractionation in alkaline buffer to prevent reassociation. Longer fragments are separated on agarose in neutral buffer. For preparative purposes, native fragments are used. For analytical runs of single-stranded DNA it is possible to maintain dissociation with 1% formaldehyde. Conditions for binding of very short reassociated fragments to hydroxyapatite have been explored and calibration measurements have been made of the binding of short fragments to DNA networks. The reassociation (Cot) curve for the binding of 50-long urchin DNA fragments to networks has been partially completed. The urchin networks themselves supply a direct and qualitative demonstration of the interspersion of single copy and repeated sequences. When long urchin DNA is incubated to a Cot such that only repeated sequences reassociate, more than 90% of the DNA is in networks (pelleted at 100,000 x g for 10 minutes). If the networks are incubated to Cot 50,000, the single copy sequences do not reassociate significantly. Apparently some of the interspersed repetitive sequences have reassociated and steric hindrance in the networks prevents further reassociation.

It has become possible to prepare shorter native DNA fragments than with other methods using the generally available Virtis 45,000 rpm blender. Crothers, Kallenbach and Zimm (1965) prepared 380-nucleotide-long fragments after 8 hours blending in 50% glycerol. We have prepared native DNA fragments with a Tm reduced by 2.5°C in 1 hour at 60,000 rpm (3.7 cm blade, 35 ml) in 67% glycerol cooled to -15°C. According to Hayes et al. (1970) (L = 440/Tm reduction), these fragments are less than 200 nucleotide pairs. The fragments are freed from glycerol and concentrated by binding to hydroxyapatite (in 50% glycerol, 0.14 M PB at 50°C). This is a valuable technical advance since these easily prepared fragments are short enough to make possible the separation of most repeated and nonrepeated DNA sequences.

**References:**


### 216. REPETITIVE AND SINGLE COPY DNA IN THE SEA URCHIN GENOME

**Investigators:** Roy J. Britten, Dale E. Graham, Michael Henerey

**Support:**

- Damon Runyon Memorial Fund for Cancer Research
- National Institutes of Health, Public Health Service
- National Science Foundation
- Charles B. Corser Fund for Biological Research
- Carnegie Institution of Washington

A broad scale analysis of the sea urchin (*Strongylocentrotus purpuratus*) genome is planned and partly under way. This includes studies of the arrangement of repetitive and nonrepetitive sequences, the characterization of nuclear RNA and polysomal messenger RNA, and studies of the frequencies and arrangement of the sequences transcribed to yield these classes of RNA (see below). The first step in this program has been to measure the amount of repetitive and nonrepetitive
DNA and the principal frequencies of repetition. A number of methods has been employed for this study including: total DNA reassociation curve for 450-long fragments (Cot curve) measured by hydroxyapatite assay (0.14 M PB, 50°C); fractionation of labeled DNA into various frequency classes; measurement of the reassociation of the classes in the spectrophotometer and with hydroxyapatite assay (minicot curve); and measurement of the reassociation of these labeled classes when added to excess total 450-long DNA (slave minicot curve).

These measurements have identified four major frequency classes, described here in ascending order of repetition frequency. Each of the numbers quoted represents the best approximation, and the frequencies, except for the single copy fractionation, are averages of a possible broad range. About 38% of the DNA is nonrepetitive. Due to the presence of a low frequency component it is difficult to identify the single copy component in the total DNA Cot curve. Therefore the most slowly reassociating fraction of the labeled urchin DNA was prepared and added to total DNA. The rate of reassociation of the added labeled DNA was exactly that predicted from the genome size for single copy DNA.

The next component appears, from its rate of reassociation when mixed with total DNA, to be present in 20 to 50 copies and probably amounts to 25% of the total DNA. This component is particularly interesting since such a small number of copies might well represent the number of related sequences required to control a battery of genes. Its kinetic complexity is very large (10,000,000 nucleotide pairs).

There are two components or ranges of components which have been termed "intermediate" and "fast." The intermediate component amounts to about 25% of the genome and is present in a few hundred copies. The fast component comprises only about 10% of the total DNA and is present in about 6000 copies. There is a probable correction of about a factor of two in each of these frequencies for the effect of sequence divergence which has not been applied.

The first results of attempts to determine the arrangement of these sequences suggest that at least some part of the intermediate and the slow components are widely interspersed throughout the DNA.

217. REASSOCIATION OF DEAMINATED E. COLI DNA

Investigators: Berney Neufeld, Roy J. Britten
Support: National Institutes of Health, Public Health Service
National Science Foundation
Carnegie Institution of Washington
Charles B. Corser Fund for Biological Research

Sequence divergence among the members of a repeated family of DNA causes a reduction of the melting temperature ($T_m$) of the reassociated material as compared with the $T_m$ of the native DNA. Repeated families of DNA are characterized by their reassociation rates so it is desirable to be able to separate rate effects due to mispairing of diverged sequences from those due to differences in reiteration frequency. A direct measure of the effect of mispairing on the reassociation rate was made in a study of the reassociation of nitrous acid deaminated E. coli DNA.

In one series of experiments the reassociation rates for the self-reactions of normal and for deaminated ($T_m$ of the reassociated DNA 10°C below normal DNA) DNA were determined. The reassociation rate of the deaminated material was approximately 0.45 that of the native DNA whether observed optically or by hydroxyapatite. As expected, the hydroxyapatite rates were approximately twice those observed optically.

In a second series of experiments the reaction of deaminated $^3$H-DNA with bulk normal DNA was examined with hydroxyapatite (criterion conditions; 50°C, 0.12 M PB). The $^3$H-DNA, reassociated with normal DNA, thermally eluted from hydroxyapatite at a temperature 15°C below that at which the normal DNA was eluted. The normal DNA had a self-reassociation rate constant of 0.101. The hybrid reaction rate constant was 0.0260. The latter has been corrected for the competition for binding sites by the faster normal self-reaction.

When these data are combined with the results of other recent measurements (T. Bonner and D. Brenner, personal communication) a reasonably good value for the magnitude of the effect can be made. The best present estimate is that sequence
divergence sufficient to cause a reduction of $10^\circ$ in the melting temperature causes a factor of two reduction in the second order reassociation rate constant.

218. XENOPUS LAEVIS DNA SEQUENCE REPRESENTATION IN XENOPUS MULLERI

Investigators: Glenn A. Galau, Margaret E. Chamberlin, Barbara R. Hough, Eric H. Davidson

Support: National Institutes of Health, Public Health Service
National Science Foundation
American Cancer Society

Experiments to determine the extent of sequence homology between the DNAs of two congeneric amphibians, *Xenopus laevis* and *Xenopus mulleri*, are in progress. These toads belong to an ancient order of amphibia, and are capable of producing viable, and occasionally fertile interspecific hybrids.

Reassociation of 450 nucleotide DNA fragments at 60°C shows that the Cot curve of *X. mulleri* is very similar to that described for *X. laevis* (Davidson and Hough, 1971), with apparently similar quantities of repetitive sequence.

Homology of the nonrepetitive DNA of the two species was investigated by reassociation of trace amounts of $^3$H-*X. laevis* nonrepetitive DNA with whole *X. mulleri* or *X. laevis* DNA. The heterologous hybrids are characterized by a $T_m$ about 14°C below that of the homologous duplex. About 15% of the hybrid has a $T_m$ of about 82°C, indicating that a portion of the hybrid molecules contain little nucleotide mismatch. A moderate rate correction (2.5) applied to the heterologous reaction in accordance with the 14°C $T_m$ depression (Biology 1972, No. 217) predicts completion of the reaction at Cot approaching $10^5$ with about 40% of the heterologous tracer reassociating compared to 88% homologous reaction. In a second exposure to *X. mulleri* DNA an additional 27% of the initial *X. laevis* tracer forms hybrids with *X. mulleri* DNA at Cot 30,000. We conclude that less than 3% of the nonrepetitive *X. laevis* DNA is represented as nonrepetitive DNA in *X. mulleri*. Preliminary experiments at a 50°C criterion indicate that little, if any, additional hybrid is formed at the lower criterion, suggesting that the remaining 25% of *X. laevis* nonrepetitive sequences is represented, if at all, in *X. mulleri* as sequences which have diverged beyond recognition. The major result obtained in these experiments is that species as closely related as these display such a large degree of nonhomology in their nonrepetitive DNA.

The presence in *X. mulleri* DNA of sequences homologous to repetitive *X. laevis* DNA was investigated by the same techniques. The extent of reassociation of tracer repetitive DNA of *X. laevis* with whole *X. mulleri* DNA is the same as that of the homologous reaction. A major difference is that the repetitive *X. laevis* DNA appears to be represented in *X. mulleri* in two degrees of sequence repetition. Some of the heterologous reaction occurs at the same rate characteristic of the major repetitive component in either organism while the other components reassociate more than an order of magnitude more slowly. Since the $T_m$ of the hybrid duplexes is invariant throughout the reaction (less than 2°C below that of the homologous reaction) it is difficult to invoke sequence divergence as an explanation of the slow heterologous rate. It would tentatively appear, therefore, that since the divergence of these congers, changes have occurred in the frequency of repetitive sequence occurrence in their genomes.

Reference:

219. SEQUENCES REPRESENTED IN SEA URCHIN EMBRYO NUCLEAR RNA

Investigators: Michael J. Smith, Barbara R. Hough, Margaret E. Chamberlin, Eric H. Davidson

Support: National Science Foundation
National Institutes of Health, Public Health Service
American Cancer Society

The aims of this investigation are to determine the amount, sequence frequency components, and sequence organization of HnRNA molecules transcribed during gastrulation in sea urchin embryos. Gastrulae were pulse-labeled for 10 minutes with tritiated uridine, nuclei were isolated, and highly radioactive HnRNA extracted therefrom. The extracted HnRNA was submitted to sucrose
gradient analyses and the fraction greater than 40S isolated. This fraction was hybridized with sheared DNA (450 nucleotides long) to Cot 40 at a DNA:RNA ratio of 300:1 which results in maximum binding of RNA to DNA. Sheared sea urchin DNA reassociates to approximately 50% by Cot 40 (see above) by which point essentially all the repetitive elements in the genome have reassociated. Hydroxyapatite chromatographic analyses of the DNA:RNA hybrids were performed under two conditions: (1) standard criterion conditions, 0.12 M phosphate buffer, 0.06% S.L.S. at 60°C; and (2) 0.2 M phosphate buffer, 8 M urea, 1% S.L.S. at 40°C (0.2 U.P.). Under standard conditions unhybridized RNA binds to hydroxyapatite unless degraded by ribonuclease; however in 0.2 U.P. at 40°C only RNA which is hybridized to DNA will bind to HAP.

In 0.2 U.P., without RNase treatment, about 25% of the pulse-labeled HnRNA cpm form DNA:RNA hybrids which bind to HAP at Cot 40. However, only about 5% of the HnRNA cpm form RNase resistant hybrids with DNA after incubation at Cot 40. These hybrids bind to HAP under standard conditions. If the HnRNA is reacted with a network formed from unsheared DNA at Cot 40, about 35% of the HnRNA cpm are bound to the DNA.

These preliminary results indicate that a minimum of 5% of the HnRNA transcribed during gastrulation is repetitive in nature (an outside figure, obtained from Sephadex chromatography, is 8%). Further, these repetitive transcripts appear to be interspersed or contiguous in the HnRNA molecule with nonrepetitive sequence transcripts.

220. ISOLATION OF MESSENGER RNA FROM ACTIVE SEA URCHIN POLYSOMES

Investigators: Robert B. Goldberg, Glenn A. Galau, Eric H. Davidson
Support: National Institutes of Health, Public Health Service
American Cancer Society

It has been well established that both repetitive and unique DNA sequences are transcribed. However, previous studies have largely dealt with whole populations of transcribed RNA, or RNAs such as transfer and ribosomal, which are readily recoverable. The experiments we have been conducting with the sea urchin Strongylocentrotus purpuratus are designed to answer the following questions: (1) from what kind of DNA sequences are the messenger RNAs translated at a given stage of embryogenesis transcribed; and (2) what is the sequence organization around the transcribed regions of DNA. In order to answer these questions trace quantities of highly labeled messenger RNA and large quantities of unlabeled messenger RNA are being isolated in pure form in order to perform both "DNA-driven" and "RNA-driven" RNA-DNA hybridization experiments.

We have isolated fairly undegraded polysomes in high yield from 36-hour gastrulae. The use of high ionic strength, low pH (6.5), and bentonite are all required to inhibit degradation of the polysomes by endogenous nucleases. Using formaldehyde fixation and CsCl isopycnic centrifugation, 25% of the polysomes are found to be membrane-bound and these are not released by a combination of detergents. The isolated free polysomes are sensitive to RNase, puromycin and EDTA.

Messenger RNA was obtained from the polysomes by the puromycin-high salt procedure of Blobel (1971) which dissociates active subunits and releases the message. Preliminary evidence indicates that membrane-bound polysomes do not release mRNA under these conditions. mRNA labeled for 3 hours with 3H-uridine and released by the puromycin technique contains less than 1% of the label as nuclear RNA and less than 0.05% of the label as rRNA.

Bulk quantities of unlabeled, active message are now being prepared. Hybridization experiments using the purified, radioactive message and designed to ascertain its content of repetitive and nonrepetitive sequence transcripts are now in progress.

Reference:
221. PERSISTENCE IN EMBRYOS OF MATERNAL RNA SYNTHESIZED DURING OOGENESIS IN ENGYSTOMOPS

Investigators: Barbara R. Hough, Eric H. Davidson
Support: National Institutes of Health, Public Health Service
National Science Foundation
American Cancer Society

In investigating mechanisms which operate in control of gene activity during early embryogenesis, it is desirable to identify and to characterize maternal RNA stored in the egg. In order to follow the utilization of the RNA during embryo development, it would be highly desirable to utilize eggs which can be labeled at the lambrush stage of their development and later, after completing oogenesis, recovered as embryos. Such eggs are produced by the Panamanian frog Engystomops pustulosus, which breeds during the rainy season. When no pools are available for mating, the female accumulates a large batch of mature oocytes, and egg maturation ceases. At the next rainy period the frogs mate; the female lays a large batch of eggs and a normal number of oocytes enter the active lamphrush stage and start to mature synchronously. This sequence of events occurs under controlled conditions in our laboratory colony (Davidson and Hough, 1969). Female frogs were injected with $^3$H-uridine just after the double egg batch was laid, sequestered for 4 weeks, and mated to produce labeled embryos. Maternally-labeled RNA is present at least through hatching stage ($>10^5$ cells). In preliminary experiments in vitro-labeled oocyte RNA was hybridized with fractions of Engystomops DNA chosen on the basis of the Cot curve previously developed. Using RNA-DNA hybridization, comparative measurements are being conducted on the maternal RNA which is present at the various stages of embryonic development.

Reference:

PUBLICATIONS


Assistant Professor: Leroy E. Hood
Senior Research Fellows: James W. Prahl, Michael D. Waterfield
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Computing Analyst: Donald E. Crede
Research Assistants: Nancy R. Carraway, Meera Dwarakanath, Vincent R. Farnsworth, Barbara Hatt, David B. Helphrey, Bertha E. Jones, Cornell C. Phillips, Marianne Wright
Laboratory Aide: Reggie P. Mireles

We are interested in studying the means by which information is stored and evolves for a variety of genetically complex systems in eukaryotes. A broad spectrum of chemical and biological approaches are employed, although major emphasis is placed on the analysis of protein molecules.

The immune system serves as a model for studying differentiation in higher organisms. Antibody information appears to be encoded in the vertebrate genome in the form of multiple germ line genes for the variable (V) portion of the antibody polypeptide chain (Biology 1972, Nos. 222 to 228). Portions of these multiple germ line genes appear to evolve in a coordinate fashion for each evolutionary line. This parallel evolution suggests that special evolutionary mechanisms such as gene expansion and contraction are employed in the evolution of this multigene family. We are engaged in defining more precisely the nature of these special evolutionary mechanisms (Biology 1972, Nos. 226, 229, 233 and 234). In addition, complex control mechanisms appear to be used in the expression of antibody molecules (Biology 1972, Nos. 225 and 230 to 232).

It is attractive to postulate that some of the general strategies employed by the immune system for dealing with information storage (e.g., separate genes for the variable and constant regions; multiple germ line V genes) and information evolution (gene expansion and contraction) may be employed by other complex systems. In this regard we are studying nonhistone chromosomal (NHC) proteins in two systems—Drosophila and the rat, and the transplantation antigens (H2 antigens) of the mouse.

We are examining NHC proteins to characterize the nature and extent of their diversity and thereby to determine whether multigene families exist in this class of proteins (Biology 1972, Nos. 236 and 237). Later, attempts will be made to determine the chromosomal distributions and functions of certain NHC proteins. The advantages of analyzing NHC proteins from Drosophila are detailed in Biology 1972, No. 235.

The study of transplantation antigens of the mouse is limited by the availability of small amounts of material. For example, current isolation methods permit us to isolate only 0.5 nanomoles of H2 antigen from one mouse (Biology 1972, No. 238). Thus we must develop new techniques for analyzing very small quantities of protein (Biology 1972, No. 239). To this end a collaborative effort has been initiated with the Dreyer group on micromethods of protein analysis. This new instrumentation and technology should in the future permit the analysis of many biologically interesting proteins which are available in limited quantity (e.g., various membrane proteins related to cancer, transplantation and cell-cell association as well as chromosomal proteins, etc.).

222. A SURVEY OF THE AMINO-TERMINAL REGIONS OF MOUSE KAPPA CHAINS

Investigators: Leroy E. Hood, Vincent R. Farnsworth

Support: National Science Foundation
National Institutes of Health, Public Health Service

The BALB/c mouse is an excellent experimental tool for the investigation of immunoglobulin diversity because myeloma tumors, each of which produce a homogeneous immunoglobulin, can be easily induced. Drawing from a bank of more than 500 tumors, we have examined 31 κ chains and compared them with 13 additional proteins from the literature. These proteins have at least 32 different sequences over their amino-terminal 23 residues. Although 9 of these sequences are identical for two or more κ chains, a majority of the proteins clearly differ from one
another by multiple nucleotide substitutions. A genealogical tree has been constructed from certain of these sequences to illustrate the genetic events required of any mechanism of antibody diversity. This analysis suggests that multiple germ line V\(_K\) genes are present in the BALB/c genome.

This work was done in collaboration with Dr. M. Potter of the National Cancer Institute, Bethesda, Maryland, and Dr. D. McKean of the Department of Medical Genetics, University of Wisconsin, Madison, Wisconsin.

Reference:

223. THE VARIABLE REGION SEQUENCE OF THREE MOUSE KAPPA CHAINS FROM ONE SUBGROUP

Investigator: Leroy E. Hood
Support: National Science Foundation
National Institutes of Health, Public Health Service

The variable regions from a single immunoglobulin family may be divided into sets (subgroups) by criteria similar to those used to divide hemoglobin chains from different species into the \(\alpha\) and \(\beta\) sets. These criteria are primarily linked amino acid residues and sequence gaps (or insertions). Most immunologists agree that proteins of differing subgroups must be encoded by separate germ line genes, but there is controversy as to how diversity within a subgroup is produced. Thus we decided to characterize the V regions of three proteins from one subgroup and compare them with a fourth already in the literature. The highly inbred nature of the BALB/c mouse permits us to minimize variation due to genetic polymorphism and accordingly this "intrasubgroup" diversity should be a direct reflection of the genetic mechanism responsible for antibody diversity.

Two of the proteins differed from one another by three residues. A third protein differed from these two by eight residues, whereas a fourth differed from the other three by 20 to 21 residues. Genealogical analysis of this data suggests that at least two and possibly three germ line V\(_K\) genes encode these four proteins. These results are consistent with the supposition that V\(_K\) regions are encoded by multiple germ line genes.

This work was done in collaboration with Dr. M. Potter of the National Cancer Institute, Bethesda, Maryland, and Dr. D. McKean of the Department of Medical Genetics, University of Wisconsin, Madison, Wisconsin.

224. IDENTICAL MYELOMA LIGHT CHAINS FROM TWO DISTINCT BALB/c TUMORS

Investigator: Paul A. Barstad
Support: National Institutes of Health, Public Health Service

One of the central questions in modern immunology centers about the mechanism responsible for antibody diversity. In its simplest form one may ask how many germ line V genes are present in a single individual? One approach to this question is that of examining random myeloma light chains (Bence-Jones proteins) from a single inbred species until two identical immunoglobulin chains are found. The total number of different myeloma genes present in the genome can then be estimated by statistical methods. We assume that myeloma proteins are randomly selected from the normal immunoglobulin pool. Even if this proves not to be the case, the gene pool size determined in this study will provide a lower limit for the number of \(\kappa\) sequences present in the BALB/c mouse.

In searching through about 60 immunoglobulin light chains, it appears that one pair may be identical. To date, peptide maps, amino acid compositions and extensive V region sequence analysis are consistent with sequence identity. If these light chains prove to be identical, our estimate of the size of the V\(_K\) library in the BALB/c mouse is more than 1000 V\(_K\) genes. Indeed, at the 90% confidence level we can say that the pool size must be greater than 700 and less than \(10^4\). This pool size will have obvious implications for theories of antibody diversity.

This work has been carried out in collaboration with Dr. P. Periman in the Department of Microbiology, George Washington University, Washington, D.C. and Dr. J. M. Hood in the Department of Mathematics, Occidental College.
225. THE SEQUENCE DETERMINATION OF TWO IDENTICAL MYELOMA ANTIBODY MOLECULES IN BALB/c MICE

Investigator: Paul A. Barstad
Support: National Institutes of Health, Public Health Service

About seven myeloma proteins in the IgA class have been found with high binding affinities for phosphorylcholine. All of these proteins appear to be identical by serological techniques; that is, antiserum made against the variable region of one of these proteins recognizes the rest, but does not recognize any of the more than 400 other myeloma proteins. Chemical studies also suggest that these proteins are identical. For example, the light chains are identical by amino acid analysis, peptide map comparison with two enzymatic fragmentations, and by a limited N-terminal sequence analysis. The heavy chains are also identical by similar criteria.

More recently we have initiated detailed sequence analysis on the two light chains and find that they are identical for their amino-terminal 35 residues. Complete sequence analysis of light chains from both antibodies is now in progress.

The implications of finding a number of myeloma tumors with identical light and identical heavy chains will raise questions about control mechanisms in the immune system. Are there special mechanisms for pairing certain kinds of light and certain kinds of heavy chains? Is there a synchronized and programmed readout mechanism for the variable genes of heavy and light chains?

This work has been carried out in collaboration with Dr. S. Rubikoff and Dr. M. Potter of the National Cancer Institute, Bethesda, Maryland.

226. EVOLUTION OF IMMUNOGLOBULIN LIGHT CHAINS

Investigator: Elwyn Y. Loh
Support: National Institutes of Health, Public Health Service

The germ line theory must explain how parallel evolution occurs in multiple germ line V genes. If one assumes that expansion and contraction events are responsible for the parallel evolution, one can ask about the frequency of these evolutionary events. Inbred strains of laboratory mice, which probably shared common ancestors several hundred years ago, provide a system to study the divergence of this multigene family. By comparing normal antibodies from several inbred lines, including BALB/c for which much myeloma sequence information exists, we hope to demonstrate the rate of this evolution.

In order to insure a similar antigenic background for the different strains of mice, a specific antibody is isolated. Large amounts of antibodies are being raised by means of ascites tumor induction after immunization. Antibodies are purified by affinity chromatography. We plan to compare the normal antibodies from different inbred strains through an analysis of the amino acid profiles of the N-terminal residues obtained with the automatic protein sequenator. Variable region evolution is also being studied by comparisons of homogeneous myeloma light chains from two inbred strains of mouse, BALB/c and NZB.

These studies are being carried out in collaboration with Dr. Noel Warner at the Walter and Eliza Hall Institute of Medical Research in Melbourne, Australia.

227. A SURVEY OF THE AMINO-TERMINAL REGIONS OF MOUSE HEAVY CHAINS

Investigators: Leroy E. Hood, Vincent R. Farnsworth
Support: National Science Foundation
          National Institutes of Health, Public Health Service

In contrast to most immunoglobulin heavy chains, those of the BALB/c mouse have an unblocked N-terminus which permits direct analysis by the automatic protein sequenator. We have examined 14 heavy chains from a variety of different classes and subclasses (G, A, F, H) and compared them with one additional sequence from the literature. Since it appears that a single family of variable regions (V<sub>H</sub>) is associated with all heavy chain C regions, it is reasonable to pool this
data for an analysis of $V_H$ diversity.

Twelve of the 15 sequences differ from one another by 1 to 11 of 20 residues. There is some suggestion that certain of these sequences can be grouped together in homologous sets ($V$ region subgroups). These data support a multigene origin for $V_H$ diversity.

**228. THE VARIABLE REGION SEQUENCE OF A MOUSE ANTIBODY HEAVY CHAIN**

**Investigator:** Leroy E. Hood  
**Support:** National Science Foundation  
National Institutes of Health, Public Health Service

The BALB/c myeloma protein, MOPC 315, has a binding constant of $10^7$ for dinitrophenol (DNP) and $5 \times 10^5$ for menadione. Because it is one of a family of myeloma proteins with anti-DNP activity and because it has recently been crystallized, the primary sequence is of interest. In addition, MOPC 315 is an IgA immunoglobulin, a class that has not heretofore been completely characterized. The sequence of the 315 light chain has been determined. We are in the process of determining the covalent structure of the heavy chain.

The heavy chain was fragmented with cyanogen bromide and an N-terminal fragment of approximately 167 residues was isolated. Essentially the entire $V$ region sequence (residues 1 to 118) has been determined. This protein differs from another BALB/c $V_H$ region already in the literature by 62/104 residues compared and two sequence gaps. This comparison supports the contention that $V_H$ regions are encoded by multiple germ line genes.

The mouse heavy chains demonstrated certain "species associated residues" which distinguished them from their human counterparts. Similar species associated residues have been seen in the comparison of mouse, human and rabbit $\kappa$ chains. In a multigene system the presence of species associated residues suggests that there are, within each of the three immunoglobulin families, evolutionary mechanisms for the contraction and expansion of antibody genes.

This work was carried out in collaboration with Drs. S. Francis and H. Eisen in the Department of Microbiology at Washington University in St. Louis, Missouri.

**229. COVALENT STRUCTURE OF A RABBIT ANTIBODY: THE LIGHT CHAIN**

**Investigators:** Michael D. Waterfield, Vincent R. Farnsworth, James W. Prahl, Leroy E. Hood  
**Support:** National Institutes of Health, Public Health Service  
National Science Foundation

The amino acid sequence of the light chains from two rabbit antibodies raised against different antigens is being determined to elucidate the relationship of functional and genetic studies to primary structure. Similar studies on the heavy chains are described in Biology 1972, Nos. 231 and 232.

This study is made possible because the two light chains are homogeneous by electrophoretic, serological and sequence criteria and are available in sufficient quantity—criteria which have only recently been achieved due to advances in immunization and antibody purification techniques. The light chains are ca. 210 residues long and have been broken into four smaller pieces by enzymatic cleavage with trypsin at arginines (3 residues/mole) after blocking of the normally susceptible lysines (7 residues/mole). These peptides have, in part, been separated by a combination of gel filtration and ion exchange procedures. The peptides obtained account for residues 1-50, 51-62, 62-138, 138-207 and 208-210.

These studies are being carried out in collaboration with Drs. T. Kindt and R. Krause at the Rockefeller University in New York and Dr. D. Hoffman at the USC Medical Center, Los Angeles.

**230. REPRESSION OF IMMUNOGLOBULIN GENETIC MARKERS IN RABBITS**

**Investigator:** James W. Prahl  
**Support:** National Institutes of Health, Public Health Service

The immunoglobulins of rabbits possess serological determinants (allootypic markers) which can be used for genetic studies. One group of these, $a_1$, $a_2$ and $a_3$, behave as alleles and are believed to be located in the variable region of the heavy chain.
Repression of these allotypic markers may be achieved by injection of the appropriate antiallotype antisera into newborn rabbits. If repression is achieved in a heterozygotic rabbit, there is a compensatory rise in the level of the immunoglobulins carrying the unrepressed allelic allotypic specificity. Repression in a homozygous rabbit is accompanied by a compensatory increase of immunoglobulins apparently bearing no determinant, that is, they are allotypically "negative." 

Structural studies of the heavy chains of such allotypically "negative" IgG immunoglobulins obtained from repressed a1, a2 and a3 homozygotes have been undertaken. Serologically and structurally the carboxy-terminal half of the "negative" heavy chains was indistinguishable from those of normal, allotype-bearing heavy chains. The N-terminal half of the heavy chains of "negative" IgG, on the other hand, revealed amino acid compositions distinctly different from their normal counterparts. Pronase digestion was employed to obtain the N-terminal tripeptides of the immunoglobulins. An initiating sequence of PCA-Glu-Gln was found in all the "negative" chains studied, although this sequence is usually observed in only a small fraction of allotypically normal heavy chains. Significant compositional differences exist between the N-terminal cyanogen bromide cleavage peptides (34 residues) of "negative" chains and those of the parent allotypes. Indeed, compositionally these peptides derived from the "negative" chains resembled each other more closely than they resembled their normal counterparts. It was concluded that "negative" heavy chains carry variable regions selected from a genetic locus other than that which encodes the group as specificities.

This work was carried out in collaboration with Drs. B. Tack and C. Todd of the Department of Immunology, City of Hope National Medical Center, Duarte, California.

231. HEAVY CHAIN SEQUENCE STUDIES ON RESTRICTED RABBIT ANTIBODIES  
Investigators: James W. Prahl, Leroy E. Hood  
Support: National Institutes of Health, Public Health Service  
National Science Foundation  
Structural studies of the variable regions of heterogeneous normal rabbit heavy chains suggest that primary sequence plays a role in determining allotypic specificity. If multiple germ line \( V_H \) genes exist, as appears to be the case, then the origin and maintenance of genetic markers in a multigene system must be explained. This problem is being approached through the use of heavy chains derived from homogeneous rabbit antibodies directed against the hapten, para-azobenzoate. It is hoped that an understanding of the nature of the determinants of allotypic specificity may be obtained from the sequence analysis of the \( V_H \) regions of a number of such heavy chains.  
This work is being carried out in collaboration with Dr. D. Hoffman of the University of Southern California, Los Angeles, California.

232. RESTRICTED HOMOGENEITY IN RABBIT ANTIBODIES: MIGHT DIFFERENT HEAVY CHAINS HAVE A COMMON LIGHT CHAIN?  
Investigators: Michael D. Waterfield, James W. Prahl, Leroy E. Hood  
Support: National Institutes of Health, Public Health Service  
National Science Foundation  
During the course of our studies on the primary structure of rabbit antibodies, one antibody was isolated which seems to have a single light chain associated with two or more different heavy chains. The light chain appears to be a single molecular species by serological, electrofocusing, and automatic sequence analysis of the N-terminal portion of this chain. The complete sequence analysis of this chain is now under way and we should soon be able to determine the extent of its homogeneity. On the other hand, the heavy chain appears to be comprised of two or more molecular species by exactly the same criteria. Serological examination suggested that there were at least two molecular species and this supposition was confirmed by electrofocusing studies. Finally, two distinct N-terminal peptides have been isolated from the heavy chain of this antibody.  
These observations suggest that a single light chain may be attached to two or more different heavy chains, giving rise to antibodies of similar specificity. This observation raises an interesting question as to whether the same or different clones of cells are producing the two or more
molecular species of antibody.
This work was carried out in collaboration

233. ANTIBODY LIGHT CHAINS OF RESTRICTED HETEROGENEITY

Investigator: Leroy E. Hood
Support: National Institutes of Health, Public Health Service

One of the most powerful modern techniques in the study of immunoglobulin structure has been the use of streptococcal vaccines to induce antibodies of highly restricted heterogeneity (and occasional homogeneity!) against simple carbohydrate determinants (e.g., glucosamine). This approach has been successfully employed in the nurse shark to generate highly restricted antibody responses. One of the responses that we have studied appears to be homogeneous by electrophoretic criteria and by automatic sequence analysis of the corresponding light chains. Gram quantities of this antibody are available. Other light chain preparations from streptococcal antibodies also demonstrated a marked restriction of heterogeneity when compared against normal shark immunoglobulin light chains. The shark light chains show a surprising degree of amino acid sequence homology with the \( \kappa \) chains of mammals. The structure of shark immunoglobulins is of interest because these animals diverged from their mammalian counterparts more than 400 million years ago. Thus sequence analyses should provide insight into the evolution of variable as well as constant immunoglobulin regions over a long period of evolutionary time.

The project was carried out in collaboration with Dr. W. Clem from the Department of Immunology and Medical Microbiology, University of Florida, Gainesville, Florida.

Reference:

234. THE PRIMARY STRUCTURE OF NORMAL GAR IMMUNOGLOBULIN LIGHT CHAINS

Investigators: Stephen M. Beverley*, Ronald T. Acton
Support: National Institutes of Health, Public Health Service

The study of gar immunoglobulin light chains is of interest in two respects: (1) one can determine whether or not two types of light chains, \( \lambda \) and \( \kappa \), are present as in higher creatures; and (2) one can ascertain the extent of divergence that has occurred since the gar, a primitive bony fish, and mammals diverged more than 300 million years ago. Since normal immunoglobulin light chains are a collection of many different variable regions, attached presumably to one or two distinct constant regions, only the primary structure of the constant region can be determined. Preliminary sequence results suggest that there is a major class of light chains in the gar which is homologous to \( \lambda \) chains in higher organisms. Currently large quantities of gar light chains are being isolated for enzymatic fragmentation followed by ion exchange separation of the resulting peptide fragments. The purified peptides will then be sequenced by automatic and manual methods.

Concurrently studies have been carried out in an attempt to obtain antibodies of restricted heterogeneity using immunization procedures described earlier (see Biology 1972, No. 233). Unfortunately, electrophoretic analysis of these samples revealed no restriction of heterogeneity and accordingly the possibility of obtaining material for \( V \) region sequence analysis seems remote at this time.

Reference:

*NSF Summer Trainee, 1972.
235. NONHISTONE CHROMOSOMAL PROTEINS OF DROSOPHILA

Investigator: Sarah C. R. Elgin
Support: Jane Coffin Childs Memorial Fund for Medical Research
         National Institutes of Health, Public Health Service
         National Science Foundation

There are several considerations which make *Drosophila* the creature of choice for the study of nonhistone chromosomal proteins (NHC proteins). Prominent among these are the available genetics (particularly chromosome morphology mutants), the possibility of being able to isolate single metaphase chromosomes in large quantities, and the available polytene chromosomes, which show visible gene activation in response to hormones, in the third instar larvae. Accordingly we have established a fly room for mass production of *Drosophila* embryos (250,000 adults maintained) according to the methods of Dr. D. S. Rogness and Dr. W. J. Peacock (Stanford Medical School, Department of Biochemistry). Such a fly room will yield from 10 to 30 g of embryos per 12-hour collection. Several methods of preparing chromatin have been explored, and the best selected on the basis of yield of chromatin and purity of the histones which can be acid-extracted from the chromatin. These techniques are currently being used to prepare chromatin from 6 to 18-hour embryos, 0 to 2-hour embryos (syncytial eggs), and polytene nuclei from third instar larvae which have or have not been treated with hormones, etc. The chromosomal proteins from these preparations are being examined by comparative gel electrophoresis to look for proteins of biological interest.

236. FURTHER STUDIES ON THE ISOLATION AND CHARACTERIZATION OF NONHISTONE CHROMOSOMAL PROTEINS

Investigator: Sarah C. R. Elgin
Support: Jane Coffin Childs Memorial Fund for Medical Research
         National Institutes of Health, Public Health Service
         National Science Foundation

We are interested in studying the protein chemistry of isolated nonhistone chromosomal proteins (NHC proteins) for several reasons. We wish to determine the extent of the microheterogeneity among proteins of a given molecular weight fraction, to look at the constancy of these proteins throughout evolution, and to try to relate these protein structures to the structure of chromatin. We hope to prepare antibodies to the purified proteins in order to explore the distribution and function of NHC proteins. We have made some improvements in our previously published method of NHC protein preparation and fractionation (Elgin and Bonner, 1972) and have explored several other methods which have recently been published. The method cited still appears to be relatively good in terms of protein recovery and efficiency; however, some of the other methods now available use solvent conditions which are less denaturing. Several types of ion exchange chromatography have been examined for the further fractionation of NHC proteins. The most promising of these is a carboxymethyl cellulose column developed with a pyridine formate gradient in salt concentration and pH (Hindennach, Stöffler and Wittmann, 1971).

References:

237. COMPARATIVE GEL ELECTROPHORESIS STUDIES OF NONHISTONE CHROMOSOMAL PROTEINS

Investigators: Flora C. Wu*, Sarah C. R. Elgin
Support: Jane Coffin Childs Memorial Fund for Medical Research
         National Institutes of Health, Public Health Service
         National Science Foundation

Nonhistone chromosomal proteins (NHC proteins) of different rat tissues (liver, kidney, spleen, lung, brain, thymus and thyroid) have been examined by comparative sodium dodecyl sulfate
(SDS) gel electrophoresis (molecular weight sieving) and Laemmli gel electrophoresis (molecular weight sieving and charge differentiation). The tissues were removed from freshly killed rats and stored in −80°C freezer until use. The procedure followed in the preparation of these NHC proteins is that given in Elgin and Bonner (1970). However, in preparing chromatin from lung and brain, it is necessary to wash the crude nuclear pellet with 1 M sucrose to remove lipid present.

The liver NHC protein was taken as a standard for comparison with other NHC protein preparations. Both the SDS and the Laemmli gels revealed that there were many protein bands common to all the tissues and that there were some tissue-specific bands. The brain NHC protein showed the most differences by SDS gel electrophoresis. The results indicate that there are tissue-specific protein bands among the NHC proteins, which are consistent with their proposed role as tissue-specific modulators of gene transcription.

Further work will be done to determine whether liver and brain NHC proteins of different organisms in the taxonomic range (Mammalia, Aves, Reptilia, Amphibia, and Pisces) have similarities and differences comparable to that shown by rat liver and brain NHC proteins. Moreover, the various regions of similarity and differences will be looked at more carefully with gel isoelectrofocusing.

Reference:

**238. THE ISOLATION OF TRANSPLANTATION ANTIGENS FROM THE MOUSE**

*Investigator:* Jack Silver

*Support:* National Institutes of Health, Public Health Service
National Science Foundation
Albert Einstein College of Medicine

During the past year we have developed a procedure for the purification of the H2 alloantigens in order to undertake chemical analysis and sequencing of these glycoprotein molecules. Although papain-digested H2 alloantigens have been prepared and extensively purified by conventional procedures, they appear to be heterogeneous, probably as a result of unspecific cleavage by papain. Also, since the alloantigens are the product of an enzymatic cleavage, they do not represent the complete product of the H2 locus. In order to overcome these disadvantages, we have purified H2 alloantigens prepared by solubilization of the membrane of whole spleen cells by the nonionic detergent NP40. The spleen cells have, prior to treatment with NP40, been cultured in the presence of 3H- or 14C-amino acid in order to label the H2 alloantigens. The crude NP40 extracts were then treated with H2-specific alloantiserum obtained from Dr. Snell (The Jackson Laboratory). Soluble antigen-antibody complexes were then precipitated by goat antimouse gamma globulin. After extensive washing, the precipitate was dissolved in sodium dodecyl sulfate (SDS) and purified by SDS acrylamide gel electrophoresis. Using H2 alloantisera of various specificities as controls, the major band obtained from SDS acrylamide gel electrophoresis was identified as the H2 glycoprotein. Using nonlabeled papain-digested H2 alloantigen of various specificities to compete with the labeled NP40 solubilized alloantigen for the specific alloantiserum, it was shown that the H2 glycoprotein was approximately 80% pure.

Subsequent manipulation allows us to purify these molecules to the 90% level. Microgram quantities of proteins from the D and K locus of two inbred mice differing at both loci are being prepared. These proteins will be investigated using microsequence techniques.

We believe that chemical analysis and sequencing of the H2 glycoproteins will serve to answer many questions concerning H2 histocompatibility antigens, e.g. (1) To what degree is there homology between the product of the D locus and K locus? (2) To what degree is there homology between the various H2 haplotypes (alleles at one locus)? (3) Are there variable and constant regions analogous to immunoglobulins? (4) Have the various haplotypes evolved from one or more common ancestors and by what mechanism?

This work was carried out in collaboration with Dr. S. Nathenson, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York.
AUTOMATIC DATA PROCESSING FOR AUTOMATIC SEQUENATOR AND AMINO ACID ANALYSES

Investigators: Donald E. Crede, David B. Helphrey, James W. Prahl, Leroy E. Hood

Support: National Institutes of Health, Public Health Service
        National Science Foundation
        Sloan Fund for Basic Research

In recent years, the amino acid sequence determination of proteins and peptides has been greatly accelerated through the use of the automatic protein sequenator developed by Edman and Begg (1967). The Beckman Model 890 Sequencer currently in use in our laboratory produces 12 samples daily, each of which requires the integration of some 25 chromatographic peaks (amino acid residues and certain of their derivative forms), the conversion of peak areas to molar ratios, the correction of the resulting yields against an internal standard, and precise quantitative comparisons from one sample to the next in order to determine the change in every amino acid yield at each position in the polypeptide chain. Protein sequencing also requires analysis of many protein and peptide hydrolysates for total amino acid composition. We have developed automated data handling procedures to eliminate errors, increase the sensitivity of the method, and free valuable research time. Further, it allows branching to programs which will do the trial-and-error data fitting peculiar to these analyses.

The automatic sequenator produces thiazolinone derivatives of amino acids which ordinarily feed into two parallel analytic pathways. On one pathway, part of the sample is converted to phenylthiohydantoin (PTH) derivatives which are run on the gas chromatograph. The Hewlett-Packard gas chromatograph integrates this data, and the peak areas and retention times are recorded on magnetic tape cassettes by a custom-built interface. On the other pathway, part of the sample is hydrolyzed to free amino acids which we analyze on one of two amino acid analyzers. The Durrum D500 analyzer integrates its chromatograms internally with a built-in PDP-8 computer, and converts the areas to molar quantities, producing both typed reports and punched paper tapes for further processing. The Beckman 121 analyzer generates only chromatograms which are recorded point-by-point on magnetic tape cassettes by another custom-built interface, leaving both integration and conversion to molar quantities to be done by further data processing.

Magnetic tapes and paper tapes are read into the PDP-10 computer at Booth Computing Center from terminals located in the laboratory for the final stage of processing. Data are integrated, converted to molar quantities, or corrected against an internal standard, depending on the source. From this point, the data may simply be printed out or fed into a variety of other programs for further refinement. Protein and peptide hydrolysate data may be fed into a molar ratio program which permits the researcher to experiment with multiplying factors to produce the set of molar ratios for which each amino acid is expressed most nearly as an integer. The data from an entire series of sequencer samples may be either tabulated or plotted graphically so that the amount of each amino acid may be readily followed through a series of cycles.

Data from a series of sequenator samples generally contain two components of "noise" resulting from incomplete cleavage of the thiazolinone derivative and random cleavage of the chain at each step. Our present effort is to adapt computer programs developed by O. Smithies and collaborators (1971) to our system so that we may filter out these noise components, permitting the sequencing of longer stretches of the protein and the determination of minor sequences in nonhomogeneous protein samples.

References:
240. L-PYRROLIDONE-2-CARBOXYLIC ACID PEPTIDASE: A SPORULATION-RELATED ENZYME IN B. SUBTILIS

Investigators: Cornell C. Phillips, James W. Prahl

Support: National Institutes of Health, Public Health Service

L-pyrrolidone-2-carboxylic acid (PCA) peptidase has been isolated from a number of bacterial species. This enzyme hydrolytically cleaves PCA from the N-terminal of peptides and proteins and thus is under investigation for protein structure studies. PCA occurs as the N-terminal residue of a number of biologically important protein molecules including the immunoglobulins, fibrinogen, and serum glycoproteins. The presence of PCA precludes the use of the Edman automated sequenator which has proved so useful in establishing the sequence patterns of heterogeneous light chains from rabbits. An enzyme which could remove PCA from peptides in the molecular weight range of 25,000 would be an extremely valuable adjunct to N-terminal studies of the heavy chains of immunoglobulins.

The enzyme, as isolated from a Polish strain of B. subtilis, appears 2 to 3 hours after the culture enters stationary phase, spikes at 5 to 6 hours, and subsequently disappears. This disappearance of enzyme activity can be correlated with the appearance of spores and refractility as followed by phase microscopy. PCA peptidase activity can also be correlated with other sporulation enzymes. The appearance of PCA peptidase activity follows the highest peak of alkaline phosphatase activity and precedes that of glucose dehydrogenase, the latter being characteristically associated with stage III of sporulation, i.e. the foresaw septum. Replacement of glutamate, normally contained in the growth medium, by aspartate, citrate or proline indicated that PCA peptidase synthesis was not being repressed by the glutamate. A late sporulation mutant of B. subtilis (kindly supplied by Dr. J. Hoch), when grown in the usual medium, synthesized neither PCA peptidase nor glucose dehydrogenase. Therefore, the correlation of PCA peptidase activity with the pattern of other sporulation enzymes and the lack of PCA enzyme production by the sporulation-defective mutant would suggest that PCA peptidase belongs to the class of sporulation-related enzymes.

This enzyme is currently being isolated to evaluate its use in removing PCA groups from proteins and peptides.

References:

241. STUDIES ON THE STRUCTURE OF CHICKEN OVOTRANSFERRIN

Investigators: Jonathan S. Fuhrman, Leroy E. Hood

Support: National Institutes of Health, Public Health Service

Transferrins are a class of nonheme iron-binding proteins. Ovotransferrin is isolated from egg white, where it apparently has bacteriostatic and fungistatic properties by virtue of its high affinity for iron. From gel chromatography, sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis, and ultracentrifugation studies on fully reduced and alkylated transferrins, several groups concluded the transferrins are monomeric proteins of approximate molecular weight 80,000. However, transferrins yield only 1/2 to 2/3 the number of tryptic peptides expected. Further, Phillips and Azari (1971) reported that upon cleavage of native ovotransferrin with cyanogen-bromide (CnBr), only half the number of fragments expected on the basis of total methionine content were produced. These workers suggested that ovotransferrin is a monomeric protein formed by a linear gene duplication, so that the molecule has two nearly identical halves.

We are currently investigating the degree of homology between the two putative duplicated halves. If this hypothesis is correct, then each CnBr fragment should be an equimolar mixture of two very similar fragments. We are attempting to separate these fragments by ion exchange chroma-
tography. Using automated sequence determination techniques, we are also determining the degree of homology in each of the paired CnBr fragments before fractionation. Preliminary results suggest that the paired sequences are quite different and accordingly militate against the tandem gene duplication hypothesis.

Reference:

PUBLICATIONS


BIOPHYSICS
CELL BIOLOGY
PHYSIOLOGICAL PSYCHOLOGY
VIROLOGY

Marine Station
Alles-Kerckhoff
(First Floor)
Professor: Robert L. Sinsheimer

Research Fellows: J. Barklie Clements, Akio Fukuda, Paul H. Johnson, Lois K. Miller, Nicole Truffaut


Research Assistants: Gloria C. Davis, Peggy A. Lockhart, Alma J. Shafer

Laboratory Aide: Jeannette Johnstone

Research has continued upon the genetics, replication, structure and transcription of bacteriophage ΦX174. Continuing study of recombination between ΦX mutants under various conditions has led to a model for ΦX genetic recombination and insight into the role of the host rec A gene.

Continuing analysis of the process of replication of the ΦX double-stranded DNA rings has provided evidence for a new intermediate in replication; however, a clear picture of the stages in this complex process is not yet available.

Considerable progress has been made in analysis of the structure of ΦX DNA and of certain intermediates in its replication through the use of deletion mutants and of various restriction enzymes. The latter, which permit degradation of ΦX DNA into specific fragments, show considerable promise for determination of the specific location of loci of interest with respect to DNA replication and transcription, and ultimately with respect to determination of DNA sequences.

Studies of ΦX transcription, both in vivo and in vitro, continue to reveal unexpected complexities presumably related to the control of gene expression during viral infection.

242. A ROLE FOR SINGLE-STRAND "BREAKS" IN BACTERIOPHAGE ΦX174 GENETIC RECOMBINATION

Investigators: Robert M. Benbow, Anthony J. Zuccarelli

Support: National Institutes of Health, Public Health Service

Bacteriophage ΦX174 parental replicative form (RF) DNA was prepared from multiply-infected cells under various conditions in which the cells were blocked in progeny RF DNA replication (Benbow, Eisenberg and Sinsheimer, 1972). Neutral and alkaline velocity sedimentation gradients, and aqueous and formamide Kleinschmidt procedures (electron microscopy) were used to examine the structure of these DNA molecules.

The structure of damaged RF DNA molecules formed from ultraviolet irradiated bacteriophage ΦX174 consists of an intact viral strand and a partially complete complementary strand extending from the origin of parental RF replication to an ultraviolet lesion.

The structure of damaged RF DNA molecules formed in thymine-deficient host strains during thymine starvation consists of nearly unit length viral and complementary strands containing random single-strand breaks or gaps.

The structure of parental RF DNA molecules formed in the presence of cistron A product consists of an intact circular complementary strand and a viral strand containing a single-strand break or gap.

The formation of wt genetic recombinants in rec" cells containing any of the above structures is stimulated up to 50-fold; in a rec A host strain there is no increase.

These data suggest that single-strand "breaks" are necessary intermediate structures in the formation of ΦX174 genetic recombinants mediated by the host rec A" enzyme.


243. A MODEL FOR BACTERIOPHAGE ΦX174 GENETIC RECOMBINATION

Investigators: Robert M. Benbow, Anthony J. Zuccarelli

Support: National Institutes of Health, Public Health Service

Based on evidence obtained primarily by electron microscopy, genetic recombination between two ΦX174 parental replicative form (RF) DNA molecules occurs by the following sequence of events: (1) a single-strand scission in one RF DNA molecule; (2) displacement synthesis; (3)
unimolecular branch migration; (4) attack; (5) formation of a hydrogen-bonded joint molecule; (6) bimolecular branch migration and further DNA (repair?) synthesis; (7) and (8) covalent bond formation and single-strand scission (order not certain); (9) restoration of the recombinant DNA molecule to the parental RF configuration.

The net result (confirmed by a “single-burst” experiment) is an asymmetric nonreciprocal recombination event yielding one parent and one recombinant. The average net DNA synthesis is less than 600 nucleotides (confirmed by a “density exchange” experiment) and breakage and reunion occurs. Single-strand insertion is (probably) the most common ΦX174 recombination event. Recombinat formation is complete at or shortly after the initiation of progeny RF DNA synthesis; the completed recombinant DNA molecule and surviving parental molecule then resume normal ΦX174 DNA replication.

A (minor) secondary recombination mechanism exists in which recombinant formation occurs between ΦX174 progeny RF DNA molecules.

244. HIGH NEGATIVE INTERFERENCE DURING BACTERIOPHAGE ΦX174 GENETIC RECOMBINATION

Investigator: Robert M. Benbow
Support: National Institutes of Health, Public Health Service

The occurrence of high negative interference during ΦX174 genetic recombination was previously established (Benbow et al., 1971). Since physical distances (in nucleotides) between genetic markers on the ΦX174 genome are known (Benbow et al., 1972) accurate determination of the high negative interference exhibited by various markers can be used to estimate the physical size of genetic exchange regions over the entire genome.

Using this method we have estimated that over 50% of all ΦX174 genetic exchanges involve regions of less than 400 nucleotides. Furthermore, multiple or successive mating events are extremely rare (<1 in 10⁴ recombination events).

In contrast, little if any high negative interference is observed in a rec A host (except the obligatory negative interference imposed by a small circular genome).

References:

245. DELETION MUTANTS OF ΦX174

Investigators: Anthony J. Zuccarelli, Robert M. Benbow
Support: National Institutes of Health, Public Health Service

A mutant of bacteriophage ΦX174 which lacks a region of the normal phage genome has been isolated. Polyacrylamide gel electrophoresis of purified virions has shown that the deletion virus coat has essentially the same protein composition as the normal virus. The mutant virus particles, however, are 0.0044 ± 0.0003 g/cm³ less dense in CsCl than wild-type ΦX174 particles. If this observed density difference is attributed to the deletion of DNA in the mutant viruses, an estimate of the extent of the deletion may be made using the relationship formulated by Weigle, Meselson and Paigen (1959):

$$\Delta \rho = \rho_0 \frac{F_m - F_v}{1 + \alpha F_v}$$

We obtain a value of 6.3% for the per cent of ΦX wt DNA deleted from the mutant virus.

DNA extracted from the deletion particles is 6.8% shorter than the DNA extracted from wt ΦX174 when viewed in the electron microscope. When heteroduplex DNA molecules are constructed by annealing DNA from the deletion viruses to the complementary strand from wt ΦX, many of the resulting double-stranded molecules have a small, single-stranded loop when observed in the electron microscope (see Plate 1). The contour length of the loops measured in 74 heteroduplex molecules was 8.2% of the wt ΦX genome.

The deletion viruses complement amber mutations in ΦX cistrons A, B, D, F, G and H but they do not complement mutants in cistron E. This genetically identifies the site of the deletion as cistron E, the cistron which controls the host lysis function.

In summary, our results indicate that we have isolated a population of deletion mutations of bacteriophage ΦX174 that are (1) genetically...
defined within the cistron E region, (2) physically defined as individual deletions with an average size of 7% of the ΦX genome (about 390 nucleotides), (3) able to propagate as efficiently as wild-type ΦX174, and (4) possess an essentially normal viral coat structure and all viral functions except host cell lysis. We have called these mutants del E.

References:

246. THE ORIGIN OF ΦX174 COMPLEMENTARY STRAND SYNTHESIS

Investigators: Anthony J. Zuccarelli, Robert M. Benbow

Support: National Institutes of Health, Public Health Service

When the single-stranded DNA from a ΦX174 bacteriophage particle penetrates a bacterial cell, a complementary DNA strand is immediately synthesized by the enzymes of the host, generating a double-stranded molecule called the replicative form (RF). However, when the virus particles are irradiated with ultraviolet light (UV) prior to infection, only a portion of the complementary strand is synthesized by the host (see Biology 1972, No. 242). We have observed only one double-stranded region in each UV-damaged RF molecule. The average length of the double-stranded region decreases with increasing UV dose, suggesting that complementary strand synthesis begins at a single site.

We have isolated the complementary strand fragments from the incomplete RF molecules and annealed them to circular viral DNA extracted from a ΦX174 mutant with a deletion in cistron E (del E, see Biology 1972, No. 245). Some of the complementary strand fragments span the lesion of the deletion DNA and form a small, single-stranded loop which can be identified in the electron microscope. Measurements are being made of such electron micrographs to determine if, indeed, complementary strand synthesis has a unique origin.

Plate 1. Heteroduplex DNA molecules containing one strand from wt φX174 and one from the deletion mutant. 153,000 X.
247. INSERTION MUTANTS OF $\Phi X174$

Investigator: Anthony J. Zuccarelli

Support: National Institutes of Health, Public Health Service

Deletion mutants of $\Phi X174$ are potentially useful tools for determining the locations of various structural features of the $\Phi X$ genome by electron microscopy. However, the loss of a portion of the $\Phi X$ genome is almost invariably lethal since only cistron E of the 9 known cistrons is dispensable to the production of infective virus particles. Even deletions in cistron E have undesirable properties. Since the cistron E product is necessary for the viruses to lyse the infected host cell, deletions in that gene (del E, see Biology 1972, No. 245) cannot form plaques. The concentration of phage in del E stocks must be determined by a time-consuming plaque assay technique requiring "helper" phage.

To avoid these disadvantages we have attempted to isolate insertion mutants, a class of mutations which have gained rather than lost a segment of DNA. Conceivably, an extra piece of DNA could be inserted between cistrons without destroying their protein-coding capability. Such mutants would have wild phenotype.

The isolation of insertion mutants was based upon their expected change in buoyant density. Phage were grown in the presence of mitomycin C, a mutagenic antibiotic, in a mitomycin-sensitive host strain (her$^+$, uvr A$^+$) infected at a multiplicity of 20 phage per cell. The resulting viruses were subjected to equilibrium buoyant density centrifugation in CsCl. Fractions from the gradient which were at least 0.005 $g/cm^3$ more dense than the position of the phage peak were pooled and the viruses in those fractions were used to infect more cells at a multiplicity of one phage per cell without mitomycin C. The progeny of this second infection were subjected to isopycnic centrifugation with selection of phage at positions of greater than wild-type density was repeated sequentially four times.

Phage selected from the final CsCl gradient were more dense than wt $\Phi X174$ when both were centrifuged to equilibrium in the same CsCl gradient. Experiments are in progress to determine if the observed increase in density is genetically transmitted and if it is the result of an alteration in the DNA content of the viruses.

248. HETERODUPLEX MAPPING OF THE $\Phi X174$ GENOME

Investigator: J. Lee Compton

Support: National Institutes of Health, Public Health Service

The heteroduplex mapping technique developed by Davis, Simon and Davidson (1971) is being used to relate the genetic map of the $\Phi X174$ genome to the physical DNA of the genome. Cistron E has already been visualized by Zuccarelli, Benbow and Sinsheimer (1972) using a deletion mutant in that cistron. Heteroduplexes are now being made between the DNAs of $\Phi X174$ and of $\Phi X174$-like bacteriophages S13, $\alpha$-3, and ST-1.

Artificial replicative form II (RF II) molecules with a single nick in one strand of the circular duplex are made from RF I (covalently closed circular duplex) by exposure to X-irradiation. On addition of alkali this gives a mixed population of denatured RF I molecules and circular and linear molecules of both positive (viral) and negative strands.

The RF mixture is mixed with the positive strand of another bacteriophage, denatured in alkali, and renatured in formamide. The renaturation mixture is then prepared and examined under the electron microscope. When the DNAs from the phages S13, $\alpha$-3, or ST-1 are hybridized to $\Phi X174$ DNA, single-stranded loops are seen in 15% of the RF II molecules. If the $\Phi X174$ RF mixture is denatured and renatured without adding additional DNA (or if $\Phi X174$ viral strands are added to the mixture) no single-stranded loops are seen.

Work is in progress to determine optimum conditions for renaturation of the hybrids and for visualization of the single-stranded loops.

References:


249. USE OF PROPIDIUM DIIODIDE-CsCl BUOYANT DENSITY GRADIENT TO ANALYZE VARIOUS FORMS OF ΦX174 DNA

Investigator: Akio Fukuda

Support: National Institutes of Health, Public Health Service

Buoyant density gradients containing intercalating dyes have been effectively used to separate open and closed forms of circular DNA and to analyze the superhelix density of closed circular DNA (Bauer and Vinograd, 1968). Propidium diiodide (PDI)-CsCl buoyant density gradients were used to analyze various forms of ΦX174 DNA and intermediates of replication. In the absence of the dye, both the closed and open circular duplex forms (RF I and RF II) have a buoyant density of 1.709 gm/cm³ and the single-stranded ΦX viral DNA of 1.725 gm/cm³ (Sinsheimer, 1968).

The relative density shift in the presence of PDI permits the separation of these forms of ΦX DNA. At a PDI concentration of 100 µg/ml, RF I banded at a density of 1.578 gm/cm³; single-stranded DNA at 1.555 gm/cm³; RF II at 1.516 gm/cm³. As expected from the above, the replicating intermediate of single-stranded DNA synthesis at the late stage of ΦX infection, namely RF II molecules with a single-stranded tail, banded in the PDI-CsCl gradient between single-stranded DNA and RF II, as a broad shoulder spreading from the RF II peak. The breadth of the shoulder is due to the heterogeneous size of the single-stranded tail. Incomplete RF II that has an appreciable segment of single-strandedness (Biology 1972, No. 250) also banded as a broad shoulder from the RF II peak.

Thus the PDI-CsCl buoyant density gradient serves as a useful method to analyze the various forms of ΦX DNA and intermediates of replication depending on the superhelicity and single-strandedness of the DNA molecules.

References:


250. STUDIES ON THE MECHANISM OF ΦX174 RF REPLICATION

Investigator: Akio Fukuda

Support: National Institutes of Health, Public Health Service

Replication of the circular double-stranded replicative form (RF) DNA proceeds during the first 20 minutes of ΦX174 infectious process to produce 20 to 30 RF molecules per host cell. Originally Knippers, Whalley and Sinsheimer (1969) reported some evidence to support the rolling circle (α) model for RF replication. They proposed an RF intermediate molecule that has a duplex tail with the complementary strand (+) longer than the unit length. Meanwhile Dressler and Wolfson (1970) suggested that the RF intermediate molecule has a duplex tail with the parental strand (+) longer than the unit length, rather than the minus (−) strand. Several experiments were carried out to clarify the above discrepancy and to study further the details of ΦX RF replication.

Freshly grown E. coli H502 HCR'thy'endo I was treated with mitomycin C (200 µg/ml) and infected at 36°C with am3, a lysis-defective mutant of ΦX174, in the presence of chloramphenicol (35 µg/ml) to prevent single-stranded DNA synthesis. At 20 minutes after infection, the infected culture was pulsed with 3H-thymidine (5 to 10 µc/ml) for 5 to 10 seconds and immediately cooled by pouring onto the crushed ice of 1/3 volume tris HCl-EDTA-poison (azide plus cyanide) in a dry ice-methanol bath. The short-pulsed DNA was extracted by lysozyme-SDS-pronase, phenol-extracted, isopropanol-precipitated, RNase-treated and further purified by neutral sucrose gradient centrifugation. The whole ΦX DNA fractions were pooled, shown to have the expected distribution of pulse counts in both plus (+) and minus (−) strands, and then banded in the neutral propidium diiodide (PDI, 100 µg/ml)-CsCl buoyant density gradient (Biology 1972, No. 249). The label incorporated into DNA in a 5-second pulse separates into 3 distinct fractions in the PDI-CsCl gradient: a peak (fraction A) at the buoyant density (1.516 gm/cm³) of the nicked RF (RF II); a broad shoulder (fraction B) that extends from the RF II peak to the position where single-stranded DNA would band (ρ = 1.555 gm/cm³); a peak (fraction D) at a density (1.597 gm/cm³) heavier than that of the normal supercoiled RF.
(RF I) \((\rho = 1.578 \text{ gm/cm}^3)\). The 5-second pulse counts distribute almost equally among these 3 fractions. The label incorporated during a 20-second pulse had these three components plus an additional peak (fraction C) at the buoyant density \((1.578 \text{ gm/cm}^3)\) of RF I. The pulse counts predominate in the RF II fraction in the 20-second pulse.

These 4 fractions above were further analyzed by sucrose velocity sedimentation and alkaline CsCl equilibrium centrifugation and electron microscopy. Fraction A contained the short-pulse label only in the linear strand of unit length and cosedimented sharply with the normal RF II \((16S)\). There were no fast-sedimenting pieces that could be the replicating intermediates of Cairns \((\theta)\) or rolling circle \((\sigma)\) model. Thus the fraction A is composed of completed RF II molecules with the pulse label only in the open, plus \((+)\) strand.

Fraction B sedimented with an S value 16 to 21 in a high-salt neutral sucrose gradient. In alkali, it released small fragments (Okazaki fragments, Okazaki et al., 1968) of 9S that were shown to be composed only of the minus \((-)\) strand. Under the electron microscope (Kleinschmidt-formamide method) fraction B was observed to contain both Cairns type molecules \((\theta)\) and rolling circle \((\sigma)\) model molecules \((\sigma)\) at similar frequencies (ca. 1 in 300 molecules). The Cairns type molecules were observed to have appreciable single-stranded segments at the branch points. The existence of the rolling circle type molecules is most likely to arise from breakage of Cairns type molecules. RF molecules with a single-stranded tail were also observed in fraction B. Since no single-stranded DNA synthesis of the late stage of infection occurs in the presence of chloramphenicol, these molecules might be similar to those observed by Francke and Ray (1972) in the \(\Phi X\)-infected \(E.\ coli rep\) mutant. However, in fraction B the majority of molecules observed under the electron microscope were incomplete RF II molecules, namely circular single-stranded DNA with duplex regions of heterogeneous size. Most probably these incomplete RF molecules are the first product of RF replication and the Okazaki fragments [minus \((-)\) strand only] were released mostly from these molecules.

Fraction C contained supercoiled molecules (RF I) that have the pulse label predominantly in the minus \((-)\) strand.

Fraction D contained supercoiled DNA molecules as judged by the buoyant density relative to the normal RF I in the neutral PDI-CsCl gradient and as observed by the electron microscope. Fraction D of the 5-second pulse DNA released, in alkali, about 50% of the pulse count as the single-stranded piece. Another 50% of the counts remained in the denatured RF I. The released single-stranded piece turned out to be composed only of plus \((+)\) strand, much longer (1.5 to 1.7 times) than the \(\Phi X\) unit length. The counts remaining in the denatured RF I were predominantly in the minus \((-)\) strand as were the short-pulse counts in the native RF I of fraction C. It therefore seems that fraction D contains the supercoiled DNA with a long single-stranded moiety \([+\text{ (+) strand}]\) hydrogen-bonded to it. The incorporation pattern of the short-pulse counts in fraction D suggests that these complex supercoiled DNAs are involved in DNA replication. It is ruled out that fraction D arises from the episomal element that might be present in the host cell.

The final elucidation of \(\Phi X174\) RF replication certainly requires the determination of the DNA structure of fraction D. However, it seems reasonable to state the following at this moment: (1) RF replication does not follow the rolling circle \((\sigma)\) model as previously thought; (2) supercoiled RF molecules are more closely involved in the replication event as intermediates than the previous idea that they were a trivial product from RF II due to the ligase action; (3) there are supercoiled RF molecules with single-stranded DNA hydrogen-bonded to them and Cairns type \((\theta)\) molecules as the possible intermediates of replication, with incomplete RF molecules as the first product of replication; (4) there are Okazaki fragments in \(\Phi X174\) RF replication.

References:


251. THE PRODUCTION OF UNIQUE FRAGMENTS OF ΦX174 RF BY SEQUENCE-SPECIFIC DEOXYRIBOENDONUCLEASES: I. THE RESTRICTION ENZYME FROM HEMOPHILUS INFLUENZAE STRAIN Rd

Investigators: Amy Shiu Lee, Paul H. Johnson
Support: McCallum Fund
American Cancer Society
National Institutes of Health, Public Health Service

Several restriction deoxyribonucleases have recently been described which produce a large number of unique fragments from high molecular weight foreign DNA, are not associated with a modification activity, and require no special cofactors other than divalent metal ions. These enzymes will be very useful in studying DNA structure.

We are specifically interested in producing sets of unique linear fragments of ΦX RF in order to establish reference points on the ΦX genome for mapping such functions as initiation of DNA replication and transcription; and in addition for the localization and isolation of genes and eventual nucleotide sequence determination of specialized regions of interest.

We have isolated and purified the restriction endonuclease from *H. influenzae* (endo R) according to the procedure of Smith and Wilcox (1970). The resulting enzyme preparation contained no contaminating exonuclease activity. Upon complete hydrolysis of purified ΦX RF I the enzyme produced at least 12 specific fragments accounting for 99% of the total ΦX genome. These fragments are well separated by electrophoresis on 5% polyacrylamide gels, and appear to be equimolar with the original DNA. The molecular weights of the fragments were determined by electrophoretic mobility and sedimentation velocity analysis on isokinetic neutral sucrose gradients. The fragments range in size from 1000 to 150 base pairs. The 5'-end groups of the fragments were determined by reacting the dephosphorylated reaction products with polynucleotide kinase and γ-32P-ATP. The labeled 5'-ends were identified as adenine (50 to 57%) and guanine (30 to 40%). These results are in agreement with the proposed recognition sequence of the restriction enzyme: G-T-PylpPu-A-C.

References:

252. THE PRODUCTION OF UNIQUE FRAGMENTS OF ΦX174 RF BY SEQUENCE-SPECIFIC DEOXYRIBOENDONUCLEASES: II. THE RESTRICTION ENZYME FROM HEMOPHILUS PARAINFLUENZAE

Investigator: Amy Shiu Lee
Support: McCallum Fund

*H. parainfluenzae* differs from *H. influenzae* in its cofactor requirements, but both strains of bacteria contain an endonuclease which produces specific double-stranded breaks in foreign DNA. We have isolated the restriction endonuclease from *H. parainfluenzae* (endo P). The enzyme was further purified from contaminating exonuclease activity by column chromatography on DEAE-cellulose. Using ΦX174 RF I as substrate this enzyme produces at least 8 specific terminal fragments, ranging in size from 2000 to 160 base pairs. The fragments produced by endo P are distinct from those produced by endo R, indicating a difference in the base sequence of the recognition site of the two enzymes.

Reference:

253. THE INITIATION SITE FOR SINGLE-STRAND DNA SYNTHESIS

Investigator: Paul H. Johnson
Support: American Cancer Society
National Institutes of Health, Public Health Service

A theory of DNA replication has been proposed by Denhardt (1972) which accounts for many of the known facts concerning DNA synthesis in vivo. It is postulated that proteins
exist called \( \tau \) which recognize specific sequences (\( \pi \) sequences) in DNA, and act in concert with an ATP-dependent replication nuclease which inserts single-strand nicks in double-stranded DNA so that the two strands may be unwound and separated during replication. One of the specific predictions of this model is the existence of gaps in newly-synthesized DNA. It is postulated that these gaps are recognition sites for the \( \pi \) proteins and the initiation sites for DNA synthesis.

In the course of studies on the structure of RF II molecules isolated during progeny single-strand DNA synthesis, it was discovered that a large proportion of these molecules contain gaps in the viral strand. In an attempt to determine the significance of these gapped molecules with respect to the mechanism of single-strand DNA synthesis we have completed the following experiments.

Gapped RF II molecules were reacted with \( E. \) \( coli \) DNA polymerase I, \( E. \) \( coli \) polynucleotide ligase and \( \alpha^{32}P\)-ATP. The repaired RF II molecules specifically labeled in the original gapped region were isolated from the reaction mixture as RF I. These molecules were degraded by endonuclease R (\( H. \) \( influenzae \)) to a set of unique terminal fragments and separated by electrophoresis on 5% polyacrylamide gels. The radioactivity profile clearly showed that all of the \( 32P \) repair synthesis counts were contained in one specific fragment (R-3) of the 12 fragments produced by endonuclease R.

The R-3 fragment has been found to map in cistron A (Hutchison, Middleton, Edgell, Jaffe and Chen, personal communication), the gene required for DNA replication. The gel profile of endonuclease R-degraded, late RF II which has not been repaired by polymerase shows that the cistron A fragment (R-3) has moved to a unique position of greater mobility (compared to the control). This is evidence for the presence of a gap, probably of uniform length, in this fragment. By quantitation of the radioactivity profile of endonuclease R-degraded, late RF II (compared to a control) the size of the gap is estimated at 200 ± 50 nucleotides.

It is suspected that the existence of gaps in late RF II may depend on the ATP content of the cell during the time the infection is stopped. This notion is currently being investigated.

**Reference:**


### 254. STRUCTURAL STUDIES OF INTERMEDIATES ISOLATED DURING \( \Phi X174 \) SINGLE-STRANDED DNA SYNTHESIS

**Investigator:** Paul H. Johnson  
**Support:** American Cancer Society  
National Institutes of Health, Public Health Service

A series of studies has been initiated to isolate and characterize unique nucleotide sequences in \( \Phi X174 \) DNA which are involved in the initiation and control of specific viral functions, e.g. DNA replication.

Double-stranded \( \Phi X174 \) DNA rings, composed of a closed complementary strand and a discontinuous viral strand are found in \( \Phi X \)-infected cells during the period of progeny single-stranded DNA synthesis (late RF II). We have studied the structure of these molecules by various physical and enzymatic methods with the following results.

Reaction of late RF II with polynucleotide phosphorylated 5'-hydroxyl group (from the result with T4 polymerase). Other molecules may contain a modified 5'-end, a hydroxyl group at the 5'-end, or some structural anomaly associated with the gapped region of the molecule. The involvement of these gapped molecules and uniquely-terminated short fragments in the mechanism of single-strand synthesis are currently being investigated.
255. A PHYSICAL GENETIC MAP OF BACTERIOPHAGE ΦX174: DIRECTION OF DNA SYNTHESIS

Investigator: Paul H. Johnson

Support: American Cancer Society, National Institutes of Health, Public Health Service

The discovery of late RF II molecules which have gaps uniquely located at or near cistron A, will allow us to order all of the endonuclease R fragments with respect to the ΦX genetic map and determine the direction of DNA synthesis.

Late RF II molecules labeled with $^3$H only in the viral strands were reacted with E. coli DNA polymerase I and $\alpha^{32}$P-TTP. The reaction was allowed to proceed beyond the repair of the gapped region and resulted in the gradual replacement of the $^3$H-labeled viral strand with a $^{32}$P viral strand. The reaction was stopped after various time periods and the repaired molecules degraded to completion by endonuclease R and analyzed by polyacrylamide gel electrophoresis. The results show that with increasing reaction times more and more of the $^3$H-labeled fragments are replaced by $^{32}$P-labeled fragments. The order in which this occurs is the order of the fragments around the ΦX genome beginning at or near cistron A and proceeding in the 5'→3' direction. These results establish that the direction of DNA synthesis follows the cistron order A B C D E F G H. Recently a similar result was found for the origin and direction of double-stranded ΦX DNA replication by Baas and Jansz (1972).

Reference:

256. FRACTIONATION OF ΦX174 DNA FORMS BY AGAROSE-ACRYLAMIDE GEL ELECTROPHORESIS

Investigator: J. Barklie Clements

Support: National Institutes of Health, Public Health Service

Recently, Dingman, Fisher and Kakefuda (1972) have described the separation of the different SV40 DNA forms on composite agarose-acrylamide gels, and Aaij and Borst (1972) have reported separation of several linear and circular duplex DNA species on agarose gels containing ethidium bromide.

Intact supercoiled circular double-stranded ΦX DNA (RF I), nicked open circular double-stranded DNA (RF II), circular and linear single-stranded DNA forms have been separated by electrophoresis on composite agarose-acrylamide gels. The degree of separation compares favorably with that obtained by sedimentation and it is hoped that this procedure will prove suitable for analysis of intermediate structures concerned with DNA replication.

References:

257. BACTERIOPHAGE ΦX174 IN VIVO MESSENGER RNA

Investigator: J. Barklie Clements

Support: National Institutes of Health, Public Health Service

As yet, studies on the size and number of ΦX-induced messenger RNA (mRNA) species have not yielded unequivocal conclusions. Hayashi and Hayashi (1970, 1972) have analyzed ΦX mRNA by polyacrylamide gel electrophoresis. They identified some ten RNA species, a major component of 0.6 x 10$^6$ daltons with others ranging from 3 x 10$^6$ daltons (equivalent to two complete rounds of transcription) to 0.2 x 10$^6$ daltons. However, the studies of Sedat and Sinseheimer (1970), in which RNA was totally denatured by sedimentation in 99% dimethyl sulfoxide, indicated a main size class of 3 x 10$^5$ daltons with a sharp upper limit of 1.5 x 10$^6$ daltons.

In the present study, the size of ΦX mRNA has been examined in an attempt to determine aspects of the transcription process such as the number of sites on RF I responsible for initiation and termination of RNA synthesis. Virus-induced RNA was identified by hybridization to ΦX RF and by a double-labeling technique in which counts specific to infection were determined by analyzing a mixture of pulse-labeled RNA from infected and uninfected cells by agarose-acrylamide
gel electrophoresis and sucrose density gradient centrifugation. This technique appears to be at least as sensitive as hybridization and also allows host-specific RNA species which are influenced by infection to be identified.

Preliminary results indicated that short pulses (45 seconds) of $^{3}$H-uracil were incorporated into $\Phi X$ mRNA of molecular weight ranging from $4.5 \times 10^6$ to $1.5 \times 10^6$ daltons. In longer pulses (2 to 5 minutes) the amount of high molecular RNA was reduced considerably and a major induced species of $0.6 \times 10^6$ daltons was detected. The detailed nature of the high molecular weight RNA currently is under study in particular to determine whether this material is a precursor of the lower molecular weight RNA.

References:

258. $\Phi X 174$ IN VITRO mRNA

**Investigator:** Lloyd H. Smith

**Support:** National Defense Education Act

Further studies on $\Phi X 174$ mRNA made *in vitro* with pure RNA polymerase and $\Phi X 174$ RF I DNA have shown that discrete species of RNA are synthesized. These are labeled with different $5'-\gamma^{32}P$ purine triphosphates. Three such species are found in DMSO gradients at molecular weight positions of $1.3 \times 10^6$, $1.7 \times 10^6$ and slightly greater than $1.7 \times 10^6$ daltons; these become end-labeled with $\gamma^{32}P$-GTP, ATP, and GTP respectively. The ATP 5'-end and GTP 5'-end molecules are made in approximately equal amounts.

The sequences of these 5'-termini and their genetic location are being studied.

259. CHARACTERIZATION OF A 22S PROTEIN COMPLEX FROM THE $\Phi X$ INFECTION

**Investigators:** Robert G. Rohwer, Robert Mayol*

**Support:** National Institutes of Health, Public Health Service

At late times in infection we find a peak of pulse-labeled protein which cosediments with the replicative intermediates of $\Phi X$ DNA. This same region of the gradient shows strong binding of pulse-labeled DNA to anti-$\Phi X$ antibodies in the radioimmune assay of Rohwer (*Biology* 1970). This suggests the existence of a $\Phi X$ DNA-protein complex. Analysis of the proteins in this particle show its composition to be as follows: 60% is a protein of approximately 65,000 molecular weight suggestive of the gene A product (Godson, 1971); 20% is the gene F capsid protein; 20% is a very low molecular weight protein. The continuity of the protein complex does not seem to be affected by a variety of harsh ionic conditions nor is it disrupted by DNase or RNase. This suggests that DNA binding in the radioimmune assay may be mediated by some other nonpulse-labeled protein or that the DNA responsible for maintaining the integrity of the complex is not subject to DNase activity. These possibilities are being investigated as is the relationship of this particle to the $\Phi X$ maturation process.

Reference:

*Mead Johnson Research Center, Evansville, Indiana.

260. THE ISOLATION AND CHARACTERIZATION OF SOME INTERMEDIATE STRUCTURES IN THE $\Phi X 174$ MATURATION PROCESS

**Investigator:** Robert G. Rohwer

**Support:** National Institutes of Health, Public Health Service

An enduring enigma in the intensive characterization of the $\Phi X 174$ infection process has centered around the lack of intermediate structures in the phage maturation process. Nascent DNA-forms and newly completed phage particles can be observed by pulse labeling, but there has been no observation of the intermediate structures which must exist between these two stages of development. Furthermore, it has been a corollary of the normal $\Phi X$ infection process that no significant pool of intracellular free single-stranded (SS) DNA exists among the pulse-labeled DNA species.
We have searched for fast-sedimenting (greater than 23S) DNA-forms which might be indicative of such intermediates by comparing sedimentation profiles of cell lysates after very short and very long DNA pulses. With careful handling and by supplying the proper ionic environment one can obtain a peak containing twice as much pulse label in the 70S region of the gradient as can be accounted for by the production of the 70S lysis artifact. Furthermore, the 114S region contains a greater proportion of protein to DNA than can be accounted for by phage particles. Analysis of labeled DNAs found in the 70S and the 114S region show significant amounts of pulse-labeled RF II and replicative intermediate species in both regions, while long-term label is found principally in SS or SS fragments.

Analysis of the proteins cosedimenting in this region demonstrates the existence of substantial quantities of gene D product and as yet uncharacterized smaller molecular weight proteins as well as the expected capsid components. These pulse-labeled species are extremely fragile; exposure to 37°C, high ionic strength, or EDTA results in their disruption to the 9, 6 and 2S protein peaks previously characterized by Mayol (Biology 1970). The relationship of these particles to one another, and to the ΦX maturation process is presently under investigation.

261. A TECHNIQUE FOR SEDIMENTATION TO EQUILIBRIUM IN LOW IONIC STRENGTH SOLUTIONS

Investigators: Robert G. Rohwer, Timothy A. Livermore*

Support: National Institutes of Health, Public Health Service National Science Foundation

In an attempt to develop new techniques for the isolation of DNA-protein complexes which are labile in high ionic strength solutions, we have characterized the equilibrium sedimentation behavior of several macromolecules relevant to the ΦX infection in low ionic strength, yet dense, solutions of the salts of 5-acetamido-2,4,6-triiodo-N-methylisophthalamic acid. In this system ΦX DNA-forms come to equilibrium at densities around 1.18 g/cm³ whereas the ΦX particle itself bands at 1.30. Proteins band at densities from 1.20 to 1.28 with different proteins having different densities. Different degrees of aggregation of a single protein also band at different densities. Surprisingly the equilibrium densities of these molecules are only very weak functions of the ionic strength of the system even for increases in ionic strength of up to 1 M. At present we are applying this technique to the isolation of DNA-protein complexes from the ΦX infection.

*NSF Summer Trainee, 1972.

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Our efforts to understand the sequence of macromolecular events leading to production of Sindbis virus have continued. We have shown that the RNA genome of Sindbis is in one piece, but that two smaller fragments of plus strand RNA are synthesized in vastly different amounts to serve (inferentially) as messengers. In addition, the isolation of conditional lethal mutants of Sindbis and the study of their defects in maturation is helping us to understand normal virus replication.

Infection of animal cells by Sindbis virus leads to extensive alteration of the cell surface. Two virus-specific glycoproteins are inserted into the cell plasma membrane and preexisting proteins are displaced; the virus nucleocapsid buds through these altered regions. Although virus-specific protein synthesis and virus production become maximal only at 5 to 6 hours after infection, major changes in the cell surface are detectable earlier, by 3 to 4 hours after infection. One result of this change is a marked increase in the agglutinability of the infected cell by the plant lectins. A second result is the failure of (extracellular) Sindbis virus to adsorb specifically to the surface of an infected cell (although virus will adsorb nonspecifically to either infected or to uninfected cells). We hope that further work with electron microscopy and isolation of plasma membranes will enable us to have a clearer understanding of the molecular structure of both the infected and uninfected cell surface.

262. STUDIES ON SINDBIS VIRUS AND SINDBIS-INFECTED CELLS USING PLANT LECTINS

Investigator: Charles R. Birdwell

Support: National Institutes of Health, Public Health Service

One method of examining changes in cell surfaces after infection with tumor viruses has been to follow changes in the agglutination of cells with plant lectins, which are proteins that bind to specific carbohydrate residues. These carbohydrate residues may be either free or bound to lipids and proteins. Transformed cells are agglutinated by much smaller amounts of lectin than are required to agglutinate normal cells (Inbar and Sachs, 1969); the exact mechanism of this change is not known, but in some cases a rearrangement of lectin binding sites appears to be involved (Nicolson, 1971).

Sindbis virus consists of an RNA-protein core surrounded by a membrane of glycoproteins and lipid. After infection the glycoproteins are incorporated into the host cell membrane and the core buds through the membrane at these points (Acheson and Tamm, 1967). Thus infection by the virus results in extensive modification of the host cell surface. Although Sindbis is not a transforming virus, experiments with this virus may also yield information about modification of cell surfaces by other viruses.

I have found that two lectins, concanavalin A (Con A) and Ricinus communis agglutinin (RCA), agglutinate Sindbis and that the binding of Con A, and probably RCA, is specific for the carbohydrate moieties of the membrane glycoproteins. Sindbis-infected cells are agglutinated by smaller amounts of Con A and RCA than are required to agglutinate mock-infected cells; this change in agglutinability occurs at 3 to 5 hours after infection. However, the infected cells bind the same amount of radioactively-labeled Con A as mock-infected cells, which could mean that a rearrangement of Con A binding sites is responsible for the increased agglutination.

I am currently preparing conjugates of both Con A and RCA bound to hemocyanin, a large protein which can be recognized under the electron microscope. Such conjugates should reveal any rearrangements of lectin binding sites which may be occurring during infection.

References:
263. EFFECT OF IONIC STRENGTH UPON THE ADSORPTION OF SINDBIS VIRUS TO INFECTED AND UNINFECTED CELLS

Investigators: John S. Pierce, James H. Strauss Jr.

Support: Damon Runyon Memorial Fund for Cancer Research
National Science Foundation
National Institutes of Health, Public Health Service
The Merck Company Foundation

A previous report (Waite and Pfefferkorn, 1970) showed that when Sindbis virus was grown in medium whose ionic strength was less than physiological (i.e., 0.15 M), almost all of the virus remained cell-associated. Incubating the cells for a short period of time in medium of physiological ionic strength resulted in the rapid release of virus from the cells. We have extended this observation and used the differential response to ionic strength to study several aspects of the interaction of the virus with the cell surface.

If Sindbis is grown in media of different ionic strengths between 0.08 and 0.30 M and the amount of infectious virus found in the culture fluid is measured, all of the virus is released at ionic strengths above 0.2, whereas little or none is released below 0.15 M. However, virus is produced under the low salt conditions and can be eluted with subsequent washes of 0.2 M ionic strength. The total yield of virus produced (cell-associated plus released virus) is constant at ionic strengths below 0.2 M; at higher salt concentrations the virus yield declines.

We have also used this ionic strength effect to study specific versus nonspecific adsorption of virus to cells. In these experiments ¹⁴C-labeled virus is adsorbed (at 4°C) to either uninfected cells or to cells infected for several hours by Sindbis. Following adsorption the monolayers are washed with buffers of various ionic strengths and the remaining adsorbed virus is measured. Two different types of virus attachment occur. One, which appears to be nonspecific, occurs only at ionic strengths less than 0.2 M; this attachment is readily reversible upon washing the cells with buffers of ionic strength greater than 0.2 M. The second type of attachment is not reversible by high salt washes and appears to be an attachment to a specific cellular receptor leading to virus infection. The amount of virus attached to cells by this second mechanism drops off rapidly between 2 and 4 hours after infection, a time period during which extensive modification of the cell surface occurs. Virus attachment by the first mechanism is little changed during this time period. Thus superinfection exclusion in the case of Sindbis apparently involves the failure of the virus to attach to a specific receptor at the cell surface after modification of the surface by virus infection.

Reference:

264. PLASMA MEMBRANE ISOLATED FROM INFECTED CHICK EMBRYO FIBROBLASTS

Investigators: John S. Pierce, James H. Strauss Jr.

Support: Damon Runyon Memorial Fund for Cancer Research
National Science Foundation
National Institutes of Health, Public Health Service
The Merck Company Foundation

We previously reported (Biology 1970, 1971) isolation of a plasma membrane (PM) fraction from Sindbis-infected chick embryo fibroblasts. This fraction contained only the virus envelope and core proteins (as detected by radioactivity). Since Sindbis maturation involves the attachment of viral cores to the inside of only those portions of the PM destined to become virus envelope, the fact that the two proteins were present in the same relative proportions as in the virion indicated that this material was in fact PM. In addition, control experiments showed that the membrane purification procedure (modified from Wallach, 1967) did not successfully purify labeled virus.

We have since looked at this fraction in the electron microscope. Electron micrographs revealed intact and broken membrane vesicles with an occasional cluster of attached structures in a size range that would identify them as virus cores. These preparations, however, showed large
amounts of clumped virus particles attached to the surface of the vesicles.

Virus particles were copurified with the PM because Sindbis attaches to the cell surface during virus growth in media of physiological ionic strength (see Biology 1972, No. 263) and is not removed from the cell surface in the buffers of low ionic strength (≤0.01 M) used in the membrane purification procedure. Treating infected cells with media of high ionic strength (>0.2 M) successfully removes virus adsorbed to the surface; however, we have not been able to prepare membrane vesicles from cells pretreated in this fashion. If other enveloped viruses also adhere to the infected cell surface under these conditions, caution must be used in interpreting comparative chemical analyses of virus envelope and the isolated PM of the infected cell.

Reference:

265. ISOLATION OF TEMPERATURE-SENSITIVE MUTANTS OF SINDBIS VIRUS


Support: The James G. Boswell Foundation for Virus Research
Research Corporation
National Science Foundation
National Institutes of Health, Public Health Service
The Merck Company Foundation

We are continuing the program of isolation and characterization of Sindbis mutants which was outlined last year (Biology 1971). We have selected primarily for temperature-sensitive mutants, following mutagenesis with a variety of chemicals. Mutants have been isolated from virus stocks treated directly with N-methyl N-nitro N’-nitroso-guanidine and with nitrous acid at both pH 7 and pH 5.5. In addition, several mutants have been obtained by growing Sindbis in chick embryo fibroblasts in the presence of the base analog aza-cytidine.

Most of our new isolates have been tested for their ability to incorporate radioactive uridine at the nonpermissive temperature (40°C). Six mutants (four from aza-cytidine treatment, one from nitric acid, and one thought to be spontaneous) make no detectable RNA at 40°C and have been designated RNA−. A few others synthesize about 10% of the normal RNA yield. However, the majority of these new isolates synthesize RNA as efficiently at 40°C as they do at 30°C, the permissive temperature, and are thus RNA+. This distribution of RNA+ and RNA− mutants differs markedly from that found by Burge and Pfefferkorn (1968), who isolated 24 Sindbis mutants, of which only 7 were RNA+.

We have begun the genetic characterization of some of these mutants by performing complementation tests between pairs of our mutants and between our mutants and the mutants of Burge. However, some of our mutants appear to be very difficult to work with and we have been able to assign only a few of them unambiguously to complementation groups. Of the RNA+ mutants we have found two which belong respectively to two of the complementation groups of Burge and one which clearly defines a new complementation group. Only two of the six RNA− isolates have been examined. One of these fails to complement any other mutant (either RNA+ or RNA−) while the other complements all groups of RNA+ mutants, as expected, but none of the RNA− groups. In general, we have found that complementation is much more difficult to demonstrate between pairs of RNA− Sindbis mutants.

In addition to the temperature-sensitive mutants, several “large plaque” mutants have been found after aza-cytidine mutagenesis. These produce plaques on chick embryo fibroblast monolayers which are several times the size of those produced by the parental Sindbis strain. One of these plaque morphology mutants is also temperature-sensitive, while the remainder are not. It will be interesting to examine these mutants more closely to determine the reason for the larger plaques. We have previously seen the reverse phenomenon, i.e. selection for a small plaque strain of Sindbis, by repeated passage of Sindbis in monolayers of hamster cells (BHK-21).

We plan to continue the program of mutant selection and characterization with the ultimate goal of defining all of the Sindbis cistrons and assigning a viral function to each. We plan to use
additional mutagens (including 5-fluorouracil and ethyl methane sulfonate) in the hope of obtaining a greater variety of mutations. It is possible, however, since Sindbis is an arbovirus, that some of the viral functions are only essential for multiplication of the virus in the insect vector and are superfluous in mammalian or avian hosts. If this is true, a complete genetic analysis of the virus may not be possible without studying the growth of Sindbis in a tissue culture system from arthropods.

Reference:

266. SINDBIS-SPECIFIC PROTEINS IN INFECTED CELLS

Investigators: John R. Bell, James H. Strauss Jr.

Support: National Institutes of Health, Public Health Service

The few structural proteins of Sindbis are easily detectable in the infected cell, as host protein synthesis is severely depressed; in fact, these structural proteins constitute about half of the protein made in the infected cell during most of the infection (Strauss, Burge and Darnell, 1969). However, upon infection at the nonpermissive temperature by any one of a class of temperature-sensitive mutants, isolated elsewhere (Burge and Pfefferkorn, 1966) and in this laboratory, the virus' structural proteins are all but absent; instead, a large amount of a high molecular weight protein is found (Strauss, Burge and Darnell, 1969). It seems reasonable to suppose that this protein may be a precursor to the structural proteins of the virion for the wild-type virus, and that the defect of this class of mutants may lie in the cleavage of this suspected precursor. The presence in infected cells of small amounts of various virus-specific protein species intermediate in size between this large protein and the virus' structural proteins, and the approximate molecular weight of this large protein support this hypothesis.

We are examining the degradation of this large protein in the hope of discovering if it is indeed a precursor to the virus' structural proteins. If it is, we shall attempt to elucidate the kinetics of its synthesis and, more interestingly, the cleavage mechanism and the integration of the cleavage products into the virion.

References:

267. REPLICATION OF SINDBIS-SPECIFIC RNAs IN CELL CULTURE

Investigators: Daniel T. Simmons, James H. Strauss Jr.

Support: National Science Foundation
National Institutes of Health, Public Health Service

As reported in *Biology 1971*, two classes of replicative intermediates (RIs) are found in RNA extracts of cells infected with Sindbis virus. One of these (RIa) directs the synthesis of 49S RNA, the single-stranded viral genome. The other (RIb) directs the synthesis of two other distinct species of single-stranded RNA (26S and 35S RNA). After digestion with pancreatic RNase, RIa is reduced to a completely double-stranded form, called RF I, with twice the molecular weight of 49S RNA. On the other hand, RNase digestion reduces RIb to two forms of double-stranded RNA (RFs II and III). RF II has twice the molecular weight of 35S RNA and RF III has twice the molecular weight of 26S RNA. The regions of RIb corresponding to RF II and RF III are most probably responsible for the synthesis of 35S and 26S RNA, respectively. The finer points of Sindbis RNA replication are presently being studied by electron microscopy. In particular the replicative intermediates are being analyzed with respect to length and position of single-stranded tails and with respect to the position of the single-stranded gap in RIb. Preliminary studies show that each RI has on the average only one single-stranded tail.

When virus labeled in the RNA is used for infection, the label in the parental strand is found in both classes of RIs shortly after infection. The distribution of parental label is approximately equal in RIa and in RIb, whereas mass label is distributed more in RIb than in RIa (2.5:1). The complete meaning of this finding is unclear, but it suggests that a conversion process exists between the two RIs. The delicate, yet reproducible
changes in the amounts of RNA in each class of RIs which occur during the infection cycle support this conclusion.

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Our concern is with brain-behavior relations in small mammals because of remarkable similarities of structure between the brains of these animals and the human brain. The main parts of the brain are the same: cortex, hippocampus, cerebellum, thalamus, hypothalamus, midbrain, and medulla. Thus small mammals provide us with inexpensive models for these parts of the human brain. Our brain behavior studies are made in conscious and behaving animals because, in these, the brain is in control of behavior. We concentrate on special problems that are not amenable to study in sleeping or anesthetized animals, particularly purposive, goal-directed behavior, memory storage, and learning. These problems are to a large degree inseparable because purposive behavior in the mammal is learned, and learned behavior is motivated, and of course, learning and memory traces are two sides of a coin.

Our main advances this year have been in neurophysiological studies of learning and memory traces. Our first aim has been to localize unambiguously central nervous system processes that underlie these. This goal was chosen because of our view that whether learning is localized or not, we still have to localize it. In other words there is no question that it goes on in the brain and few of us would agree that it goes on equally everywhere. If it is at the nerve cell synapses but not the nucleus, this would be localized. If in neurons but not glia (or vice versa), this would be localization. If in some neurons or synapses more than others, this would be localization. If dynamic traces are in some places more than others, this would be localization.

Lesions have failed to localize the relevant processes. We presume this is because there is such a diffuse location that lesions always leave enough of the relevant tissues to permit the animal to still solve the learning problem.

In recording experiments, neuronal activity correlated with conditioning has been recorded for about two decades. But there are many neural correlates of every behavior and because there is new behavior after training there are new neuronal responses. The problem is the difficulty to separate primary events that are at the site of memory traces from secondary and tertiary events: some fed back from behavior; others just internally secondary (i.e., fed back or forward from antecedent primary brain events).

Our argument is that progress toward specification of the primary events can be established by timing. Two time series occur in every conditioning experiment. One time domain is the series of trials. With these trials along the baseline we get what is called a learning curve. Changes get bigger or performance gets better from trial to trial. Some animals learn before others. Happily, some parts of the brain learn before other parts of the brain.

Another time series is the latency domain: the succession of events that intervenes in the millisecond chain between stimulus and response.

Our model of the learning process can be approximated very roughly (too roughly) by a set of sensory events called 1, 2, 3 and 4, and a set of motor processes called 5, 6, 7, 8. In the 1 to 4 chain and the 5 to 8 chain there is rigid coupling. That is to say, 1 implies 4, and 5 implies 8. This is by genetic coding or some other kind of prior connection. At the 4 to 5 interface, however, the coupling is labile. That is, the 1 to 4 chains could be coupled by learning to 5 to 8, or if another behavior were learned, to a different sequence (say 15 to 18). The question is: how would we localize the 4 to 5 coupling by recording during a Pavlovian experiment?
If the model were veridical, the answer would be simple. (1) All elements of the 1 to 4 chain would give the same responses before and after conditioning, (2) all elements of the 5 to 8 chain would give changed responses, (3) among these changed responses, those at the 4 to 5 interface would have the shortest latency.

Actually the model is bad because it is too simplistic but there are two saving graces. (1) It provides a basis for separating the new responses into primary and secondary on a basis that has an important relation to the problem of primacy in learning, and (2) there is one other feature of the simple model that provides a basis for a more complex attack, namely, all the elements 5, 6, 7 and 8 would give evidence of learning at the same time in the series of trials if the rigid coupling model were fulfilled. This is a major clue to solving the problem for the more complex model.

What is the more complex model? It is that a chain like 1, 2, 3, 4 may exist before training (where 1 implies 4). But (1) connections in the 1 to 4 chain are labile, (2) branching into learned pathways can occur at every step, and (3) once the message is moving in a new path, there can be secondary changes as the message moves along it.

The method to unravel the complex model is based on using the two time series in tandem. Without going into details, let me suggest the lines of the solution. It is possible to divide neurons on the basis of our studies into groups that are rigidly coupled to one another but loosely coupled to other groups. A rigidly coupled system "learns together." Within any system such as this that learns all at once, there is a shortest latency subset. Logic gives us good reason to treat this as a switching point, a locus at which the brain message is rerouted during the course of learning.

Using this kind of logic we have proceeded now to canvas about 2000 brain points. These have led us to the preliminary specification of several systems and 3 or 4 switching points. The details of these data are given in some of the reports from our group. It is important to observe, however, that while the argument is sound, the data are tentative until a fair sample has been made of the parts of the brain that might be involved. The logic is heavily based on the belief that a fair sample can be made and we do not expect to have that made for a considerable period.

The most interesting preliminary facts are these: (1) the shortest latency new responses after conditioning, so far, appear in cortex itself, (2) the largest number of new responses appear in the association nuclei of the thalamus and these are likely independent of the cortex response changes, because the thalamus changes come earlier in the series of trials, (3) nevertheless, cortical "reverberating" background activity is changed at this early period and it might be responsible for the changed thalamic responses, and finally (4) the only parts of the brain that "learn" prior to the behavioral signs of conditioning in the trial series seem to be located in those areas such as hypothalamus, previously linked to motivation by experiments of quite a different nature.

268. UNIT RESPONSES IN THE AUDITORY SYSTEM AND POSTERIOR THALAMUS OF RAT DURING CLASSICAL CONDITIONING AND EXTINCTION

Investigators: John F. Disterhoft, Duncan K. Stuart
Support: National Institutes of Health, Public Health Service

Previous mapping of chronically-recorded unit changes in rat brain indicated that "learned responses" during classical conditioning, measured in terms of response latency from CS onset in the trained animal, occurred earlier in a small percentage of cortical units than in thalamic units (Olds et al., 1972). However, it was not possible to assign functional primacy within the thalamic or cortical regions except to note that learned responses were particularly numerous and large in posterior nucleus of thalamus units and seemed to be more heavily concentrated in posterior regions of cortex. One purpose of the present work is to determine more precisely the temporal chain of learned responses set up within the various regions of thalamus and cortex when a rat learns a classical association between tone and food. Since a tone is being used as the CS, the auditory regions of thalamus and cortex are being closely examined, especially in regard to their interactions with posterior nucleus of thalamus. Also, since it is possible that conditioned changes occur in more peripheral regions of the auditory system, unit responses in inferior colliculus are being examined.
In the previous work, the development of the learned responses in thalamus and cortex were also studied in the trial-to-trial sequence of training. This analysis indicated that the learned responses in the thalamus emerged earlier than those in cortex. However, background changes in cortex may have preceded the response changes in thalamus. This phenomenon was particularly evident in the posterior regions of cortex. Thus the second major focus of the present work involves a closer examination of the development of the responses which occur during the trial-to-trial sequence of training to determine more precisely the succession of involvement of thalamic and cortical regions in the conditioned changes which occur. Also, cortical and thalamic background firing rates are being further examined to determine whether dynamic shifts in cortical background firing may be causally related to learned responses which are exhibited in thalamic units.

The paradigm being used involves pairing one of two tones (CS+) with food. Another tone serves as the CS−. Unit and behavioral responses to the tones during successive pseudoconditioning, conditioning and extinction periods are examined. The data gathered thus far indicates that functionally significant learned changes are seen in medial geniculate with a latency of 7.5 msec and in posterior nucleus with a 12.5 msec latency. Detectable but small changes have been seen in inferior colliculus with a latency of 4.5 msec but their importance remains in doubt. Very interesting increases in firing have been detected in medial geniculate and posterior nucleus neurons after the beginning of extinction. This phenomenon has not been seen in inferior colliculus.

Reference:

269. SHORT-TERM CHANGES IN THE LATERAL HYPOTHALAMUS DURING CLASSICAL CONDITIONING

Investigator: Marianne E. Olds
Support: National Institutes of Health, Public Health Service

A novel approach in the study of neuronal mechanisms of learning has relied on methods for recording unitary activity in the chronic preparation during the elaboration of a skill rewarded by food (Hirano, Olds and Best, 1970). Two main measures of changes in frequency of firing of neurons have been (1) the latencies of the modifications in discharge brought about during repeated presentation of a tone signifying subsequent reward with food, and (2) the number of trials given before such modification makes its appearance in different anatomical regions. The assumption behind this strategy was that in some regions the latencies would be very short and therefore reflect earliest loci of rerouting of the auditory signal due to its informational properties, and some regions would reflect changes after very few trials, regardless of latencies, thereby signifying their early participation in the transformation of a “meaningless” auditory signal into one having cognitive and emotional value. With these methods and these measures of changes in neuronal discharges it has been reported that the cortex and the thalamus seem to have important roles as processors of auditory signals in a classical appetitive task (Olds et al., 1972). The midbrain region seems to participate, but in a less clear-cut fashion, at least so far as these measures were concerned. Linseman (1972) and Kornblith (1972) have preliminary data suggesting that the anterior hypothalamic region and the most posterior hypothalamic region may be implicated in a more crucial manner in the transformation of the auditory signal into one signifying subsequent food reward. Because no data were obtained for the middle hypothalamus, especially in those regions which are said to participate in the regulation of food intake and positive reinforcement, and because, in the final analysis, learning does not take place without reinforcement, we investigated the changes which occurred in the hypothalamic medial forebrain bundle and regions immediately adjacent to it during a classical appetitive task.

So far as latencies of changes in rate of discharge to the presentation of the conditioned stimulus are concerned, the main findings were two: (1) there were several instances of innate and sensory responses to the auditory signal with latencies of 20 msec or less, but there were no instances of “learned responses” with such short latencies; (2) the innate responses had latencies distributed between 0 and 80 msec range after the onset of the stimulus; none were in the 80 to 1000 msec range after the onset of the stimulus.
contrast, latencies of learned responses tended mostly to fall between 60 to 80 msec and there were a number of learned responses with latencies as long as 250 to 500 msec.

Such changes in neuronal activity during the elaboration of a skill were diffusely distributed throughout the lateral hypothalamus starting from the anterior hypothalamic region to the lateral premammillary region. There was no particular region which was characterized by the appearance of "learned neuronal responses" of a particular shape, duration, or latency.

Although they were found throughout the hypothalamic region, they did not represent nonspecific changes in the rate of discharge of this region because similar changes were not observed during pseudoconditioning tests and not during background activity immediately prior to the presentation of the CS. Thus, the modification in neuronal activity was a specific response to the CS only when it functioned as a cue in the acquisition of a skill. Furthermore in a given animal, it was possible to observe a large modification at a particular site of recording and a total lack of responsiveness simultaneously recorded from another site in the same lateral hypothalamic region.

The behavioral index of learning as measured by the increase in the movements of the animal after the onset of the tone during the conditioning tests, but not during the pseudoconditioning tests, revealed that these "behavioral-learned responses" had latencies ranging between 100 to 160 msec. 100 msec appeared to be the minimal delay necessary for the initiation of movements correlated with the response. Consequently, on the basis of this measure it would appear that even though learned neuronal responses in the hypothalamus had mostly long latencies, those falling within the 40 to 60 msec range clearly preceded the behavioral response. These relatively long-delayed modifications of neuronal discharge were observed after a comparatively few number of trials compared to other regions of the central nervous system. The magnitude of these large increases in rate of discharge in the hypothalamus was underscored by the fact that during tests of sensitization the response to the auditory signal was a decrease in the spontaneous rate of discharge.

Our conclusions based on these results are that neuronal activity in the lateral hypothalamic region, more than that in other regions thus far sampled, bears some relation to the performance of the skill, and thus may be the basis for the early trial-to-trial appearance of changes in this area. On the other hand, because the changes of neuronal activity were sudden, dramatic and considerably earlier than the gradual changes in behavioral performance, we tend to think that they represent a combination of changes reflecting information, reward and performance properties of the CS, reflected as changes in the discharge rate of these neurons. This is further suggested by the fact that even though the latencies of the changes in rate of discharge were long, they were nevertheless considerably shorter than the latencies of the behavioral changes.

References:
located largely near the intersection of hypothalamic and striatal structures. A transient increase in rate of background firing over trials was recorded following the onset of conditioning among hypothalamic units, suggesting they may temporarily represent a dynamic trace of the new learning. No significant differences were found between areas studied in order of appearance over trials of the conditioned responses. However, as a group, the conditioned responses studied here appeared significantly earlier than those of a group of cortical neurons studied under similar conditions. There was greater generalization of response to the CS- by units of the basal ganglia than of other areas, suggesting they may be of importance in inhibition of response to the CS-. 23 units, located mostly in the ventrolateral forebrain, showed inhibitory conditioned responses. Most often these had latencies of about 80 to 150 msec, and terminated prior to the UCS application. Further tests of two of these units revealed the inhibitory responses were present whether the UCS was food or shock. They were interpreted as possibly representing a transient increase in arousal of the animal following CS application.

271. MECHANISMS OF CONDITIONING IN THE LIMBIC TELENCEPHALIC SYSTEM OF THE RAT

Investigators: Menahem Segal, James Olds

Support: Earle C. Anthony Fellowship
National Institutes of Health, Public Health Service

The behavior of units in the components of the hippocampus and in areas afferent to it in the rat was monitored during a conditioning experiment. Rats were trained by correlating one of two tones presented in random order with a food pellet. The responses to the tone were recorded during pseudoconditioning, conditioning, and extinction sessions. Medial septal units (afferent to hippocampus) had unconditioned short latency phasic responses to the tone. These responses were prolonged during conditioning. CA-3 and CA-1 units (in hippocampus) had short latency conditioned responses to the tone. Entorhinal units (a second set of afferents) had a long latency conditioned response; the same was true for dentate units which conveyed most of the entorhinal input to the hippocampus. Posterior cingulate units (outside of hippocampus but closely linked to outputs and inputs) had relatively short latency conditioned responses. The data suggested that sensory unconditioned input entered the hippocampus from the septal side and was able to trigger the CA-3 units effectively only after conditioning. Messages from entorhinal area fed into the hippocampus via the dentate may have carried reinforcing information which helped to set the hippocampal units to respond to septal and perhaps to cingulate inputs during conditioning, with short latency and long duration.

272. UNIT ACTIVITY IN THE BRAIN STEM RETICULAR FORMATION OF THE RAT DURING LEARNING

Investigators: Carol L. Kornblith, James Olds

Support: National Institutes of Health, Public Health Service

The reticular formation has been suggested as a possible region where changes occur in neural connections as a result of learning. Changes have been observed during conditioning in reticular EEG, multiple unit activity, and single unit activity. While these studies showed that important changes occur in the activity of the reticular formation during conditioning, they do not indicate whether these are primary changes representing the learning experience or secondary changes resulting from alterations in neural activity in other parts of the brain. Changed neural activity has been observed in many other brain regions as a result of conditioning. This may reflect a sequence of events during learning which progresses from one region of the brain to another, or a simultaneous processing of information in several regions.

The aim of the present study was 3-fold: (1) to sample points in the brain stem to determine the regions where short latency conditioned responses occur; (2) to determine when in the course of learning the conditioned response develops so that comparisons might be made with other brain regions; and (3) to study the relative permanence of these responses with additional training and during extinction.

Unit activity was recorded from the midbrain
and pons of 40 freely-moving rats in an appetitive classical conditioning situation. Responses to auditory stimuli were recorded from 100 units before and during a conditioning procedure in which presentation of food occurred 1 second after the onset of the auditory stimulus. Very short latency conditioned unit responses were recorded from 7 probes located in the midlateral part of the ventral brain stem. Conditioned unit responses of considerably longer latencies were recorded from 80 probes in other regions. With additional training, units in the “reticular activating system” tended to yield stabilized responses in the early portion of the CS-US interval, while the responses of units in the limbic midbrain tended to stabilize in the later part of this interval. During extinction many of the responses were reduced to their pre-conditioning levels. The data were interpreted as indicating a local correlate of learning in the reticular formation of midbrain and pons with a separation of the midbrain system into two functionally different areas.

273. PHYSIOLOGICAL PROPERTIES OF RETICULAR NEURONS INVOLVED IN LEARNING

Investigators: Jacques Montplaisir, James Olds

Support: Medical Research Council of Canada National Institutes of Health, Public Health Service

Early studies (see Gastaut, 1958) have demonstrated the participation of brain stem reticular formation in the establishment of a positive conditioned reflex. More recently several authors (Yoshii and Ogura, 1960; Sparks and Travis, 1968; Olds et al., 1972) have described changes in the response of reticular units to conditioned stimulus as a result of learning. But as similar changes occur also in many other brain areas we were looking more specifically to short latency learned responses which could represent the result of local reticular processes involved in learning. The first part of this work was then to complete the mapping of learned responses in various parts of the reticular formation and more specifically at the midbrain level where a relatively small number of neurons has been sampled previously. All the experiments have been done on chronically implanted rat during classical conditioning. Preliminary results have shown that a large number of neurons in the midbrain reticular formation present learned response during conditioning. Learned response means the appearance of a new response or a significant increase in the amplitude or the duration of an unconditioned response present prior to conditioning. These learned responses in the reticular formation could be divided in two categories. Many neurons present a long latency learned response (80 msec and more) which appears to be closely related to the behavioral performance both during conditioning and extinction procedures (these responses could represent an efferent response preceding and possibly participating in the elaboration of the conditioned motor response). Another group of neurons presents short latency learned responses (9 to 40 msec) closely related in time to the conditioned stimulus.

The next step in this study will be to get more information about the physiological properties of these reticular neurons involved in learning. More precisely we want to correlate the behavior of these neurons during conditioning with (1) their location within the reticular formation, (2) their pattern of spontaneous discharges, (3) the variation of their spontaneous and evoked activity with changes in the level of arousal and after injection of pharmacological agents, (4) the nature of their responses to stimuli of different sensory modality (visual, auditory and somesthetic). It could be interesting for example to see if multisensorial units are more involved in learning than unimodal or unresponsive neurons. It could be interesting also to know if the same neurons would present the same learned response if we use conditioned stimuli of different modality.

The reticular formation is a very complex and heterogeneous structure both anatomically and physiologically and we think that any further information about localization and physiological properties of neurons which present learned response will increase our understanding of the role of this structure in learning mechanisms.

References:


274. DISSECTING A POSSIBLE SHORT-TERM MEMORY CIRCUIT IN THE RAT BRAIN

Investigators: Menahem Segal, Karen V. Roberts*

Support: National Institutes of Health, Public Health Service
Earle C. Anthony Fellowship

Previous studies suggested that the entorhinal cortex may be involved in predetermining learned responses in the hippocampus of the rat by tonically bombarding some synapses and thereby setting them up for function. The present experiment searched for a possible short-term effect of entorhinal activity upon hippocampus. Rats were trained that a tone is followed by food pellets. Unit activity in the hippocampus and its afferents, i.e. entorhinal cortex and septal area, was monitored during and between presentation of the conditioned stimuli. It was discovered that entorhinal units are “turned on” by the application of the food pellets and return gradually to the prestimulus firing rate after approximately 1 minute. No similar phenomenon was observed in septal or hippocampal units. If the next trial was applied within 1 minute, the response of hippocampal units to the conditioned stimulus was bigger than their response to this stimulus when applied later, when the entorhinal units are less active. Further experiments are under way to test the dependence of hippocampal units on entorhinal activity when the time factor is parceled out.

*NSF Summer Trainee, 1972.

275. HIPPOCAMPAL UNIT RESPONSES TO PERFORANT PATH STIMULATION IN A CHRONIC PREPARATION

Investigator: Menahem Segal

Support: National Institutes of Health, Public Health Service
Earle C. Anthony Fellowship

There are two anatomical pathways an impulse can travel from the entorhinal cortex to field CA-1 of the hippocampus: a short monosynaptic pathway and a long 3-synaptic one. The long one can be potentiated by impulse repetition and is susceptible to certain drugs. Data from acute, anesthetized animals have left in doubt the question of whether the short one could evoke spikes or even “EPSPs.” In the present study the coexistence of short (4 to 5 msec) and long (8 to 10 msec) latency-driven CA-1 unit responses to the combined pathways was demonstrated in an awake, chronic preparation. The long latency response was enhanced with increasing frequency of stimulation (suggesting its mediation by 3-synaptic path), and was abolished after inhalation with H2S (which is supposed to block the 3-synaptic path). These treatments did not affect the short latency-driven response. It was proposed that the short latency response is carried through the short monosynaptic pathway. It was proposed, too, that the two pathways may have different functional significances.

276. CONDITIONED UNIT ACTIVITY IN THE ABSENCE OF PURPOSIVE BEHAVIOR

Investigators: Menahem Segal, David E. Hiatt

Support: National Institutes of Health, Public Health Service
Earle C. Anthony Fellowship

One of the functions ascribed to the hippocampus is control of voluntary, purposive behavior. In some studies we found that hippocampal units did not respond to a conditioned stimulus which preceded either an aversive electric shock or a flash of light. No purposive behavior was initiated by the animal in either case. However, when a conditioned stimulus preceded food, hippocampal units did respond. In these cases purposive behavior was initiated by the animal. Question: Is it the “rewardness” or the movement involved that make the hippocampal units respond to the stimulus? To answer this we selected a situation in which a reward was available, but no movement was needed to get it; i.e. electrical brain stimulation in positive brain areas (M.F.B.). Would hippocampal units respond to a tone that precedes the brain shock? They did.
INVESTIGATIONS OF OPERANT CONDITIONING OF SINGLE UNIT ACTIVITY IN RAT BRAIN

Investigators: David E. Hiatt, James Olds

Support: National Institutes of Health, Public Health Service

The aim of these studies was to show that the capacity for operant responses is distributed differentially in the brain and that such capacity is maintained in the absence of feedback from movement in specific parts of the brain. The experimental subjects were rats chronically implanted with microelectrodes for single unit recording from several different brain structures. There were three experimental paradigms. In Experiments I and II positive reinforcement was applied following bursts of activity of an arbitrarily selected unit during periods indicated by a discriminative stimulus. All such units in cerebellum and brain stem displayed significant conditioned rate increases while only about half those in hippocampus, midbrain and superior colliculus did so indicating that operant conditioning is more a property of "motor" units. Experiment II was a direct continuation of Experiment I with some of the rats which had conditioned units. The contribution of the bodily movement which seemed inevitably correlated with the conditioned unit response was determined by inducing skeletal muscle paralysis with flaxedil. Conditioned responses were maintained under paralysis in all 5 rats with an experimental unit in the brain stem, but in only one of the 6 rats with an experimental unit in the cerebellum, and none in the other 7 rats with experimental units divided among hippocampus, midbrain and superior colliculus. This indicated that the conditioned responses of most of the units were fed back from movement which the conditioned activity of the brain stem units probably preceded. A control experiment with noncontingent reinforcement showed that these conditioned responses were probably not entirely due to operant conditioning. This ambiguity was absent in Experiment III which showed clearly operant activity. The rate of units, predominantly in the cerebellum, was increased or decreased depending upon the contingency of reinforcement. However, Experiment III used active animals and tested no units in the brain stem. A final experiment demonstrated clearly operant activity of a brain stem unit under paralysis. Reinforcement was made contingent upon rapid alternation between activity and inactivity of the unit. After acquisition, this behavior was brought under the control of a discriminative stimulus, and then maintained under paralysis, which eliminated the alternation of stereotyped movements that had been correlated with the unit activity.

OPERANT CONDITIONING OF UNIT FIRING PATTERNS IN THE COCKROACH, PERIPLANETA AMERICANA AND LOCUST, SCHISTOCERCA GREGARIA

Investigator: John F. Disterhoft

Support: National Institutes of Health, Public Health Service

Recent behavioral studies have indicated that intact, headless, and ganglionic cockroach and locust preparations exhibit behavioral conditioning when placed in a punishment situation which requires that a leg-lift response be learned in order that shocks to the leg be avoided (Disterhoft, 1972; Disterhoft, Haggerty and Corning, 1971; Eisenstein and Cohen, 1965; Horridge, 1962). Hoyle (1965) has also showed that motor impulses of one of the leg-lifting muscles may analogously be trained to increase or decrease in firing rate by the appropriate application of shock to the leg.

Exploratory studies are being done to determine whether units recorded from the metathoracic or sixth abdominal ganglion of cockroach and locust may likewise be induced to change their firing patterns in order to avoid shock. The purpose of the work is to develop a physiological analog to operant conditioning within a ganglionic preparation. This type of simplified preparation could be very powerful for study of the physiological basis of learning and memory.

References:


279. NORADRENERGIC AND CHOLINERGIC ACTION ON NEURONAL ACTIVITY DURING SELF-STIMULATION BEHAVIOR IN THE RAT

Investigators: Marianne E. Olds, Muneyuki Ito*

Support: National Institutes of Health, Public Health Service

Pairs of drugs—of which the first in each pair suppressed responding and the second one reinstated it—were tested in rats during self-stimulation behavior while effects on neuronal activity correlated with this behavior were determined. Two types of neuronal responses were studied. The first was the occurrence of spikes with fixed latencies in relation to stimulation applied in the posterior-lateral hypothalamus. The first type of response was studied in the cingulate, the preoptic and the ventral midbrain regions. The second type was studied in the middle hypothalamus.

The drugs with adrenergic action were tetrabenazine and d-amphetamine; the drugs with cholinergic action were eserine and scopolamine. The second drug in each pair was administered 30 minutes after the first one and at the time of maximal effect of the first drug on self-stimulation behavior.

Tetrabenazine reduced spike activity with fixed latencies in the ventral midbrain region, but not in the cingulate and the preoptic regions at the time when it suppressed self-stimulation behavior. Amphetamine partially restored the frequency of spikes with fixed latencies in the midbrain region at the time when it reinstated self-stimulation behavior. Tetrabenazine reduced the suppressant effect of stimulation in the posterior hypothalamus (disinhibition) on neuronal activity in the middle hypothalamus. Amphetamine partially reinstated the suppressant action of stimulation on neuronal activity recorded in the middle hypothalamus at the time it reinstated self-stimulation behavior.

Physostigmine and scopolamine produced the identical effects of tetrabenazine and amphetamine on behavior, but they were ineffective on neuronal activity.

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280. A CANNULAR SYSTEM FOR THE DIRECT LIQUID REINFORCEMENT OF FREELY-MOVING RATS

Investigator: John F. Disterhoft

Support: National Institutes of Health, Public Health Service

We have done, and are presently continuing, extensive research (Olds et al., 1972) examining changes in unit responses which occur during classical conditioning in the freely-moving rat. This work has utilized a paradigm in which one of two tones (CS+) is repeatedly paired with the presentation of a food pellet in a food magazine in order to effect classical conditioning. The rat, in this situation, learns that the CS+ signals the presentation of food and is reinforced by retrieving the pellet from the magazine.

In practice, there are sometimes large individual differences between animals in their manner of retrieval and in the number of reinforcements actually received. These differences may be causing some variation among the patterns of unit changes seen between animals. Also, the main interest of the research is to determine those unit changes which underlie the learned association between tone and food and not those unit changes which may be connected with food pellet retrieval. It thus seemed worthwhile to develop a system by which a natural reinforcement could be presented directly to the animal which would assure that the animal would be reinforced on every trial and which would not require the animal to perform any behavior in order to be reinforced. A cannular system by which liquid reinforcements may be injected directly into the mouth of freely-moving rats has been developed to meet these requirements.

Pilot work indicates that presentation of a glucose solution via the cannula, when paired with a tone, is effective in causing classical conditioning to occur. Both gross movement of the animal and tongue licking may be used as behavioral indices of conditioning which appear in the CS-US interval. This system will be used to determine whether the pattern of unit responses between brain regions seen after conditioning with the direct reinforcement is different from that seen when presentation of food pellets is used as the reinforcement. Brain regions which show primary changes with the same characteristics with both modes of reinforcement
should prove most involved with the learned association rather than with the performance or nonperformance of food pellet retrieval. The system should also be useful in reducing variability in the behavioral situation. Finally, it offers the potential for developing a fixed preparation for electrophysiological recording during learning in the chronic rat with movable extracellular or intracellular microelectrodes.

Reference:

281. EFFECTS OF d-AMPHETAMINE, SCOPOLAMINE, CHLORDIAZEPoxide AND DIPHENYLHYDANTOIN ON SELF-STIMULATION BEHAVIOR AND BRAIN ACETYLCHOLINE

Investigators: Marianne E. Olds, Edward F. Domino*

Support: National Institutes of Health, Public Health Service

The effects of d-amphetamine (0.25 to 8), scopolamine (0.25 to 8), chlordiazepoxide (2.5 to 40), and diphenylhydantoin (25 to 75), given i.p. or s.c. on a mg/kg basis, were studied on self-stimulation behavior in the male albino rat. The dose-effect relationships, the role of baseline rates of responding and their effects on brain acetylcholine (ACh) were determined in rats trained to self-stimulate for electrical reward in the lateral posterior hypothalamus. The effects of d-amphetamine were both dose and baseline rate-dependent. Low-moderate doses (0.5 to 2.0 mg/kg inclusive) facilitated self-stimulation and larger doses (2.0 to 8.0 mg/kg) depressed responding. Baseline rates before d-amphetamine administration were extremely important in the effect observed. Low rates of responding were facilitated and high rates were depressed by this agent. The effects of scopolamine in a wide range of dosage were less consistent. A small dose (0.5 mg/kg) only transiently facilitated self-stimulation and larger doses (1 to 8 mg/kg) tended to depress this behavior. Baseline-rate effects were less important but high-rate responders were usually depressed by scopolamine.

The effects of chlordiazepoxide were dose-dependent. A dose of 5 mg/kg caused facilitation but larger doses (10 to 40 mg/kg) produced depression of self-stimulation irrespective of baseline rates. However, high-rate stimulators showed the most dramatic increases with 5 mg/kg of chlordiazepoxide. In contrast, diphenylhydantoin (25 to 75 mg/kg) usually depressed self-stimulation. Low-rate self-stimulators showed the most marked depressant effects.

Brain ACh was progressively reduced by handling of naive animals, injection of saline, and ½ hour of self-stimulation and escape behavior. Animals not allowed to self-stimulate but given d-amphetamine (2.0 mg/kg), scopolamine (2.0 mg/kg) showed a significant decrease in brain ACh. Self-stimulation, in addition to medication with the various drugs, showed a trend for further reduction in brain ACh but the differences were not statistically significant.

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282. ASSIMILATION OF RHYTHM PRODUCED BY CHOLINERGIC ACTIVATION OF THE CEREBRAL CORTEX

Investigator: Jacques Montplaisir

Support: Medical Research Council of Canada
National Institutes of Health, Public Health Service

Rhythmic sensory stimulation can produce in the primary area of the cerebral cortex a nonspecific increase in the rate of firing of cortical neurons which persists several minutes after the end of the stimulation (Bindman and Baisaqq-Schepens, 1966). However the reinforcement of a rhythmic stimulus in a great variety of behavioral situations produces in many cortical and subcortical structures a rhythm activity of the same frequency which persists during intertrial period (see John, 1967). This phenomenon has been called assimilation of rhythm. On the other hand, a similar phenomenon has been produced in acute preparations by coupling rhythmic stimulation with cortical polarization (Morrell, 1961) or tetanic cortical stimulation (Chow and Dewson, 1964). One interpretation of this result is that cortical polarization or stimulation would represent analogs of natural reinforcement.

There are many hypotheses about the neural
mechanism responsible for the reinforcement of cortical sensory inputs. One of these hypotheses postulates the importance of the ascending reticular activating system (Jasper and Doane, 1968). On the other hand, there is an increasing amount of evidence (see Krnjevic, 1969) suggesting that acetylcholine would be the neurotransmitter of the reticulo-cortical projections responsible for cortical activation. Following this hypothesis we wanted to know if the injection or the local application of cholinomimetic drugs such as eserine could produce persistence of a rhythmic cortical response.

Previous experiments have suggested that an optimal dose of eserine producing EEG activation in cat isolated forebrain could produce such a phenomenon. We will make a more extensive study of this problem using chronic preparation in rat. The technique has been developed but results are not yet available.

References:
Bindman, L. J. and Baisacq-Schepens, H. (1966) J. Physiol. 185: 14P-17P.

283. CATECHOLAMINES AND SELF-STIMULATION BEHAVIOR: EFFECTS ON BRAIN LEVELS AFTER STIMULATION, AND PRETREATMENT WITH DL-α-METHYL-P-TYROSINE

Investigators: Marianne E. Olds, Arthur Yuwiler*

Support: National Institutes of Health, Public Health Service

In a previous study the authors sought to compare the changes in levels of brain catecholamines during self-stimulation behavior with the changes produced during two other tasks, noncontingent or forced stimulation of brain-reward regions of the hypothalamus and aversive stimulation applied to the dorsal midbrain tegmentum. A fall in the concentration of hypothalamic and brain norepinephrine was observed during self-stimulation behavior but not in the other two situations. Our conclusion was that norepinephrine levels were reduced during the voluntary brain-reward task probably because of increased utilization, but not during the nonvoluntary activation of the same neuronal pathway and not during the aversive task despite the stressful elements in it. However, there were differences in the tasks themselves which might have accounted for the failure to obtain depletion during forced stimulation. One difference was the large number of electric shocks taken by the subject during self-stimulation behavior within a given period compared to the much smaller number of shocks received during forced stimulation. Since more electrical stimuli were applied to the hypothalamus during the limited period of self-stimulation than during forced stimulation of the same region or of another region where stimulation was at least equally stressful, the concentration of brain norepinephrine in self-stimulations could have been reduced because synthesis might have been insufficient to match release. On the other hand, synthesis might have kept pace with utilization accompanying forced stimulation which was given at a lower rate. The problem therefore was to test for catecholamine depletion under conditions where small changes in utilization would be observed and where the number of stimuli applied to the reward region of the hypothalamus would vary minimally between subjects and conditions. The total number of stimuli applied to a given region during the test however would have to be identical to that received by subjects which were high self-stimulators. These conditions were met by two procedures. One involved the use of forced or noncontingent stimulation, when this was part of the test, delivered at a rate matching the rate of self-stimulation. The second involved the use of drugs to stabilize the content of brain catecholamines so that small changes produced by the different procedures could be observed. The effects of stimulation on norepinephrine were examined, in the present studies, in animals in which catecholamine biosynthesis was inhibited through prior treatment with α-methyl-para-tyrosine (α-MPT). It was reasoned that changes in the content of hypothalamic and brain amines due to electric activation of hypothalamic loci would be magnified under such conditions, and accelerated synthesis with use would be precluded. We report here results of such experiments. They were designed to determine (1) the degree of depletion of brain norepinephrine during stimulation of the reward pathway in the hypothalamic medial
forebrain bundle, and (2) the degree of depletion during such stimulation when synthesis was inhibited.

Three main findings were made. The first was that without drug treatment forced stimulation of the hypothalamic reward pathway did not lower the concentrations of brain norepinephrine or dopamine. This was so despite the fact that the parameters of stimulation were identical to those during self-stimulation behavior. The second was that following inhibition of norepinephrine synthesis, noncontingent stimulation of the hypothalamic reward system did lower the concentration of brain norepinephrine and dopamine, whereas stimulation of the hypothalamic nonreward pathways or of the caudate nucleus did not. The third was that a substantially larger reduction of hypothalamic norepinephrine occurred in implanted animals pretreated with \( \alpha \)-MPT when the electrodes were in the hypothalamus than in \( \alpha \)-MPT-treated animals in which the electrodes were in the caudate nucleus, or in nonimplanted \( \alpha \)-MPT-treated animals.

Thus the present results confirm earlier findings that forced stimulation of the hypothalamic reward region in non-\( \alpha \)-MPT-treated animals does not appreciably lower hypothalamic norepinephrine, yet elicits an adrenal stress response. In experiments in which norepinephrine synthesis was blocked, the results suggest that hypothalamic norepinephrine turnover is increased in animals in which noncontingent stimulation is applied to the hypothalamic reinforcing region. Although stimulation at rewarding sites was not clearly differentiated from stimulation at nonrewarding sites, there was a tendency for norepinephrine concentrations to be lower in the former both after \( \alpha \)-MPT alone and especially after combined drug treatment and forced stimulation. The variance in both behavioral and biochemical responses in the latter condition were attributed to activation of adjacent, but functionally distinct, hypothalamic neurons in the region of probe implantation. Finally, the technique of prior blockade of synthesis appears useful in permitting detection of small absolute changes in concentration by magnifying the relative change.

**PUBLICATIONS**


Olds, J. (1972) (1) The evolution of man's behavior (or: Phylogeny of behavior), (2) Modern behavior theory and its relation to the brain, (3) The neuropsychology of motivation, (4) The neuropsychology of learning, (5) The social implications of neuropsychological findings and
prospects (or: Important social implications of neuropsychological research). In: *Brain Mechanisms and the Control of Behavior*, O. Guth (Ed.), The Science Foundation for Physics within the University of Sydney, Sydney, Australia. In press.


Evidence has accumulated which indicates that the bending movements of cilia and flagella are generated by a sliding filament process, similar to the sliding filament process responsible for muscle contraction. Flagella and cilia also need a control mechanism which is responsible for rhythmic bending and for the phase differences between the bending of different regions which are required for propagated bending waves. I have suggested that the basic feature of this control mechanism may be a simple feedback process, whereby the active sliding process at a particular point on the flagellum is regulated by the curvature of the flagellum at that point. In its simplest form, this model for the control of flagellar bending can be represented by an equation,

$$\frac{dM_a}{ds} = m_o \kappa.$$ 

$M_a$ is a measure of the active bending moment at any point on the flagellum, $s$ is a measure of distance along the flagellum, $\kappa$ is a measure of the curvature of the flagellum, and $m_o$ is a constant. The movement of the flagellum must also satisfy an equation which balances the active bending moment against the bending moments which resist movement, all along the length of the flagellum:

$$M_a + M_r = 0.$$ 

The resisting moment, $M_r$, will be a complicated function of the shape and rate of bending of the flagellum, as well as the physical properties of the flagellum and its surrounding medium, and, as a result, the differential equation resulting from these equations cannot be solved analytically under conditions which match the movement of real flagella. A computer program for numerical solution of this problem has been developed which satisfactorily simulates the movement of this flagellar model, and demonstrates that the suggested control process is a sufficient explanation for the spontaneous initiation and propagation of flagellar bending waves. Current work, described in the following sections, is attempting to determine whether this is a realistic model for the mechanisms of movement of real flagella.

**284. SELECTIVE ACTIVATION OF BENDING IN DISTAL OR PROXIMAL REGIONS OF SPERM FLAGELLA***

**Investigator:** Charles J. Brokaw

**Support:** National Science Foundation
Rockefeller Foundation

Our working model for flagellar movement suggests that elements distributed along the length of the flagellum contribute equally to the initiation and propagation of bending, that there is no need for anything special at the base of a flagellum to initiate bending waves, and that isolated segments of flagella should initiate and propagate bends if they are of sufficient length.

Experimental observations on several kinds of flagella indicate that isolated segments of flagella can continue to generate rhythmic movements, but previous experiments with sea urchin sperm flagella have suggested that bend initiation is a special property of the basal region of the flagellum. We have found a method for selectively activating the distal region of a sea urchin sperm flagellum, which clarifies this disagreement.

These experiments were carried out with spermatozoa from a Hawaiian sea urchin, *Colobocentrotus atratus*, which display remarkably normal movements when reactivated by ATP after membrane removal with Triton X-100. When these spermatozoa were demembranated with slightly suboptimal concentrations of Triton, some spermatozoa were found which retained a visible membrane on either the proximal or distal portion of the flagellum. When these spermatozoa were observed at low ATP concentrations, bending movements were restricted to the demembranated portions of the flagellum. More satisfactory localization of bending was obtained when the spermatozoa were observed in ADP solutions. In ADP solutions, the membrane appears to act to retain ATP formed from ADP by a flagellar adenylate kinase, so that the membrane-covered region experiences a much higher ATP concentration than a demembranated region. Numerous examples were observed where the distal, membrane-covered region of the flagellum was beating regularly at 8 to 10 beats per second, while the proximal, naked region was motionless, or was generating very slow bending waves (less than 1
beat per second). This result shows that sea urchin sperm flagella, like other flagella, can initiate bending waves in the distal half of the flagellum, without any activity in the basal region.

In many of the cases where rapid movements were observed in a distal region and slow bending waves in a proximal region, the movement of the distal region was intermittent. The beating of the distal region stopped and started in synchrony with the slow bending cycle of the proximal region, suggesting that events occurring in the proximal region can modulate the activity of a distal region. The internal basis for this modulation is not yet known, but it suggests explanations for previous results, such as Goldstein's observation that a distal region removed by laser irradiation was unable to initiate new bends.

*This work was carried out at the Pacific Biomedical Research Center, Honolulu, in collaboration with I. R. Gibbons.

285. VISCOUS RESISTANCES IN FLAGELLA

Investigator: Charles J. Brokaw
Support: National Science Foundation
         Rockefeller Foundation

Computer simulation of the sliding filament model for flagellar movement showed that the wavelength of the bending wave pattern could be controlled by including in the model a term which was mathematically equivalent to an internal viscous resistance to bending. The regulation of wavelength by internal viscosity can be analyzed explicitly for small amplitude sinusoidal waves, and this analysis predicts that if the wavelength is regulated by the presence of internal viscosity, the wavelength and frequency should each be inversely proportional to the fourth root of the external viscosity. This prediction agrees well with previously collected experimental data for three species of invertebrate spermatozoa, which showed that the bend propagation velocity was inversely proportional to the square root of the external viscosity. The computer simulation method is now being used to make a detailed comparison between the predictions of the model and the experimental data. So far, the results appear to be completely consistent with the idea that the wavelength of the movement of these sperm flagella is regulated by the internal viscosity of the flagella. If the wavelength is regulated by internal viscosity, the internal viscosity must be relatively large, such that it accounts for about 75% of the total viscous resistance which must be overcome by active processes within the flagellum. Use of this simple method for regulation of the wavelength of movement might therefore explain the relatively low energy efficiency which has been observed for flagellar movement.

286. CONTROL OF THE AMPLITUDE OF BENDING IN FLAGELLA

Investigator: Charles J. Brokaw
Support: National Science Foundation
         Rockefeller Foundation

Flagella also need a mechanism which controls the amplitude of flagellar bending. In the computer models studied so far, amplitude regulation has been achieved by introducing terms which are mathematically equivalent to nonlinear flagellar elasticities. The amplitude of bending is then determined by a balance between a delayed component of the active bending moment and the moments resulting from elastic resistances.

This method for amplitude regulation predicts that the amplitude of bending should decrease when movement is inhibited by factors which decrease the active moment. This prediction disagrees with many experimental observations of normal-looking bending waves which can be generated at very low frequencies, for example, at low ATP concentrations, or when the movement is inhibited with thiourea. Control of bend amplitude by nonlinear elasticities does not, therefore, appear to be a realistic feature of the flagellar model which is being developed, and a variety of approaches are being explored in an attempt to find out more details about the regulation of the amplitude of flagellar bending, so that a more realistic mechanism can be incorporated into the model.
PUBLICATIONS


THE FOLLOWING REPORTS ARE BY GRADUATE STUDENTS IN THE DIVISION OF BIOLOGY WHO ELECTED TO DO THEIR THESIS WORK IN THE DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING.

287. PURIFICATION OF TOXIC COMPONENTS FROM CONE SNAIL VENOMS AND CHARACTERIZATION OF THEIR MODES OF ACTION

Investigators: Ellen J. Elliott, Michael Raftery*

Support: National Science Foundation

Neurotoxins from animal sources have proved to be very useful tools for investigation of the functioning of nervous systems at the molecular level. For example, specific components of the venoms of the cobra and a related snake, Bungarus multicinctus, appear to block acetylcholine action at neuromuscular junctions by binding to the acetylcholine receptor. Tetrodotoxin from the Japanese puffer fish and saxitoxin from a dinoflagellate block the Na⁺ channels of nerve and muscle membrane, preventing the Na⁺ influx of normal action potentials.

Marine cone snails possess a venom, contained within an elaborate venom apparatus, which can be lethal to mammals, fish, and other molluscs. Preliminary investigations of the venom of a Conus species found off the California coast have indicated the presence of factors which interfere with nervous function in some molluscan preparations (Cottrell and Twarog, 1972).

Purification of the toxic components of the cone snail venom by gel filtration, hydroxypatite absorption, and ion exchange chromatography is currently in progress. The effects of venom components are being observed on the activity, both spontaneous and serotonin-excited, of the excised heart of the clam. Preliminary results indicate that some components of the venom may be acting upon molecular processes which have not previously been studied biochemically. These components may thus constitute new tools with which to dissect nervous function.

Reference:

*Division of Chemistry and Chemical Engineering, California Institute of Technology.

288. PROPERTIES OF FRAGMENTED SARCOPLASMIC RETICULUM ISOLATED FROM RABBIT SKELETAL MUSCLE

Investigators: Bentson H. McFarland, S. I. Chan*

Support: National Science Foundation

Contraction of skeletal muscle is controlled by the concentration of free calcium within the sarcoplasm. The membranes of the sarcoplasmic reticulum regulate this concentration by sequestering calcium while hydrolyzing adenosine triphosphate (ATP). Contraction is initiated by release of calcium from the reticulum in response to depolarization of the sarcolemma. By means of differential centrifugation, fragmented sarcoplasmic reticulum (FSR) may be isolated from homogenized muscle in the form of microsomes. These vesicles retain their enzymatic properties and will actively accumulate calcium at the expense of ATP. Fragmented sarcoplasmic reticulum is of interest since it is a central part of the as yet poorly understood mechanism coupling excitation to muscle contraction and because it may serve as a model for other, more complicated active transport systems.

To date, our efforts have been directed towards characterizing the microsomal preparation—particularly with respect to homogeneity. Using density gradient centrifugation, we have established that FSR isolated by differential centrifugation is an essentially uniform fraction of the homogenized muscle. This method has also enabled us to determine the density of these vesicles. As might be expected from the low protein to lipid ratio of the membranes, muscle microsomes are much lighter than other cell fragments. In addition, we have developed methods which permit the rapid and routine determination of the enzymatic activities associated with FSR. With these procedures, we have shown that there are no substantial biochemical differences between membranes isolated by differential centrifugation and those prepared using density gradient methods. Finally, the scanning electron microscope (SEM)
has been employed to give a three-dimensional view of the vesicles. With the aid of techniques developed in our laboratory, we can efficiently fix, wash, and coat FSR samples for examination in the scanning microscope. Since we are working close to the limit of SEM resolution, we have devised controls which assure us that our subject is, in fact, the fraction of interest. Again, no significant morphological heterogeneity is apparent within the FSR. Hence, we feel that vesicles isolated by differential centrifugation from skeletal muscle consist primarily of sarcoplasmic reticulum fragments with little contamination from other cell structures.

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Fragmented sarcoplasmic reticulum seen through the scanning electron microscope. a,c: native microsomes; b,d: vesicles purified on a sucrose density gradient. In c and d the microsomes have filled with a calcium oxalate precipitate during active calcium uptake. Bar represents one micrometer.
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William Hayes, University of Edinburgh, Scotland: Genetic and Biochemical Studies of Transcription in *E. coli*, with Special Reference to RNA Polymerase.

Raphael P. Gruener, University of Arizona Medical School, Tucson: Steroid-Induced Myopathy: Effects of Membrane Properties.

John Abelson, University of California, San Diego: Bacteriophage Mu, a Lysogenizing Phage Which Causes Mutations in Its Host.

Samuel Ward, MRC Laboratory of Molecular Biology, Cambridge, England: Nematode Chemotaxis.

G. Georgieff, Institute of Molecular Biology, Academy of Sciences, USSR, Moscow: The Organization of Transcription Units in Eukaryotes.

L. Rakic, Department of Physiology and Biochemistry, University of Belgrade, Yugoslavia: Biochemical Correlates of Paradoxical Sleep Deprivation.

Bertil Hille, Department of Physiology and Biophysics, University of Washington: Sodium and Potassium Ionic Channels in Nerve Membranes.

N. A. Newby, Department of Psychobiology, University of California, Irvine: Looking and Learning by the Skin of *Aplysia*.

Don L. Caspar, Children’s Cancer Research Foundation, Boston, Massachusetts: Molecular Organization of Lipid-Containing Viruses and Myelin Membranes.

Rosalie Hoyt, Bryn Mawr College: A Stored Charge Model for the Sodium Channel.

Giovanna Ferro-Luci Ames, Department of Biochemistry, University of California, Berkeley: Biochemical and Genetic Studies of Histidine Transport in *E. coli*.

John G. Nicholls, Department of Neurobiology, Harvard Medical School, Boston, Massachusetts: Regeneration and Changes in Synaptic Transmission Between Individual Nerve Cells in the Central Nervous System of the Leech.

E. Roy John, New York Medical College: Neural Readout from Memory.

Russell Pemberton, Department of Biology, Massachusetts Institute of Technology: Measurement of Reiteration Frequency of Genes for Hemoglobin in Duck Erythrocytes.

David Premack, Department of Biological Sciences, University of California, Santa Barbara: Symbolic Communication in Chimps.


D. Junge, Department of Physiology, UCLA School of Dentistry: Post-Stimulus Hyperpolarization in Molluscan Neurons.

H. Collewijn, Department of Physiology, New University of Rotterdam, The Netherlands: The Role of the Superior Colliculus in Smooth Eye Movements in the Rabbit.


Paul Berg, Stanford University: Dissections and Reconstructions of SV40 DNA Molecules.


Neal Smith, Smithsonian Tropical Research Institute: Oropendolas, Cowbirds, Flies, and Wasps; The Importance of Being Parasitized.

George W. Beadle, Nobel Laureate: Teocentli: Madre de Maiz.

Gunther Stent, University of California, Berkeley: Prematurity and Uniqueness in Scientific Discovery.

Richard McCaman, Division of Neurosciences, City of Hope Medical Center: Net Casting in the Field of Neurobiology of Marine Invertebrates.

Lucy Shapiro, Albert Einstein College of Medicine: Bacterial Differentiation.

Marcel Kinsbourne, Duke University Medical Center: Neuropsychology of Hemispheric Differences.

B. R. Ware, Department of Chemistry, University of Illinois: Measurements of Transport Properties of Biological Macromolecules in Solution by Laser Light Scattering.

Harvey Kartin, Massachusetts Institute of Technology: Neocortical Constituents: Unique to Mammals?

Eric Frank, Harvard Medical School, Boston, Massachusetts: Control of Facilitation at the Neuromuscular Junction of the Lobster.

David Ingle, McBean Hospital, Boston, Massachusetts: Tuning of Tectal Units in the Frog.

Roger Weil, Institute of Molecular Biology, University of Geneva, Switzerland: A Study on Polyoma- and SV40-Induced Chromosome Replication and Mitosis.

Donald Kennedy, Stanford University: Ontogenetic Factors of Receptive Field Organization.

P. M. A. Broda, Department of Molecular Biology, University of Edinburgh, Scotland: Types of Recombination of Sex Factors with the Chromosome and with Other Sex Factors.

Thomas Thompson, University of Virginia: Bilayers and Biomembranes.

Charles Thomas, Harvard University Medical School, Boston, Massachusetts: The Cyclization of DNA Fragments and Its Implications to Chromosome Structure.

John Sutherland, Lawrence Radiation Laboratory, Livermore, California: Are All Porphyrins Squares? The Magnetic Circular Dichroism of Porphyrins and Metalloproteins.

Gary Freeman, Department of Biology, University of California, San Diego: Studies on Cytoplasmic Localization in the Eggs of Ctenophores.

Gordon Tompkins, University of California Medical Center, San Francisco: Control of Gene Expression in Animal Cells.


G. V. Rama Reddy, Department of Medicine, University of California, San Diego: The DNA Synthesis in *E. coli*.

Sir John Eccles, State University of New York, Buffalo: The Principles of Operation in the Central Nervous System.

Roger Kornberg, Department of Chemistry, Stanford University: The Diffusion of Phospholipids in Membranes.

William Rutter, University of California Medical Center, San Francisco: RNA Polymerase and Transcripive Regulation in Eukaryotes.

Robert M. Lebovitz, Department of Physiology, University of Texas, Southwestern Medical School: Role of ATP-Dependent Metabolism in Hippocampal Penicillin Seizure.


Roger Eckert, Department of Zoology, University of California, Los Angeles: Calcium Gates and Excitation-Response Coupling in *Paramecium*: Electrical Studies of Wild Type and Mutant Membranes.

Alan Garen, Yale University: Genetic Programming for Imaginal Cell Development in *Drosophila*.
David Freifelder, Department of Biochemistry, Brandeis University: Physical Studies on Lysogenization and Induction of Phage λ.

Rolf J. Mehlhorn, Department of Genetics, University of California, Berkeley: Spin Labels and Membrane Mutants.

Harold J. Morowitz, Yale University: Mycoplasma Membranes.

Matthew S. Meselson, Department of Biology, Harvard University: Bacterial Nucleases and Restrictions.

Austin L. Taylor, University of Colorado Medical Center: Integration of Phage Mu at Random Chromosomal Sites in E. coli.

Ann Stuart, Department of Physiology, UCLA School of Medicine: Identified Cells, Synaptic Connection and Neurotransmitters in the CNS of the Leech.


Douglas Brutlag, Department of Biochemistry, Stanford University Medical School: Role of RNA in the Initiation of DNA Synthesis.

Majorie Grene, Department of Philosophy, University of California, Davis: People and Other Animals.

Thomas Wegmann, Harvard University: Immunologic Tolerance and Tetraparental Mice.

David Y. Thomas, Department of Genetics, University of Washington: Mutants of Mitochondrial DNA in Yeast.

Phillip A. Sharp, Cold Spring Harbor Laboratory, New York: Transcription of SV40.

Geoffrey Zubay, Columbia University, New York: Cracking the Metabolic Code.

Takuji Kasamatsu, Department of Psychiatry, UCLA School of Medicine: Neural Excitability Changes of Visual Cortical Units in Relationship to Vigilance Levels.
GRADUATES

Forty-four students in Biology were awarded the B.S., M.S. or Ph.D. degree in June, 1972. The names, degrees conferred, the titles of doctoral theses, and the plans of the recipients for future work are as follows:

Bachelor of Science

Steven William Battelle, B.S. with honor.
John Ralph Cameron, B.S. with honor. Graduate work in biology at Stanford.
Loring G. Craymer III, B.S. Graduate work in biology at the University of Wisconsin.
Peter Lynn Davis, B.S. with honor. University of California Medical School, Los Angeles.
Ratchford Clark Higgins, B.S. with honor. Graduate work in biology at the University of California, Davis.
Jeffrey Bishop Hurm, B.S.
Jonathan Paul Jacky, B.S. with honor. Graduate work in biology at the University of Washington.
Roger Allen Lighty, B.S.
Paul Raymond Morand, B.S. with honor.
Mark Ray Morris, B.S. Graduate work in biology at Stanford.
Ken Donald Pischel, B.S. with honor. Washington University Medical School.
George Allen Rappolt, B.S. with honor.
Tad Edward Reynales, B.S. Graduate work in biology at the University of California, Santa Barbara.
Harvey Alan Risch, B.S. with honor.
Brian Sarsfield Seed, B.S. Graduate work in biology at C.I.T.
Gary Dean Stormo, B.S. Graduate work in biology at the University of Colorado.
Paul Studenski, B.S.
Harley Yau-Shuin Tse, B.S. with honor. Graduate work in biology at the University of California, San Diego.

Master of Science

Gregory John Del Zoppo, M.S. Graduate work in biology at C.I.T.
William Aro Hill, M.S.
Jane Elinor Latta, M.S.
Amy Shiu Lee, M.S. Graduate work in biology at C.I.T.
Bentson Hayes McFarland, M.S. University of Washington Medical School.
Thomas Joseph Quinlan, M.S. Graduate work in biology at the University of Chicago.
Gary Carl Scheidt, M.S.
Margaret MacMorris Swanson, M.S.

Doctor of Philosophy

Kostia Bergman, Ph.D. Thesis: Sensory Responses of Phycomyces: I. Blue-Light Control of Sporangiophore Initiation. II. Classification of mad Mutants. Postdoctoral appointment, University of Seville.


Lee-Ming Kow, Ph.D. Thesis: Study of Ion Movements in Isolated Chicken Retinas During Spreading Depression. Postdoctoral appointment, Rockefeller University.


HONORS AND AWARDS

Seymour Benzer received the Albert Lasker Award in Basic Medical Research.
Roy Britten was elected to membership in the National Academy of Sciences.
A. J. Haagen-Smit received the first Cottrell Award of the National Academy of Sciences and the American Chemical Society Award for Pollution Control sponsored by the Monsanto Company. He was given an Honor Scroll for achievements in the field of chemistry by the American Institute of Chemists and the Man of Science of the Year Award by the ARCS Foundation.
Ray Owen was elected a member of the Board of Directors of the American Society of Human Genetics.
Jean-Paul Revel was elected President-Elect of the American Society for Cell Biology.
Roger Sperry received the Honorary Degree of Doctor of Science from Cambridge University. He was also named California Scientist of the Year. He received the first William Thomson Wakeman Basic Research Award of the National Paraplegia Foundation and the Distinguished Scientific Contribution Award of the American Psychological Association.
Jerome Vinograd gave the first Jesse W. Beams Lecture at the University of Virginia.
William Wood was elected to membership in the National Academy of Sciences.

APPOINTMENTS

Norman Horowitz was appointed to the Space Science Board of the National Academy of Sciences.
Ray Owen was appointed Member-at-Large of the National Research Council, Executive Committee of the Division of Biology and Agriculture.
Robert L. Sinsheimer was appointed Chairman of the Editorial Board of the Proceedings of the National Academy of Sciences.
Felix Strumwasser was appointed Associate Editor of the Journal of Neurobiology and a member of the Editorial Board of Physiological Reviews.
Jerome Vinograd was appointed to the Working Group for Molecular Control of the National Cancer Institute.
FINANCIAL SUPPORT

The financial support available for the work of the Division of Biology comes from many sources: from general institute endowment and from special endowment funds for broad areas of work; from grants or contracts with individuals, companies, foundations and U.S. governmental agencies for specific projects; from unrestricted annual gifts; from fellowships for the support of individual scholars; and from contributions to general funds provided by Industrial Associates and Institute Associates, as follows:

**Fund—Research Support**

- American Cancer Society
- John E. Barber Fund for Biological Research
- Beckman Instruments, Inc.
- Bing Endowment Fund
- James G. Boswell Foundation
- Cambridge University
- Lois Gosney Castle Fund
- Louise Jane Church Fund
- Norman W. Church
- Commonwealth Fund
- Corser Fund for Biology
- Damon Runyon Memorial Fund
- Josephine V. Dunke Fund
- Herman Frasch Foundation
- The Goodyear Tire and Rubber Co.
- Frank P. Hixon Fund
- Irma Hoefly Fund
- Lasker Award Fund
- Eli Lilly Foundation
- Clara Lockwood Fund
- Merck Company Foundation
- National Aeronautics and Space Administration
- National Science Foundation
- The President’s Fund
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- Research Career Program
- Research Corporation
- Rockefeller Foundation
- Gordon S. Ross Foundation
- Albert Billings Ruddock Fund

**Purpose**

- Cancer research
- Research, particularly as related to the brain
- Funds for equipment
- Professorship in behavioral biology
- Virus research
- General research
- Research in behavioral biology
- Research in biology
- Research in chemical biology
- General support
- General support
- C.I.T.-Huntington Hospital Joint Seminar
- Cancer research
- Plant growth research
- Biochemistry research
- Neurobiology, physiological psychology, and related research
- Cancer research
- Chemical genetics
- C.I.T.-Huntington Hospital Joint Seminar
- General support
- Faculty development
- Hibernation studies
- Studies in animal physiology, biochemistry, biophysics, developmental biology, genetics, neurobiology, plant and cell biology; undergraduate research participation program
- Research and development at JPL
- Medical science
- Research
- Genetic research
- Chemical biology research
- Medical research
- Professorship in biology
Edwin H. Schneider Fund
Sloan Fund for Basic Research
Stone Foundation
Sturtevant Memorial Fund
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Union Carbide Corporation
U.S. Atomic Energy Commission
U.S. Public Health Service

Joseph G. Venable Fund for Arthritis Research
Martin Webster Fund
Jean Weigle Memorial Fund
Wright Foundation

Study and research in genetics
General support
Psychobiology research
General support
Chemical biology research
Annual lectureship in biological research
General support
Research in radiation genetics, protein synthesis
Studies in basic experimental biology, animal physiology, biochemistry, biological systems analysis, biophysics, developmental biology, genetics, neurobiology, plant and cell biology, psycho­biology, and virology; graduate research training, biomedical sciences support grant
Arthritis research
Immunology and virology
General support
Medical science

Fund—Fellowship Support

American Cancer Society
Earle C. Anthony
Mary K. Beschorman Fund
California Foundation for Biochemical Research
California State College
Centre National de la Recherche Scientifique, France
Jane Coffin Childs Memorial Fund for Medical Research
Lucy Mason Clark Fund
Dartmouth College
Deutsche Forschungsgemeinschaft
Deutscher Akademischer Austauschdienst
Gloria Gartz Fund
E. S. Gosney Fund
Guggenheim Foundation
Malaysian Government
Helen G. and Arthur McCallum Fund
Medical Research Council, Canada
National Aeronautics and Space Administration
National Defense Education Act
National Science Council, Republic of China
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Veterans Administration
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Reflections on Seymour Benzer's Fly Paper, "From the Gene to Behavior."

Then operation Big Bang geneticists blastulated — Grigorous Mendel first eclosed. His immutant penchant for longshortstem sweetpeas, of Red-Cross blooms made him Father. Organisms galore (rotedervishers, paradigmecia, mnematodes, Escherichia colonic, phycemo-Myces and mice) in biochem-anatomical searches more'n morecular, in codings And structures were used, for philo- and ontogene-antics with nevarious degrees o' Sexexess... Caltech "chromato" selected my left acrostician to put me right on the map. He'n his breed with techerous mutagens my urgenitors hunted (never heard'm cry ouch!) Under strained, subcoding conditions he kept'm, invoking a neuronically-linked alibi — Normal behavior for Homo Sapiens! ... Little did we know that our trial was trivial Til Zer begat Seymour—then ... why, plus sex begat to fly! ... I metamorphomosaic-ed in a Ménage morganatic: my melanopater ethyl-methane-subformated, mom with no savoir-rhythm. Oh what freaks abound me! Slugalong, Grounded, Eggdropper, Twins-in-onebody ... (I ask yo u!) Rut-time for Stuck, for Drop-dead, for cuz'n who thinks he knows how when he doesn't, Grave misery did prove — all for the voyerg ratification of a night owl's generotic a! Avec this superenergencic sexdemoniac ("fun"-he-calls-it!) S. B. predestinarian, No blim'd prefractionized drosophilhomme can ever betrussed to keep his fly shut!

D. m.

alias Adina Cherkin Codray
Procion-dye identified amacrine cells in the frog retina. The amacrine cells whose somata lie in the proximal layer of the inner synaptic layer send their processes toward the internal synaptic layer. A similar amacrine cell was first shown by Cajal using the Golgi method. Amacrine cells are thought to participate in complex information processing within the retina. (Photo by Mobuyoshi Matsumoto, Research Fellow, Applied Science, 1970-71.)

Electron micrographs of hybrids of the heavy (H) strand of HeLa cell mitochondrial DNA with mitochondrial 12S and 16S ribosomal RNA and 4S RNA-ferritin conjugates. The RNA-DNA duplex regions can be distinguished from the single-stranded DNA portions for their thicker appearance. As illustrated in the explanatory tracings, each filament has two such duplex regions of unequal length separated by a single-stranded spacer, which correspond to the 12S and 16S RNA genes hybridized with the complementary RNA sequences. The locations of the nine 4S RNA genes (H1 to H9) are recognized from the positions of the electron opaque ferritin labels along the DNA strand, and are mapped relative to the ribosomal RNA sites.