The eyes of wild-type and visual mutants of Drosophila melanogaster as viewed under oil by the Franceschini technique.

Normal fly: Seven photoreceptors visible in each facet, six outer and one central

Mutant isolated at Caltech: central photoreceptor missing

Mutant isolated by Jane Koenig at UCLA: outer photoreceptors missing
A Report for the Year 1973-74

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 1973-June 1974, are listed separately, at the end of the research reports of each group.
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INTRODUCTION
NEW FACULTY

Two new appointments have been made to our faculty in neuro/behavioral biology, to take effect during the 1974-75 academic year. Dr. John Allman, a Ph.D. from the University of Chicago who has been working at the University of Wisconsin, and Dr. Ronald Konopka, a Caltech Ph.D. who has been working at Stanford University, will become Assistant Professors of Biology. Dr. Allman is interested in the cortical basis of visual perception; he has discovered that in primates the visual field is represented multiply, in several different areas of the visual cortex, which presumably have distinct modes of analysis and transformation. He is seeking to understand these distinctions and to learn how an organism may make use of these several modes of vision.

Dr. Konopka is interested in circadian rhythms. Working with the fruit fly Drosophila he has discovered that certain of its circadian rhythms are subject to genetic modification; with this lead he has traced the origin of these rhythms to a small cluster of cells in the Drosophila brain.

With these additions we continue to broaden our program in this exciting field.
FACULTY HONORS AND APPOINTMENTS

The following notable honors and appointments were received by the faculty during 1973-74:
Seymour Benzer was awarded an Honorary Degree of Doctor of Science from Columbia University.
Sterling Emerson was elected a foreign member of the “Matematisk-naturvidenskabelige Klasse” of The Royal Danish Academy of Sciences and Letters.
A. J. Haagen-Smit gave the first Raymond R. Tucker Memorial Lecture on March 29, 1974, entitled “A Lesson from St. Louis.” He was also one of the first recipients of the John and Alice Tyler Ecology Award, presented in February. On June 5, Dr. Haagen-Smit received the Rhineland Award for 1974 from environmental organizations of Western European nations.
Robert L. Sinsheimer was elected to membership in the Institute of Medicine of the National Academy of Sciences. He was also appointed to the Board of Advisors of the Jane Coffin Childs Fund.
R. W. Sperry was elected to the American Philosophical Society. He also received an honorary membership in the American Neurological Association.
RETIREMENT

Professor Anthonie Van Harreveld retired in June 1974 after forty years on the faculty of this Division, and has been appointed Professor Emeritus. Professor Van Harreveld’s contributions to our understanding of the vertebrate nervous system, his studies of the phenomenon of spreading depression, his demonstration of the existence of extracellular space in the CNS, and his recent hypotheses concerning the structural basis of synaptic change in memory deposition are widely known in neurobiology. Fortunately we will continue to have the benefit of his association and his wide knowledge.

NEW BUILDING

The Mabel and Arnold Beckman Laboratories of Behavioral Biology were dedicated on January 14, 1974. Speakers included President Brown, Professor Huttenback, Chairman of the Division of Humanities, Professor Olds, Bing Professor of Behavioral Biology, Professor Sinsheimer and Dr. Beckman. In this year, the Beckman Laboratories will be occupied by the research groups of Professors Allman, Konopka, Olds, Pettigrew and Russell; additional appointments will be made in the near future.
LECTURES

The Division sponsored two Jennifer Jones Simon Lectures on Biomedical Research to bring to Caltech and the neighboring medical community an insight into some of the promising areas of medical research.

Dr. Edgar Haber, Professor of Medicine at Harvard and Chief of the Cardiac Unit at Massachusetts General Hospital, spoke on “The Future of Antibodies as Specific Therapeutic Agents.”

Dr. Arno Motulsky, Professor of Medicine and Director of the Center for Inherited Diseases, spoke on “Current Status and Future Trends in Human and Medical Genetics.”

STUDENTS

Interest among Caltech undergraduates in the Biology program has continued at the high level of recent years. Eighty-seven of the 565 students in the upper three classes (15%) were enrolled in the Biology option during the winter quarter.

Thanks to the (perhaps temporary) reprieve of the USPH training grant program, we have been able to maintain our graduate program at nearly its customary size. Our future capability to support such a program clearly depends upon government policies.
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Research in this laboratory is concerned with the molecular basis of gene regulation in higher organisms. Recently, we demonstrated an ordered pattern of DNA sequence arrangement in the genome of the African toad *Xenopus*. We found that in general repeated DNA sequences about 300 nucleotide pairs long are interspersed with nonrepetitive DNA sequences of two general size classes, short sequences of about 1000 nucleotide pairs and much longer nonrepetitive sequences of 4000 nucleotide pairs or more. We believe that sequence organization probably provides the physical basis for the system properties of genetic control in higher organisms. We have now extended our investigations to other animals and have developed new techniques for the study of sequence interspersion and of DNA reassociation. An integral part of the work involves messenger RNA (mRNA), as a direct product of active structural genes. The mRNA studies are being carried out in a number of related projects designed to increase our understanding of regulatory processes at the molecular level. For example, we have already developed evidence that the gene product in question is usually transcribed from nonrepetitive DNA, that mRNA does not include both repetitive and nonrepetitive transcript on the same molecules, and that at least 80% of the mRNA that is nonrepetitive transcript is homologous to (and therefore presumably transcribed from) sequences adjacent to repetitive sequences in the sea urchin genome.

1. **INTERSPERSION OF REPETITIVE AND NONREPETITIVE SEQUENCES IN XENOPUS DNA AS STUDIED BY ELECTRON MICROSCOPY**

*Investigators:* Margaret E. Chamberlin, Eric H. Davidson  
*Support:* National Institutes of Health, Public Health Service  
National Science Foundation

An extensive examination by electron microscopy was undertaken to visualize reassociated repetitive DNA of *Xenopus laevis*. *Xenopus* DNA was sheared to 2700 nucleotide fragments, stripped of foldback regions and incubated until most of the repetitive sequence had reassociated. This material was visualized in the electron microscope by the Kleinschmidt procedure from formamide. 1600 molecules were photographed, analyzed and measured. Figure 1 shows examples of some of the structures seen. 28% of the mass of the DNA (362 structures) contains one repetitive duplex region and four single-strand ends. 24% of the DNA mass (276 structures) is in molecules containing more than one duplex region. The mean size of the DNA containing duplex regions is 2400 nucleotides. 38% of the DNA analyzed appears to have no duplex region. The mean size of this class of DNA is 1200 nucleotides indicating that it is mainly a breakdown product.

The repetitive sequence length is 300 nucleotides. This length is not affected by the length of the strand on which it occurs. The mean distance between two duplex regions on the same strand is 1100 nucleotides. An interspersion curve generated from the length of the interduplex regions as a
function of component strand length is consistent with the interspersion curve derived from hydroxyapatite binding data. Only four structures were found where two repetitive regions of one strand were paired with two regions of one other strand. This argues against homologous adjacent repeats. Very few slipped circles (5) were observed, from which we conclude most repeats are not long or tandemly repeated.

This study, utilizing electron microscopy, has independently confirmed the fine scale interspersion of repetitive sequence in *Xenopus laevis* DNA.
2. GENOME ORGANIZATION OF APLYSIA CALIFORNICA

Investigators: Robert C. Angerer, 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

We have undertaken studies on the DNA sequence organization of the mollusk Aplysia californica in order to determine whether the pattern of interspersion of repeated and single copy sequences previously found in two deuterostomes (Strongylocentrotus purpuratus and Xenopus laevis) is also observed in this protostome, and is thus a common feature of the genomes of members of these two major evolutionary lines.

Measurement of the rate of reassociation of total Aplysia DNA demonstrates the presence of families of an extremely wide range of repetition frequency. At our standard criterion, reassociation of 400 nucleotide fragments is observed between Cot values of \(10^{-6}\) and \(10^{-4}\).

7% of the genome forms S-1 nuclease-resistant duplexes at the earliest Cots measured \((10^{-6})\) and may represent tandem inverted repeats. When excised by means of S-1 nuclease from reassociated 2000 nucleotide fragments this class of sequences is found to range from several hundred to more than 1000 nucleotides long as measured by agarose gel filtration.

Measurement of the extent of base pairing in long DNA fragments incubated to a Cot where there is negligible reassociation of nonrepetitive sequences indicates a high degree of sequence interspersion in the Aplysia genome. At Cot 5, where only 25 to 30% of the nucleotides are base-paired, 50% of 400 nucleotide fragments and 90% of 2000 nucleotide fragments contain a duplex region as measured by their ability to bind to hydroxyapatite. Repetitive sequences reassociating between Cots \(10^{-5}\) and 2.5 were excised from 2000 nucleotide-long fragments by S-1 nuclease and their size as determined by agarose gel filtration was approximately 300 nucleotides. Thus, the presence of a short interspersed repetitive sequence is a feature of the genome of this protostome, as well as of the two deuterostomes previously studied.

3. FURTHER CHARACTERIZATION OF CLUSTERED AND INTERSPERSED REPETITIVE SEQUENCES IN THE SEA URCHIN GENOME

Investigators: Francine C. Eden, 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

Reassociation of 2000 nucleotide fragments of sea urchin DNA to a Cot at which only repetitive DNA sequences have formed duplexes (Cot 4), followed by S-1 nuclease digestion and collection of duplexes on hydroxyapatite, yields a DNA fraction (20% of the genome) consisting of repetitive duplexes with no single-stranded tails. These duplexes can be separated into two classes by column chromatography on Agarose A50. One-third of the duplexes (6 to 7% of the total DNA) consists of structures longer than 2000 nucleotides and is excluded from the column, presumably because of the reassociation of clustered repetitive sequences; most of the remaining duplexes are 300 nucleotides in length, and represent repetitive sequence elements interspersed with other (single copy) sequences in the sea urchin genome.
The clustered sequences (sheared to a fragment size of 300 nucleotides) are characterized by a thermal stability (Tm) within 1° of native DNA, whereas the Tm of the interspersed fraction is 10 to 11° lower, indicating extensive sequence divergence. Both classes of duplexes have essentially native hyperchromicity, as expected for enzyme-trimmed structures.

Duplexes formed after dissociation and self-reassociation of the clustered repetitive fraction have a Tm 3 to 5° lower than that of native DNA; in contrast, the thermal stability of interspersed repetitive duplexes formed after self-reassociation remains about 10° lower. This difference may be the result of different sequence arrangement within these two classes of repetitive DNA.

Measurements of the kinetics of reassociation show that both classes of repetitive DNA contain a component which reassociates very rapidly (Cot 1/2 = 0.04), and a second component reassociating more slowly (Cot 1/2 = 2.5). Reassociation of trace amounts of labeled DNA from each fraction with an excess of unlabeled DNA from the other fraction indicates extensive sequence homology between these two classes of repetitive DNA, although the sequence classes in these two populations are not identical.

These data suggest that the major difference between the clustered and interspersed repetitive sequences may lie in their sequence arrangement rather than in the particular nucleotide sequences represented.

4. DISTRIBUTION OF REPETITIVE AND NONREPETITIVE SEQUENCE TRANSCRIPTS IN HeLa mRNA

Investigators: William H. Klein, Eric H. Davidson, Roy J. Britten

Support:
Damon Runyon Memorial Fund for Cancer Research
National Institutes of Health, Public Health Service
National Science Foundation
Carnegie Institution of Washington
Charles B. Corser Fund for Biological Research

Polyadenylated messenger RNA (mRNA) extracted from HeLa cells was hybridized with a mass excess of HeLa DNA. The kinetics of the hybridization reaction demonstrated that most of the mRNA is transcribed from nonrepetitive DNA. The amount of mRNA hybridized to DNA was measured both with and without prior RNase treatment. Comparison of the results indicates that within the limits of detection, HeLa mRNA does not contain repetitive sequence elements covalently linked to nonrepetitive sequence transcripts. However, a small fraction of the HeLa mRNA preparation is transcribed entirely from repetitive DNA sequences. This fraction represents about 6% of the total polyadenylated mRNA preparation.

5. MEASUREMENT OF SEQUENCE COMPLEXITY OF POLYSOMAL mRNA IN SEA URCHIN EMBRYOS

Investigators: Glenn A. Galau, Eric H. Davidson, 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

The first measurement has been made of the number of diverse messenger RNA (mRNA) sequences (mRNA sequence complexity) in the total polysomes of a eukaryotic system, the sea urchin gastrula. mRNA was purified of nuclear RNA and any other heterogeneous RNA contaminants by release from polysomes with puromycin. Trace quantities of labeled nonrepetitive DNA fragments were hybridized with an excess of mRNA. The hybridization reaction followed ideal
first-order kinetics in mRNA concentration. At completion of the hybridization reaction, 1.35% of the nonrepetitive DNA was present as mRNA-DNA hybrid. The hybridized DNA was extracted and was at least 70% hybridizable with mRNA, demonstrating a 50-fold purification of the expressed sequences. This purified DNA fraction reassociated with excess unfractionated sea urchin DNA at a rate identical to that of the total nonrepetitive DNA tracer. The mRNA had therefore been hybridized to nonrepetitive DNA sequence, and the amount of hybrid could be used as a direct measure of the mRNA sequence complexity. The complexity of the gastrula mRNA can be calculated as about 17 million nucleotides, sufficient to comprise some 14,000 distinct structural genes. This result also provides an estimate of the number of diverse proteins being translated in the gastrula. From the rate of mRNA-DNA hybrid formation, we estimate that about 8% of the mRNA belongs to this complex class, and that less than 500 copies of each species of message in this class exist per embryo. Most of the mRNA population consists of a relatively small number of diverse species represented a much larger number of times. Since there are only 600 cells in the embryo, the most likely implication is that small subsets of the embryo cells contain most of the mRNA molecules of each type in the complex class. That is, we visualize a mosaic of mRNA distributions. Such an organizational pattern would nicely parallel the functional organization of the embryo which, at this stage, contains a number of distinct, differentiating cell lineages and tissue primordia.

We have found that a 600-cell embryo requires the transcripts of some 14,000 diverse structural genes. It seems obvious that in the entire developmental and physiological history of the organism a far larger number of structural genes would ultimately be required.

6. HYBRIDIZATION OF mRNA WITH NONREPETITIVE DNA CONTIGUOUS TO REPETITIVE SEQUENCES

Investigators: Barbara R. Hough, William H. Klein, Eric H. Davidson, Roy J. Britten
Support: Damon Runyon Memorial Fund for Cancer Research
National Institutes of Health, Public Health Service
National Science Foundation
Carnegie Institution of Washington
Charles B. Corser Fund for Biological Research

The sea urchin genome consists of repetitive and nonrepetitive DNA sequences, arranged so that nonrepetitive sequences are interspersed with repetitive sequences. It is also known that most polysomal messenger RNA (mRNA) is transcribed from nonrepetitive DNA, i.e. most structural genes are present only once in the DNA. The relationship between the interspersed repetitive sequences and structural gene sequences is unknown. The object of this investigation was to determine whether mRNA is transcribed from nonrepetitive sequences which are contiguous to repetitive sequences in sea urchin DNA.

A fraction of sea urchin DNA containing repetitive sequences and their contiguous non-repetitive sequences was selected by incubating long (2000 nucleotide) DNA fragments to a low Cot, so that repetitive sequences reassociated but nonrepetitive sequences did not. The DNA which contained repetitive sequences was isolated by hydroxyapatite binding. This DNA was sheared to 450 nucleotide-long fragments, and a nonrepetitive fraction isolated. Its complexity was about one-third that of the nonrepetitive component of whole DNA; thus this DNA fraction represented a 3-fold enrichment of sequences located close to repetitive sequences in the genome. This DNA was used in DNA excess hybridization reactions with purified, labeled mRNA isolated from sea urchin gastrulas (Goldberg et al., 1973).
mRNA reacted with about the same rate, relative to that of the DNA-DNA reassociation, in incubations with whole DNA and with the DNA fraction. At very high Cots, about 65% of the mRNA hybridized with whole DNA. In reactions with the enriched DNA fraction, 50% of the mRNA hybridized. Calculations based on the size distribution of sea urchin embryo mRNAs show that 80% or more of the hybridizable RNA is transcribed from structural genes which are contiguous to repetitive sequence elements.

Reference:

7. SEA URCHIN BLASTULA mRNA

Investigators: Barbara J. Wold, Eric H. Davidson
Support: National Institutes of Health, Public Health Service
          National Science Foundation

Techniques have been developed to obtain messenger RNA (mRNA) in high yield from blastula stage sea urchin embryos preliminary to further studies of mRNA sequence representation (Galau et al., 1974) and sequence organization. The procedure employs puromycin-induced release of mRNA from polyribosomes, and is modified from the method of Goldberg et al. (1973).

References:

8. SYNTHESIS KINETICS OF SEA URCHIN GASTRULA POLYSOMAL mRNA

Investigators: Glenn A. Galau, Eric H. Davidson
Support: National Institutes of Health, Public Health Service
          National Science Foundation

Studies of the sequence content and complexity of sea urchin gastrula polysomal messenger RNA (mRNA) indicate that there is heterogeneity in the frequency of the gene transcripts in the polysomes. The majority of the genes are represented only rarely while the large part of the mass of the mRNA represents a relatively few sequences. It was thus of interest to study the kinetics of mRNA synthesis at the same developmental stage to discern if heterogeneity might also exist in the rates of gene transcription.

Embryos were incubated with 3H-guanosine and the specific activity of GTP was measured utilizing thin-layer chromatography. The accumulation of label in mRNA and thus the net accumulation of mRNA was measured using puromycin release of mRNA from isolated embryo polysomes with corrections for the small quantity of label in ribosomal RNA in these preparations.

Preliminary results indicate that the entire embryo mRNA population turns over with a 4-hour half-life during the 27 to 45 hour period of gastrulation. While this particular study lacks the precision needed to indicate whether the 8% of the mRNA mass which contains the majority of the gene sequences turns over with a half-life much longer than that of the bulk mRNA mass, it does show that the half-life cannot be very much shorter than that of the whole mRNA since no such minor fast component was observed. The hybridization experiments of Goldberg et al. (1973) with 3-hour labeled mRNA, may then be interpreted as indicating a similarity in turnover rates for these classes of mRNA which differ about 100-fold in sequence concentration. This difference in concentration must then be a direct reflection of differences in transcription.

Reference:
9. ANALYSIS OF DNA SEQUENCE ARRANGEMENT USING HYPERPOLYMER FORMATION

Investigators: Dale E. Graham, Roy J. Britten
Support: National Institutes of Health, Public Health Service
National Science Foundation
Damon Runyon Memorial Fund for Cancer Research
Carnegie Institution of Washington
Charles B. Corser Fund for Biological Research

Hyperpolymer formation results from the pairing of single-stranded “tails” of DNA fragments already containing duplex regions. As such reassociations occur, average particle size increases. This process is limited for the reason that the hyperpolymer formed cannot exceed sequence length; a hyperpolymer formed from T4 DNA fragments would not be expected to be longer than the T4 genome. Sequence arrangement also affects hyperpolymer formation. If the sequence is virtually nonterminating; that is, if it is terminally repetitious (as are concatenated DNAs) or if it is tandemly repetitious (as are satellite DNAs), then the maximum length of the hyperpolymer is not limited. Hyperpolymer formation occurs at Cots that are 10 or more times the Cot ½ for the reassociation of a particular sequence.

The assay for hyperpolymer formation simply entails the monitoring of the increase in average particle size as measured by agarose gel filtration (A-50m). Using this assay, we have demonstrated that 300 nucleotide-long DNA fragments of T4 and E. coli can form particles containing more than 10 fragments at Cots much greater than their Cot ½s.

If the interspersed repetitive sequences of the sea urchin genome (which comprise more than 75% of the total repetitive sequences) were terminally or internally repetitious, they would be expected to form hyperpolymers. In Biology 1973 (No. 161) we reported that sea urchin repetitive sequences did not form hyperpolymers. We have expanded this study to include: (1) total repetitive DNA, (2) the fast and intermediate repetitive fractions, and (3) the interspersed repetitive sequences isolated by using S-1 nuclease digestion of reassociated, long DNA fragments. In each case, the great majority of the sea urchin sequences demonstrated no increase in particle size at 100 times the Cot ½. This suggests that the interspersed repetitive sequences of the sea urchin are homologous elements with discrete terminations and no internal repetitions.

10. DNA REASSOCIATION: MEASUREMENTS OF RATE

Investigators: Michael J. Smith, Eric H. Davidson
Support: National Institutes of Health, Public Health Service
National Science Foundation

The DNA reassociation reaction has been described as a bimolecular reaction with second-order reaction kinetics. A variety of methods are utilized to determine the extent of DNA reassociation reactions. Among these are hydroxyapatite chromatography (HAP) in which the HAP binds all DNA fragments containing duplex, hyperchromicity measurement which indicates the fraction of nucleotides base-paired, and digestion of the DNA with S-1 nuclease which, under the appropriate conditions, will enzymatically degrade to nucleotides those portions of the DNA that are in the single-strand form.

We are investigating the apparent deviations of DNA reassociation reactions from second-order kinetics when the reaction is assayed by various methods. HAP assays very closely follow ideal second-order kinetics even though HAP columns trap all DNA fragments with only some portion of their length in duplex form. We are determining the fraction of HAP-bound DNA which is still in single-strand form. HAP-bound DNA structures have been eluted and treated with S-1 nuclease which digests that portion of the fragments in the
single-strand form. Results to date indicate that a substantial portion of the DNA trapped by hydroxyapatite consists of single-strand DNA “tails.” For example, when *E. coli* DNA (sheared to 900 nucleotide pairs average length) is 95% reassociated as judged by HAP chromatography, approximately 25% of this DNA is still in the single-strand form as tails on duplex DNA structures. The fraction of the DNA \((1 - C/C_0)\) which is in actual duplex, as judged by S-1 nuclease at a given point during the reassociation reaction, equals \(1/(1 + kCot)^n\). We have empirically determined that the exponent \(n\) has a value of 0.44 in this system.

These studies are being extended to the analysis of the kinetics of hybridization reactions between DNA and complementary RNA. We are interested in determining the differences between the rate of DNA:RNA hybridization and DNA:DNA reassociation as well as the effect on hybridization rates of single-strand DNA tails in DNA duplex structures.

11. THE KINETICS OF THE DNA REASSOCIATION REACTION

*Investigators:* Roy J. Britten, Eric H. Davidson

*Support:* National Science Foundation
National Institutes of Health, Public Health Service
Carnegie Institution of Washington
Charles B. Corser Fund for Biological Research

The kinetics of reassociation of DNA are being examined in detail, since DNA duplex formation and DNA-RNA hybridization underlie many of the present approaches to the study of the genome and its arrangement and expression. An understanding of the kinetics of reaction is required to establish the extent of reaction and to determine the “complexity” or number of different sequences present in fractions of DNA or RNA.

Measurements made a number of years ago showed that DNA reassociation is an approximately second-order reaction (Wetmur and Davidson, 1968). Calculations have not accounted completely for aspects of the reaction which result from the random fragmentation of the DNA however. The first step in reassociation produces paired fragments which contain on the average about 50% duplex, with single-strand ends remaining which can take part in further pairing events. Thus, particles made up of many reassociated fragments can be formed. The single-strand ends may exhibit excluded volume and steric hindrance effects which would change their rate of reassociation.

These phenomena are being studied experimentally as well as by mathematical calculation and computer modeling. Reassociation of labeled fragments of single-strand DNA with particles which have been isolated on hydroxyapatite at various stages of completion of their reassociation has been measured. Early results indicate that the rate of reassociation of single-strand ends attached to particles is lowered only slightly compared to that which would be observed if they were free in solution. We are also studying the reassociation of particles with particles. Mathematical approaches show that reassociation should not follow the second-order form exactly, but should terminate significantly more sharply. It appears that there are components in the DNA which reassociate more slowly than the majority—possibly as a result of secondary structure.

*Reference:*
PUBLICATIONS


Professor: Max Delbrück
Visiting Associate: Richard P. Sutter
Research Fellows: Arturo P. Eslava, Edward D. Lipson, Klaus-Sten Thomson, Hans J. Weber
Graduate Students: Ernst-Peter Fischer, Lily Jan, Y. N. Jan
Research Staff: Maria Isabel Alvarez, Peggy A. Lockhart
Laboratory Staff: Jeanette Navest, Doris Tsongas, Michael P. Walsh

The motivation of the Phycomyces work continues to be the desire to see how far molecular biology can take us in the understanding of an organism, especially in the understanding of its integration into its environment, its intake of information, its processing of such information, and its eventual responses.

On the descriptive side, Lipson (Biology 1974, No. 13) has completed a study of the light growth response channel, using the “white noise” method developed earlier by Marmarelis and Naka at Caltech, and found to be especially well applicable to our system (see Foster and Lipson, Biology 1973).

Several other studies concern some presumed links in this channel. Jan and Forgac (Biology 1974, No. 14) showed that the cAMP level is involved and could demonstrate an in vitro effect of light on the activity of chitin synthetase (Biology 1974, No. 15) (cp. Jan, Biology 1973, No. 156). Our quest for the chemical identification of the elusive “blue” receptor pigment made a big leap forward through the construction, by Lipson and Walsh, of an elegant spectrometric facility and the demonstration with its aid of transient light-induced absorption changes, both in vivo and in vitro (Biology 1974, No. 16).

Meanwhile, genetic analysis has established 5 complementation groups related to this channel (Biology 1974, No. 18). The clear initial successes of this genetic work have encouraged us to push much harder in this direction: Eslava has embarked on a systematic study of mutagenesis (Biology 1974, No. 17), and has used auxotrophs, isolated last year, for sexual crosses (Biology 1974, No. 19). Some of these crosses yield sex heterokaryons with very interesting properties (Biology 1974, No. 20).

Parallel to this work using sexual crosses runs work on the physiology of the sexual reaction, a fascinating problem opened up by Burgeff half a century ago. The interplay of the sex hormones has been studied by Sutter and Mitchell (Biology 1974, No. 22), its relation to carotene metabolism by Sutter (Biology 1974, No. 23), and the latter’s regulation by Eslava et al. (Biology 1974, No. 21).

The avoidance response, which has baffled us for so many years, we now have good reasons to believe involves an exceedingly sensitive device for measuring random air movements (Biology 1974, No. 12), possibly via the effects of such air movements or the losses of a volatile substance.

The study of a light receptor pigment in a bacterium (bacteriorhodopsin) has led to results which challenge one of the oldest dogmas of visual physiology; namely, that it is triggered by an isomerization reaction (Biology 1974, No. 24).

12. THE AVOIDANCE RESPONSE OF PHYCOMYCES

Investigators: Y. N. Jan, Max Delbrück
Support: California Foundation for Biochemical Research
National Science Foundation
Ruddock Fund

If an object is placed about 1 mm from the growing zone of a Phycomyces sporangiophore growing in air, in about 2 minutes the sporangiophore starts to bend away at a rate of about 2°/minute for as long as half an hour or more. This is called the avoidance response of Phycomyces. The avoidance response is found to be dependent on the size and the distance of the nearby objects and to be independent of the composition and surface properties of the objects.

An air current parallel to the barrier and the sporangiophore can eliminate the avoidance response. The avoidance response is reversed by the addition of cAMP to the agar block, and it is eliminated by cAMP added to the medium.
In conjunction with this key observation, the Phycomyces responses to wind (rheotropic response and wind growth response) and to enclosure (house growth response) were characterized. The key parameter in all these responses seems to be the air movement in the vicinity of the sporangiophore growing zone. All observations are compatible with the assertion that a positive growth response is associated with a drop in wind velocity. To specify in which way air movement can become a signal directly received by the sensor, we propose that the avoidance is mediated by a volatile growth effector emitted somewhere along the sporangiophore and sensed by the growing zone (the chemical self-guidance hypothesis). Alternative forms of the hypothesis were tested experimentally. The only remaining viable one is that the sporangiophore emits growth-promoting molecules continuously. The majority of the molecules are readsobered by the growing zone before they diffuse away, and the barriers modify the distribution of the molecules exclusively by altering the ambient wind pattern, not by adsorption or reflection of the molecules.

13. WHITE NOISE ANALYSIS OF THE PHYCOMYCES LIGHT RESPONSE SYSTEM

**Investigator:** Edward D. Lipson

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

White noise studies (*Biology 1973, No. 154*) of the light growth response have been extended to different intensity ranges and to mutant strains. The wild-type response was examined at four absolute intensity levels spanning the full visual range. At a mean intensity level of $10^{-6}$ W/cm² ("normal range") the Wiener kernels show maximal amplitude and a latency of about 3 minutes. At lower intensity levels the amplitude diminishes gradually and the latency increases. At a mean level of $10^{-3}$ W/cm² ("high intensity range") the amplitude decreases to 32%. This result implies that pigment bleaching overtakes regeneration at an intensity of $0.5 \times 10^{-3}$ W/cm² and provides a relation between these two pigment parameters. At $10^{-6}$ W/cm² the power spectrum of the response record shows a rise of 20 dB/decade at low frequency and a rolloff of 80 dB/decade beyond the cutoff frequency of 0.15 min⁻¹.

In addition Wiener kernels have been obtained for a number of photomutants for the five known complementation groups (*Biology 1974, No. 18*) associated with the light response pathway. Above their threshold the madA (class 1.1) mutants show essentially normal response. On the other hand the madB (class 1.1) and madC (class 1.2) mutants show a weak and distorted response even above their thresholds. Output mutants (class 2) in groups madD and madE have responses with the same shape as wild type at $10^{-6}$ W/cm² but attenuated by factors of 30% and 60% respectively. Furthermore, examination of their response at low intensity ($10^{-9}$ W/cm²) indicates that their absolute threshold is close to that of wild type, as suspected.

14. THE LIGHT EFFECT ON THE INTRACELLULAR CONCENTRATION OF cAMP IN STAGE IVb SPORANGIOPHORE

**Investigators:** Y. N. Jan, Michael Forgac

**Support:**
- California Foundation for Biochemical Research
- National Science Foundation

Using Gilman's protein kinase binding assay, we found that the intracellular concentration of cAMP in stage IVb sporangiophores is about 2 µM and that light decreases the cAMP concentration in the sporangiophore. The concentration decreases by about 30% within 2 minutes after the onset of a
step up in light intensity from darkness. The concentration returns to the normal dark-adapted level within 15 minutes if the light is stepped up to 10 µW/cm². The concentration stays at 30% lower than the dark-adapted level for at least 30 minutes if the light is stepped up to 20,000 µW/cm². The time course of this light effect is analogous to the light growth responses of Phycomyces, i.e. a transient response to a step up of light intensity to $10^3$ µW/cm² and a steady-state response to a step up of intensity to 20,000 µW/cm².

Reference:

*Undergraduate, California Institute of Technology.

15. THE LIGHT EFFECT ON CHITIN SYNTHETASE ACTIVITY IN VITRO

Investigators: Y. N. Jan, Michael Forgac*, Edward D. Lipson
Support: California Foundation for Biochemical Research
National Science Foundation
National Institutes of Health, Public Health Service

In the genetic dissection of the sensory transduction mechanism of Phycomyces, one useful approach would be the chemical transfer experiments. If one can supply a mutant with certain cellular components from wild type and thus cure the abnormality of the mutant behavior, one would be in a good position to identify the molecular events which produce that behavior. In Phycomyces, such experiments are practicable only in vitro, because the sporangiophore takes up very few chemicals. The enzyme chitin synthetase was studied (Biology 1973, No. 156). To find out whether chitin synthetase activity is indeed affected by sensory stimuli, light was chosen as the stimulus because it is easy to quantitate and also because the light growth response is by far the best characterized sensory response.

The chitin synthetase activity of a homogenate of wild-type mycelium was $21 \pm 5\%$ greater when illuminated with 20,000 µW/cm² 488 nm blue light than in the dark. No significant difference was found between assays in room light (10 µW/cm²) and in the dark. This result is compatible with the physiological data on the light growth response. A step up in light intensity from darkness to room light intensity causes a transient positive growth response. Over the period of the enzyme assay, the total growth of sporangiophore under room light is only about 3% more than the total growth of sporangiophore kept in the dark; whereas a step up in light intensity from darkness to high intensity (20,000 µW/cm²) causes a 30 to 40% increase in the steady-state growth rate.

Thermal artifacts were ruled out, both by controlling the temperature of the sample illuminated with high intensity light, and by comparison of the light effects of wild type and of an albino mutant C2 which absorbs much less light.

In contrast to these findings with mycelial extracts, the chitin synthetase activity in a homogenate of sporangiophores exhibits a light effect of only marginal significance, probably because of the large contribution of the central vacuoles to the total volume of homogenate.

Reference:
*Undergraduate, California Institute of Technology.
16. LIGHT-INDUCED ABSORBANCE CHANGES IN PHYCOMYCES

Investigators: Edward D. Lipson, Hans J. Weber, Y. N. Jan, Klaus-Sten Thomson, Michael P. Walsh

Support: National Science Foundation
National Institutes of Health, Public Health Service
Deutsche Forschungsgemeinschaft
California Foundation for Biochemical Research

Phycomyces shares with a number of organisms an unidentified blue-light photoreceptor (Bergman et al., 1969) with an action spectrum suggesting a flavin chromophore. A promising assay for identifying and localizing this photoreceptor is offered by the measurement of light-induced absorbance changes (LIAC). Recently LIAC measurements have been reported on Dictyostelium and Phycomyces by Poff and Butler (1974) and on Neurospora by Munoz and Butler (1974). The combined spectrophotometric results for all three systems suggest a scheme involving flavin-mediated photoreduction of cytochromes.

To enable LIAC measurements in our laboratory we have constructed a spectrophotometer which operates in two modes. In the scanning single-beam mode the system records absolute and difference spectra of samples before and after exposure to actinic light and/or chemicals. In the dual-wavelength mode the system measures the kinetics of the relative absorbance change (down to 10^-4 OD) between two fixed wavelengths.

For in vivo measurements the optimal preparation seems to be young mycelia from petri dishes or from liquid culture. A rough LIAC action spectrum indicated blue-light sensitivity. Presently attention is being focused on fractionating the receptor using the LIAC as the ultimate assay. In particular, purification and testing of plasma membrane and mitochondrial fractions are being emphasized. In order to establish the connection between the LIAC and the photophysiology it will be important to demonstrate common defects in appropriate photomutants.

References:

17. MUTAGENESIS IN PHYCOMYCES

Investigator: Arturo P. Eslava

Support: National Science Foundation
Commission for Educational Exchange Between USA and Spain

Phycomyces mutants have been induced mostly by nitrosoguanidine (NG) treatment. Botstein and Jones (1969) found that after treatment of stationary-phase cultures of E. coli with NG the temperature-sensitive mutations are limited to one-third of the genome; Guerola et al. (1971) have shown that NG produces co-mutation in E. coli about 1% per neighboring cistron. The existence of parts of the genome not reached by NG and the production of double mutants in single treatments introduce big errors in biochemical and genetic studies.

To compare different mutagens and mutagenesis procedures we are trying to measure forward and reverse mutation rates induced in the wild type and in several mutants. First results are given in Table I.

References:
### Table I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Frequency of revertants</th>
<th>Resists to 5-fluorouracil</th>
<th>Resists to 8-azaguanine induced by NG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del (-)</td>
<td>-</td>
<td>-</td>
<td>1.7</td>
<td>13</td>
</tr>
<tr>
<td>S102 nicA101(-)</td>
<td>&lt;0.3</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1 leu-51(-)</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2 sul-51(-)</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4 nicA51(+)</td>
<td>&lt;0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

18. COMPLEMENTATION BETWEEN MUTANTS OF PHYCOMYCES WITH ABNORMAL PHOTOTROPISM

**Investigators:** Tamotsu Ootaki*, Ernst-Peter Fischer, Peggy A. Lockhart

**Support:** National Science Foundation
Deutscher Akademischer Austauschdienst
Gosney Fund

Fifteen mutants of Phycomyces with abnormal phototropism (phenotype mad) were tested for genetic complementation using heterokaryons produced by grafting stage I sporangiophores. Five complementation groups were found, defining five genes, designated madA to madE. This grouping of the mutants is completely consistent with the phenotypic classification scheme of Bergman et al. (1973), based on sensory responses of the sporangiophore other than those to light (geotropism and avoidance), and two responses of the mycelium to light. At two points the complementation grouping refines this scheme. The groups A, B and C coincide with the class 1 mad mutants of Bergman et al. (1973) which have a high threshold for the photoresponses but show normal geotropism and avoidance. Class 1 can be subdivided further into 1.1 (groups A and B) and 1.2 (group C), depending on whether the mutants do not or do show normal mycelial responses. The groups D and E coincide with class 2 which is defective on the output side of the sporangiophore growth response.

**Reference:**

*Education Faculty, Biology Department, Yamagata University, Yamagata, Japan.

19. SEXUAL GENETICS OF PHYCOMYCES

**Investigators:** Arturo P. Eslava, Maria Isabel Alvarez, Max Delbrück

**Support:** Commission for Educational Exchange Between USA and Spain
National Science Foundation
Ruddock Fund

Sexual genetics in Phycomyces has been little studied because of the long dormancy of the zygospores (6 months), the difficulties of manipulation of the progeny and the absence of suitable markers. We have used a wild-type (+) strain with a much shorter dormancy (less than 2 months) when crossed with our standard (-) strain (E. W. Goodell, unpublished) and a set of auxotrophic
markers. Crosses were performed to study the segregation of several markers and to start a series of backcrosses with the hope of constructing a (+) strain with short dormancy and isogenic with the (-) wild type commonly used for physiological studies. Crosses and some general properties are given in Table II.

From the analysis of the progeny several tentative conclusions are drawn:

1. *nicA101* and *nicA51* are alleles of the same gene, the frequency of intragenic recombination is about 0.3%.
2. *nicA, leu-51* and mating type are in different linkage groups.
3. The spores of a single germsporangium are the result of more than one meiosis in a few cases at least.
4. Very few or no apogamic nuclei contribute to the progeny.
5. There is a rather high proportion (~5%) of sexual heterokaryons in the progeny (see following report).

<table>
<thead>
<tr>
<th>Cross</th>
<th>Dormancy (days)</th>
<th>Germination rate of zygospores (%)</th>
<th>Germsporangia with fertile spores (%)</th>
<th>Average spores/germsporangium</th>
<th>Germination rate of heat-shocked germspores (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) x (+)</td>
<td>50</td>
<td>91</td>
<td>54</td>
<td>7,500</td>
<td>16</td>
</tr>
<tr>
<td><em>nicA101</em>(-) x <em>nicA51</em> (+)</td>
<td>53</td>
<td>83</td>
<td>54</td>
<td>9,000</td>
<td>12</td>
</tr>
<tr>
<td>(-) x <em>nicA51</em> (+)</td>
<td>52</td>
<td>69</td>
<td>42</td>
<td>6,500</td>
<td>24</td>
</tr>
<tr>
<td><em>nicA101</em> (-) x (+)</td>
<td>52</td>
<td>79</td>
<td>35</td>
<td>7,800</td>
<td>15</td>
</tr>
<tr>
<td><em>leu-51</em> (-) x (+)</td>
<td>57</td>
<td>72</td>
<td>37</td>
<td>6,500</td>
<td>19</td>
</tr>
<tr>
<td><em>leu-51</em> (-) x <em>nicA51</em> (+)</td>
<td>54</td>
<td>58</td>
<td>30</td>
<td>4,700</td>
<td></td>
</tr>
</tbody>
</table>

20. STUDY OF THE COMPONENTS OF SEX HETEROKARYONS OF PHYCOMYCES DERIVED FROM A THREE-FACTOR CROSS

**Investigators:** Maria Isabel Alvarez, Max Delbrück

**Support:** National Science Foundation
Ruddock Fund

We have studied the descendants of a three-factor cross (sex, nicotinic acid requirement and leucine requirement) by analyzing samples of 40 segregants from each of 36 germsporangia. Pooling the results from all sporangia we recovered all possible recombinants. However, individual germsporangia in most cases show only one of the eight possible genotypes. In 10 out of 36 fertile germsporangia we have found sex heterokaryons in addition to the apparently homokaryotic segregants. Are these sex heterokaryons formed by two viable genotypes which appear among the segregants? Or are they formed by two genotypes that do not appear because they are lethal in homokaryosis? To answer these questions we analyzed the genotype of 100 vegetative spores derived from each of 8 sex heterokaryons.

In two cases all 100 colonies were again sex heterokaryons. We assume that both components of these sex heterokaryons are lethal in homokaryosis. In each of three cases we found two segregating homokaryotic components, similar to those which had been isolated from the germsporangium in which the sexual heterokaryon had been found. In three cases we found only one homokaryotic segregant. In each case this segregant had also been seen in the germsporangium of origin. In these cases one of the components of the sex heterokaryons appeared to be recessive-lethal. We did not find any sex heterokaryons with
viable components different from those seen in the germsporangium of origin. These results suggest that the majority of the segregants in each germsporangium are lethal, and that some of the lethals are recessive and can proliferate in some heterokaryotic situations.

21. REGULATION OF CAROTENE BIOSYNTHESIS IN PHYCOMYCES BY VITAMIN A AND β-IONONE

Investigators: Arturo P. Eslava, María Isabel Alvarez, Enrique Cerdá-Olmedo*

Support: Commission for Educational Exchange Between USA and Spain
National Science Foundation

The presence of vitamin A in the medium results in a large increase in β-carotene content in wild-type *Phycomyces blakesleeanus*; in *carB* mutants, which accumulate phytoene, and *carR* mutants, which accumulate lycopene, the presence of vitamin A results in an increase in these intermediates. In *carA* mutants, which usually form no carotenoids, vitamin A partially counteracts the mutational block and allows β-carotene synthesis. Vitamin A is not itself converted to β-carotene. β-Ionone, long known as a stimulator of carotenogenesis, can also counteract *carA* mutations. The effects of vitamin A and β-ionone do not occur in the presence of the protein synthesis inhibitor cycloheximide. Vitamin A and β-ionone stimulate carotenogenesis through the normal pathway, probably by interaction with the *carA* gene product.

*Department of Genetics, University of Seville, Spain.

22. ANALYSIS OF THE "SEX HORMONE" MADE BY (+) BLAKESLEA TRISPORA

Investigators: Richard P. Sutter, Herschel K. Mitchell

Support: National Science Foundation
West Virginia University Sabbatical Program

Trisporic acids—oxidized, unsaturated derivatives of 1,1,3-trimethyl-2-(3’-methylene)cyclohexane—stimulate zygophore (sex cell) development in *Mucor mucedo*, *Phycomyces blakesleeanus*, and presumably in all other mucoraceous fungi including *B. trispora*. Plus cultures of *B. trispora* synthesize trisporic acids from precursors (*M*) made by (−) cultures; (−) cultures synthesize trisporic acids from precursors (*P*) made by (+) cultures. We have developed a defined medium to grow (+) cultures. Small amounts of *P* accumulate in the culture medium after growth has ceased. A neutral fraction extract from a liter of 7-day (+) culture medium contains *P* for (−) cultures to synthesize 1 mg of trisporic acids. Experiments are in progress to prepare enough (+) culture medium to isolate, purify and determine the structures of *P*.

23. MUTATIONS AFFECTING SEXUAL DEVELOPMENT IN PHYCOMYCES

Investigator: Richard P. Sutter

Support: National Science Foundation
West Virginia University Sabbatical Program

The hyphal tips of mycelia of opposite sexes [(+) and (−)] of wild-type *Phycomyces* swell when they grow near each other. Upon contact, the swollen hyphae (zygophores) intertwine and eventually give rise to zygotes (zygospores). Mutants with abnormal carotene synthesis are defective in
sexual development. Zygospores are formed at a reduced level in crosses of wild-type(+) with carA(−) mutants and are not formed in crosses of wild-type(+) with carB(−), carR(−), carAcarR(−), and super-carotene-producing(−) mutants. Zygospheres (swollen hyphae) are formed in crosses of wild-type(+) with carB(−), carR(−), and carAcarR(−) mutants, but they are formed only by the mutant. In contrast, zygospheres are formed by both sexes in crosses of wild-type(+) with super-carotene-producing(−) mutants. If only trisporic acid initiates zygosphere development, these observations are consistent with the hypotheses that: (1) β-carotene is a precursor of trisporic acid; (2) trisporic acid biosynthesis proceeds via mating-type specific precursors received from the opposite mating type; and (3) trisporic acid acts intracellularly rather than intercellularly.

These observations were made initially with cultures grown on a medium containing potato extract. Some observations were not reproducible when new lots of potato extract were used. A defined medium was developed and the initial results were verified. Experiments are in progress to determine if all available car mutants behave as described.

24. THE ISOMERIC CONFIGURATION OF RETINAL IN BACTERIORHODOPSIN

Investigator: Lily Jan

Support: California Foundation for Biochemical Research  
National Science Foundation

The isomeric configuration of the chromophore of bacteriorhodopsin was studied by thin-layer chromatography of the retinal and of the retinal oxime derivatives (Oesterhelt et al., 1973). It was found that substantial thermal isomerization of retinal occurs unless the temperature is controlled to below 4°C throughout the extraction procedure. Under physiological conditions the dark-adapted bacteriorhodopsin (R560) contains 13-cis retinal, in contrast to the 11-cis retinal of rhodopsin. Upon illumination, R560 is bleached to a form absorbing at 412 nm (M412), which regenerates to a 570-nm form (R'570) with a half-life of 5 msec in high salt solution (Oesterhelt and Stoeckenius, 1973) or a half-life of 13.5 seconds in ether-saturated high salt solution at room temperature (Oesterhelt and Hess, 1973). R'570 is also light sensitive and is converted by light to M412 (Oesterhelt and Hess, 1973). In darkness, R'570 is slowly converted to R560. (The sequence of light and dark reactions of bacteriorhodopsin in ether-saturated high salt solution at room temperature was checked by E. D. Lipson and Lily Jan in this laboratory.) The chromophores in both M412 and R'570 were found to be all-trans retinal. Since the bleaching of R'570 to M412 includes a series of photochemical reactions very similar to those of the bleaching of rhodopsin (W. Stoeckenius, personal communication), the finding that apparently isomerization of retinal is not involved in this case raises questions about the presumption that isomerization of 11-cis retinal is the triggering event in the bleaching of rhodopsin.

References:
PUBLICATIONS


Professor: Herschel K. Mitchell
Visiting Gosney Professor: Alfred Tissières
Research Associate: Richard P. Sutter*
Graduate Students: Anne Chomyn, William J. Driskell, John E. Geltosky, Galina Moller, Wayne K. Warzecha
Research Staff: Julia E. Del Zoppo, Loveriza S. Lipps, Ursula M. Weber-Tracy

*See Biology 1974, Nos. 22 and 23.

The central interests of our group are concerned with questions related to the biochemical genetics of the regulation of development in Drosophila. Investigations continue at levels of enzyme production and regulation, such as with phenol oxidase and chitinase, at the level of membrane transport and permeability and at the level of gene activation as in induction of chromosome puffing.

25. EFFECTS OF PHOSPHOLIPASE A₂ ("MINIMINE") ON DROSOPHILA

Investigators: Peter H. Lowy, Herschel K. Mitchell
Support: Josephine V. Dumke Fund
National Science Foundation

Phospholipase A₂ is an important enzyme in membrane metabolism and occurs with entirely different amino acid compositions in two well-studied sources—snake venom and porcine pancreas. A third phospholipase A₂ in bee venom is a glycoprotein with a single chain of 129 amino acids of known sequence and is identical with minimine described by us earlier.

Injection of bee venom phospholipase A₂ into Drosophila larvae causes them to stop feeding and growing and to develop into miniature adult flies weighing ¼ of normal flies.

The assay of the enzyme is difficult because the degree of dispersion of the substrate and the presence of divalent cations like Ca²⁺ can affect the apparent activity 1000-fold.

With regard to the miniaturizing effect we would like to know whether the injected bee phospholipase acts in the larvae as an enzyme or by some specific binding, and also whether endogenous enzyme levels change during normal larval development and metamorphosis.

Michel Philippart, M.D., of UCLA Neuropsychiatric Institute has very kindly assayed for us bee venom enzyme and larvae of different ages. Very tentatively we estimate that the effect is caused by injection of 100 times the endogenous level. Hoping to devise an improved assay procedure, we have synthesized the water-soluble radioactive substrate dihexanoyl lecithin, labeled in the 2-position only, with ¹⁴C-hexanoic acid. All labeled hexanoic acid released from this substrate by enzyme should be due specifically to phospholipase A₂ activity.

We have also prepared tritiated bee venom phospholipase A₂ and studied its binding to larval membranes prepared by sucrose gradient centrifugation. About 30% of the label was bound and not removed by repeated saline washes but was released by SDS or HCl. The selective binding could be due to differences in the carbohydrate portion of the enzyme.

References:
Lowy, P. H., Sarmiento, L. and Mitchell, H. K. (1971) 
26. PROTEIN SYNTHESIS IN SALIVARY GLANDS OF DROSOPHILA: RELATION TO CHROMOSOME PUFFS

Investigators: Alfred Tissières, Herschel K. Mitchell, Ursula M. Weber-Tracy

Support: National Science Foundation
Gosney Fund
University of Geneva

The salivary glands and other tissues from Drosophila melanogaster were dissected at various times throughout the prepupal period, as well as after heat shocks and ecdysterone treatments, and the proteins labeled by incubating the isolated tissues with $^{35}$S-methionine were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel. The labeled band patterns from salivary gland, as seen on the autoradiograph of the gel, showed striking variations, in a manner remarkably similar to variations in puff patterns during the same prepupal period. In proteins from Malpighian tubes, the pattern of bands varied to a lesser extent and in brain only a few components were modified.

Heat shock brought about the appearance of a number of new bands, while others were reduced in intensity. This effect was observed with all the tissues examined, salivary glands, brain and Malpighian tubes, as well as wing imaginal discs, tissue lacking polytene chromosomes. The six most heavily labeled bands induced by heat shock represent about 30%, and one component alone represents over 15%, of the total label in the sample, as seen in salivary glands, brain and Malpighian tubes. The synthesis of RNA at puff sites was investigated after heat shock by $^3$H-uridine labeling. By correlating the amount of $^3$H-uridine in some puffs with the level of $^{35}$S-methionine in some bands a tentative relation is suggested in a few instances.

The effect of ecdysterone treatment was also studied in the salivary glands. Changes in a number of protein bands were noticed, though they were much less pronounced than those following heat shock.

Reference:

27. RAPIDLY LABELED CHROMOSOMAL PROTEINS IN SALIVARY GLANDS OF DROSOPHILA

Investigators: Herschel K. Mitchell, Loveriza S. Lipps

Support: National Science Foundation

Following the earlier observations (Biology 1974, No. 26) that messenger RNA produced at puff sites on salivary gland chromosomes of Drosophila is rapidly translated to proteins, we have investigated the question of which, if any, of these proteins is found in the cell nuclei. It should be noted that neither DNA nor histones are in process of synthesis in salivary glands during the periods of our experiments. We have observed that: (1) salivary gland chromosomal proteins become maximally labeled between 15 and 20 minutes after a 3-minute pulse and a chase with cold amino acid; (2) the chromosomes become labeled throughout but there are some rate differences in specific regions; and (3) the nuclei from glands from different ages of prepupae have different complements of pulse-labeled chromosomal proteins.

At the present time no specific relation has been established between any specific chromosome puff and any specific chromosomal protein but it is clear that the system is suitable for examining the question of how one puff product may be involved in the subsequent orderly program of gene activity.
28. SALIVARY GLAND FUNCTION IN DROSOPHILA

Investigators: Herschel K. Mitchell, Ursula M. Weber-Tracy

Support: National Science Foundation

Larval salivary glands in Drosophila melanogaster survive for about 16 hours after puparium formation and they continue to show a specific sequence of chromosome puffs during this period (Ashburner, 1967). Since specific RNA and protein synthesis results from puffing (Tissières and Mitchell, 1974), it is reasonable to suppose that the salivary glands may have important functions during the major metamorphosis of the animals that occurs during the first 12 hours after puparium formation. Investigations directed at verification of this supposition have shown that: (1) Cytoplasmic secretions are discharged massively at least three more times after the ejection of “glue” (Lane et al., 1972) which occurs at puparium formation. Documentation of the details of these cycles by light and electron microscopy is in progress. (2) Throughout the period of existence of the larval salivary gland there is a rapid exchange of materials between the nucleus and the cytoplasm. By pulse-chase experiments and EM autoradiography we have shown that the nuclear “blebs” (Lane et al., 1972) do not involve RNA exchange. The “blebs” have been observed to be most frequent during later stages of prepupal life but it is not yet clear whether they show movement of materials in or out of the nucleus.

References:

29. HEAT-SHOCK PROTEINS IN DROSOPHILA MELANOGASTER PREPUPAE

Investigators: Anne Chomyn, Herschel K. Mitchell

Support: National Institutes of Health, Public Health Service

Subjecting Drosophila melanogaster prepupae to heat shock induces 9 bands on the chromosomes of salivary glands to puff (Ashburner, 1970). Tissières et al. (1974) have labeled heat-shocked salivary glands with $^{35}$S-methionine. The autoradiographs of the SDS gels of these gland proteins show at least 7 new bands. The animals were heat-shocked at 37.5°C for 20 minutes and the glands dissected out immediately after the heat shock. We have found that the characteristic pattern of heat-shock proteins remains constant for about an hour after the heat shock, and that the normal protein pattern of the animal is affected for as long as 3 hours after the heat shock. Heat shock for 1 minute at 37.5°C is enough to induce the heat-shock pattern.

These heat-shock proteins can also be extracted with a glycerol-Tween buffer and electrophoresed on non-SDS gels. We intend to isolate and study one heat-shock protein in particular which accumulates to 17% of the total protein label in heat-shocked glands (Tissières et al., 1974).

References:


30. STUDIES ON BINDING OF LECTINS TO CELL SURFACES

Investigators: Susan S. Brown, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

Studies of the cell surface using markers which can be visualized in the electron microscope have demonstrated a heterogeneous distribution of binding sites for concanavalin A on many cell types. This distribution can be altered by low temperature, metabolic inhibitors, and cytochalasin B but not colchicine. Fixation of the cell before labeling, as well as an examination of the pattern after a very short period of labeling has demonstrated that the distribution of binding sites in untreated cells is homogeneous. One cause for the rearrangements is the cross-linking which occurs between multivalent label molecules, forming patches of label. However, additional explanations must be found to account for the preferential clearing of label from the edges, microvilli, and sometimes the central region of the cell.

We have found that the peripheral areas of the cell which ruffle are also the areas which are clear of label. Treatments which affect ruffling also affect clearing. Secondly, we have found that the behavior of labeled ricin differs from that of concanavalin A. Ricin binding sites clear from the edges of the cell only when washed after application of lectin and can be relabeled immediately when they have cleared. Concanavalin A binding sites can recede from the edges even in the presence of the label and thus do not relabel immediately. This finding demonstrates that the appearance of unlabeled areas is not simply due to the insertion of new, unlabeled binding sites. The third finding is that when new sites appear, they are often first found in association with a structure which may be the precursor of a ruffle.

An explanation for these diverse findings could be that ruffling and insertion of new membrane are associated, and that this activity is involved in the clearing of these regions of label. The differences between concanavalin A and ricin could then be due to different rates of replacement of their binding sites.

31. DISTRIBUTION OF RHODOPSIN IN THE ROD OUTER SEGMENT MEMBRANES: COMPARISON OF RESULTS FROM PEROXIDASE IMMUNOLABELING AND FREEZE-FRACTURING EXPERIMENTS

Investigators: Lily Jan, Jean-Paul Revel

Support: California Foundation for Biochemical Research
National Institutes of Health, Public Health Service

We have shown that rabbit antibodies specific for bovine rhodopsin were able to cause clustering or redistribution of the membrane particles in freeze-fractured rod outer segment (ROS) cell membrane, suggesting that many of the intramembrane particles of the ROS cell membrane reflect the presence of rhodopsin. Since a drop in the level of rhodopsin and opsin in the vitamin
A-deficient mouse retina is accompanied by a drop in the number of membrane particles in the freeze-fractured disc membranes, the membrane particles on the disc membrane should also represent rhodopsin, or membrane structures resulting from the presence of rhodopsin.

The distribution of rhodopsin on the ROS cell membrane was shown to be uniform in Fab-peroxidase labeling experiments, whereas the intramembrane particle distribution in ROS cell membrane as revealed by freeze-fracturing was patchy. The freeze-fracturing experiment seems to give a better approximation of the rhodopsin distribution: prefixation of the samples in glutaraldehyde at 4°C should not cause any rearrangement of rhodopsin owing to the clearly demonstrable low membrane fluidity at 4°C. Once the membranes are fixed, rhodopsin is deprived of the freedom of lateral and rotational diffusion so that redistribution of rhodopsin during the subsequent rapid freezing step is also not likely. In the immunolabeling experiment, however, the membrane can be shown to be still fluid at room temperature after formaldehyde fixation. Thus redistribution of rhodopsin as a consequence of the attachment of large label molecules could take place.

32. DISTRIBUTION OF RHODOPSIN IN MOUSE RETINAL ROD: ANALYSIS OF RESULTS FROM INDIRECT FERRITIN IMMUNOLABELING ON THIN SECTIONS OF RETINA

Investigators: Lily Jan, Jean-Paul Revel

Support: California Foundation for Biochemical Research
National Institutes of Health, Public Health Service

In experiments where a tissue block is treated with antibodies and label molecules, the labels often do not penetrate deeply into the tissue. The cell membrane and the membranes of organelles may also impose a barrier to the diffusion of the label molecules intracellulary. For this reason a new technique was used to treat thin sections of retina with antibodies and label molecules (Biology 1973, No. 96). Rhodopsin and/or its cross-reactive molecules (opsin, precursors of rhodopsin) (Papermaster and Converse, 1974) can thus be found on the disc membrane, the cell membrane of the rod outer and inner segment, the connecting cilium, inside the cytoplasmic bridge (Richardson, 1969) and the rod inner segment. The level of ferritin label in the outer nuclear layer is not significantly higher than that in the inner nuclear layer or that in the outer nuclear layer of the control retina.

References:

33. DISTRIBUTION OF RHODOPSIN ON THE SURFACE OF MOUSE ROD OUTER SEGMENT: HEMOCYANIN IMMUNOLABELING IN A SCANNING ELECTRON MICROSCOPE STUDY

Investigators: Jean-Paul Revel, Lily Jan

Support: California Foundation for Biochemical Research
National Institutes of Health, Public Health Service

In the experiments described in the previous two sections (Biology 1974, Nos. 31 and 32), we observed the distribution of ferritin or peroxidase staining in cross-sections of the rod outer segment cell membrane but views of the true cell surface were hard to come by. Freeze-fracturing experiments allow us to study the distribution of membrane particles over a large area of the hydrophobic face of the fractured ROS cell membrane but not of the cell surface itself. Therefore we attempted to study the distribution of rhodopsin on surface views of the outer segment membrane.
as seen in the scanning electron microscope. Glutaraldehyde-fixed mouse retina was first treated with rabbit IgGs specific for bovine rhodopsin, then with hemocyanin-labeled goat IgGs specific for rabbit IgG. The presence of hemocyanin label all over the rod outer segment cell membrane thus establishes the fact that rhodopsin in the cell membrane exposes its antigenic sites to the extracellular space.

34. ISOLATION AND ANALYSIS OF GAP JUNCTIONS

Investigators: John R. Duguid, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

We are currently investigating the structural components of the gap junction, a specialized membrane structure found in many tissues, which is believed to be the connection between electrotonically-coupled cells.

An analysis of purified gap junctions by SDS polyacrylamide gel electrophoresis shows that the junction protein migrates as a single band of molecular weight 27,000. We find that a recently reported protein of lower molecular weight ("connexin") is due to enzymatic degradation during the isolation of the junctions. A lipid analysis by thin-layer chromatography shows a relative enrichment in cholesterol and also in phosphatidyl choline over whole liver membranes.

35. SCANNING ELECTRON MICROSCOPY OF MOVING CELLS IN SITU

Investigators: Gregory Nelson*, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

The embryonic chick cornea at Hamburger and Hamilton stage 20 consists of an epithelium backed by a collagenous layer, the primary stroma. Juxta-retinal mesenchyme cells invade the back surface of the stroma from the periphery, moving inward until it is all covered. Because the general direction of the movement of cells is known and the movements occur with very precise timing, one can use the scanning electron microscope to take a series of "snapshots" recording the migration of cells in their normal environment and allowing comparison with the behavior of cells moving in tissue culture systems. The cells in the intact embryo seem to move without the dramatic ruffling activity so characteristically seen in cells in culture (Ciba Symposium, 1973). One does find small lamellipodia and ruffles but also lobopodia, filopodia and other structures which may be involved in the forward movement of the cells. The appearance of the cells suggests random movements on the stromal surface which has many folds and ridges.

The stroma represents a surface on which cells would have been expected to exhibit the mechanotactic properties which can be demonstrated in model systems. The moving cells in the embryonic cornea, however, seem to pay no attention to the topography that they encounter, suggesting that contact guidance does not play a major role in determining the direction of their movement. Concepts such as contact inhibition of movement in colliding cells appear sufficient to account for the behavior of the migrating mesenchyme cells.

Reference:

*Undergraduate, California Institute of Technology.
36. MOVEMENT OF CELLS IN VITRO

Investigators: Jean-Paul Revel, Frances Janssen*, Jean Edens

Support: National Institutes of Health, Public Health Service

The movement of cells in vitro is being analyzed both with the light and the scanning electron microscopes. Cells are grown on coverslips imprinted with a grid pattern by evaporating gold on their surface. These coverslips can be used for time lapse studies with Nomarski optics so that the behavior of cells can be followed for extensive periods of time. The same cells can then be examined for their morphology in the scanning electron microscope. We have found that what appears as "ruffling" activity in the light microscope is seen in the scanning electron microscope as widely different structures. LA9 cells, for example, form large lamellipodia (thin protoplasmic extensions of the forward edge of the cells which extend forward, and after attempting to make contact with the substrate are pulled back). In other cell types such as chick heart fibroblasts the areas of the cells which seem to be ruffling when examined in the light microscope contain many long microvilli which extend from the forward edge of the cell. It is likely that these microvilli play the same role in cellular locomotion as the lamellipodia of other cell types. It is interesting to note that in all cases, contact with the substrate at the forward edge of the cell is made by a microvilli with a short radius of curvature, supporting the ideas of Bangham (1964) on the morphology needed to overcome repulsion between two approaching surfaces.

Reference:

*Undergraduate, California Institute of Technology.

37. BEHAVIOR OF LA9 CELLS ON COLLAGEN SURFACES

Investigators: Jean-Paul Revel, Marcia M. Miller, Jean Edens

Support: National Institutes of Health, Public Health Service

In order to compare the behavior of cells in vivo and in vitro we sought to approximate conditions in vivo more closely by growing some cells on collagen layers. Using LA9 cells we found that they have a widely differing morphology, assuming extremely low profiles and becoming very flattened. This would suggest that adhesion of these cells is greater on the collagen substratum than it would be on glass. After about 12 hours in culture, however, it was found that the cells regained their normal morphology. Upon examination in the scanning electron microscope it was found that the collagen had been digested locally where cells had been and that the cells were now resting once more on the glass surface. The possibility that the collagenase was secreted by these cells in a culture system is presently under investigation (see Biology 1974, No. 38).

38. BEHAVIOR OF LA9 CELLS ON FIBROUS COLLAGEN

Investigators: Marcia M. Miller, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

In a broader attempt to investigate the relationship between cells and their substrates we noted that LA9 cells, a line of transformed mouse fibroblasts, produce holes in thin collagen substrates. At first small holes in the collagen substrate are found at the margins of the cells. After longer periods of time in culture the holes become larger and the cells are frequently found between the coverglass and the sheet of collagen fibers. The fact that the collagen fibers appear to remain
intact over the cell surface may indicate that the ability of the cells to disrupt the collagen may be confined to the lower cell surface. Our initial observations were made with a crude preparation of acid-soluble rat-tail collagen dried onto coverslips from a dilute acetic acid solution. Similar results are obtained using rat-tail collagen, purified by repeated precipitation in NaCl, dried onto coverslips at neutral and acid pH. Since other cell lines we examined (BHK, 3T3, SV3T3) do not behave in the same manner on the collagen substrate and these observations may indicate the presence of a collagenolytic activity of the cells in the presence of serum, we are now trying to detect the presence of collagenolytic products in the culture medium.

39. CLOSURE OF THE NEURAL TUBE IN THE YOUNG CHICK EMBRYO

Investigators: Jean-Paul Revel, Michael L. Rees

Support: National Institutes of Health, Public Health Service

Examination of the neural tube at various stages of development in the young chick embryo reveals some interesting aspects of the actual closure mechanism. As the lips of the neural tube become approximated there appears to be surface ruffling activity with long flat lamellipodia extending from the cells of one side of the lip to those of the other lip (Revel, 1974). This activity takes place at a time when the two lips are already approximated. This is a case where ruffling activity does not take place at the edge of a cell sheet as would be predicted by current ideas about contact inhibition. The observation is also difficult to reconcile with the idea that ruffling activity is involved in cellular locomotion. It is easiest to explain the surface activity observed at the lips of the closing neural tube as part of a mechanism for promoting the adhesion between the newly apposed cells. The phenomenon is very similar to what can be observed during the fusion of the palatal shelves.

Reference:

40. SCANNING ELECTRON MICROSCOPY OF EPITHELIA PREPARED BY BLUNT DISSECTION

Investigators: Marcia M. Miller, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

Of the techniques introduced for the preparation of tissues for examination in the scanning electron microscope, we have found that blunt dissection is most suitable for examining epithelia because it favors cleavage at cellular interfaces. After fixation in situ in glutaraldehyde followed by OsO4, we cut or tease the tissue apart prior to dehydration and critical point drying. With this technique we can expose and examine all the surfaces of epithelia—apical, basal and lateral. Three-dimensional images of the surface elaborations, the intercellular spaces between cells and the underlying basement membrane can be obtained. We have used this technique to study the epithelia in small intestine, kidney, gall bladder and embryonic Xenopus skin.

With this approach one can recognize the region occupied by the occluding and adhering zonules on the lateral surface of the epithelia cells. Differences in the depth of the zonules between cells of epithelia with different permeabilities are readily seen. For example, the band of close adhesion between cells in the distal tubule epithelium is much deeper than the corresponding region in the proximal tubule epithelium. Lateral elaborations are most often seen as sheets and ridges and are not finger-like projections as one might assume from thin sections. Basal interdigitations of cells can be seen either when viewed from the side or the base. It is possible to study the relationship of the cells within the epithelium to the underlying basement membrane. With this technique one
should be able to study the activities of the lateral cell surface. For example, we have identified the sites of chylomicra secretion on the lateral cell surface of the small intestine. We are also able to visualize concanavalin A (con A) binding sites in tissues by combining this technique of tissue preparation with the labeling technique introduced by Smith and Revel (1972) for examining the con A binding sites of cells in culture.

Reference:

41. THE FINE STRUCTURE OF THE BASEMENT LAMELLA IN XENOPUS TAIL DERMIS

Investigators: Marcia M. Miller, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

The dermis of amphibian larvae is of particular interest because the epithelium is known to deposit collagen within a basement lamella in a number of orthogonal plies, a relatively simple morphogenetic event. There are two theories on the manner in which deposition may occur, the shingle theory and the ply theory. In the former the strands are thought to actually interdigitate at a shallow angle with one another in a manner similar to shingles stacked on one another. In the latter the collagen strands are thought to be deposited first in one ply and then in another in an array turned 90° from the first. We are using the scanning electron microscope and blunt dissection to look carefully at the basement lamella in the tails of larval Xenopus. This approach provides the advantage of letting one look at broad expanses of both the basement lamella and the undersurface of the epithelial cells from which it originates. We have found an intriguing system of grooves and ridges on the basal surface of these epithelial cells; thus the collagen, at least superficially, appears to be in a true orthogonal ply. We find however that at some stages of development the collagen fibers actually appear woven in a basket-weave pattern making it necessary to suggest a third pattern of collagen deposition. We would now like to see if we may relate the pattern on the undersurface of the cells to the pattern of collagen deposition.

42. AN INVESTIGATION OF TIGHT JUNCTIONS IN NERVES OF DIFFERENT SPECIES

Investigators: Bill Beutel*, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

We have previously reported (Biology 1973, No. 103) on the existence of tight junctions in nerve myelin, and in Torpedo electric organs. We have been able to confirm these findings and to extend our observations to the electric eel, and mouse, rat, hamster and guinea pig sciatic nerve. Tight junctions are much less prevalent in the mammalian systems although they can be found in a few places at the level of the external mesaxon. There seems to be a great disparity in the extent of the tight junction between electric eel and Torpedo electroplaques. This may be correlated with the physiological mechanism involved in the firing of the plaques. Besides tight junctions between layers of the myelin, extensive tight junctions are also found in the epineurial sheath surrounding the nerve.

*Occidental College, Los Angeles, California.
43. AN OPTICAL SPECTROPHOTOMETRIC METHOD FOR DETERMINING THE THICKNESS OF GOLD COATING ON SCANNING ELECTRON MICROSCOPE SPECIMENS

Investigators: J. Lee Compton, Jean-Paul Revel

Support: National Institutes of Health, Public Health Service

The relationship between the optical density of a gold coating on a glass coverslip and the thickness of the coating has been determined. The thickness of the coating on a sample may be determined by including a blank coverslip in the coating step and then measuring the optical density of the blank. Approximate thicknesses may be estimated by standardizing the position of the coverslips relative to the gold source and measuring the amount of gold before evaporation on a standard curve.

A series of coverslips with different thicknesses of gold coatings was prepared and the optical density and X-ray fluorescence of each coverslip measured. A second series of coverslips with thicker coatings of gold was prepared, the X-ray fluorescence of each slip measured and the thickness of the coating measured with an optical interferometer. The relationship between X-ray fluorescence and thickness of the gold coating obtained from the second series of coverslips was combined with the relationship between optical density and X-ray fluorescence obtained from the first series of coverslips to yield the final relationship between optical density and thickness of the gold coating.

PUBLICATIONS


The two species of single-stranded RNAs produced after infection by Sindbis virus, 49S RNA (the viral genome) and 26S RNA, have been shown to have messenger function. 26S RNA, a one-third fragment of the genome, is translated, in vitro and presumably in vivo, into the three structural proteins of the virus (or into their precursors). 49S RNA also serves as messenger; in addition this RNA is packaged into nucleocapsids, which bud through modified regions of the cell plasmalemma to form infectious virions. As expected from their messenger activity, both 49S and 26S RNA contain poly A tracts, of about 80 nucleotides in length.

The budding of nucleocapsids through the cell surface has been further investigated. The virus glycoproteins appear in the cell surface starting early after infection, and accumulate as the infection cycle progresses. At all times the distribution of these glycoproteins is fairly uniform over the cell surface, despite the fact that the virus buds extensively through processes which are located primarily at the cell periphery. We have also found similar virus-specific processes on cells infected with VSV, a virus unrelated to Sindbis.

We have also studied the budding process by using a mutant of Sindbis virus defective in maturation. Although many wild-type virions mature by budding directly from the cell surface as single particles, the nucleocapsids of this mutant bud exclusively from virus-specific processes. These processes differ markedly in morphology from those produced in wild-type infection. The virus yield in the case of the mutant consists primarily of large, infectious particles possessing more than one nucleocapsid in a single envelope.

The receptor sites for adsorption of Sindbis virus by uninfected cells have been studied. These sites are evenly distributed over the surface of the cell, and their concentration varies from cell to cell.

We are continuing our studies on the structural proteins of the virus.

44. IN VITRO TRANSLATION OF 26S RNA AND 49S RNA OF SINDBIS VIRUS

Investigators: Daniel T. Simmons, James H. Strauss Jr.

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

The two principal messenger RNAs of Sindbis virus found in infected cells, 26S RNA and 49S RNA, have been translated in vitro, using a lysate of rabbit reticulocytes.

Wild-type 26S RNA was translated primarily into two polypeptides which appear to be the Sindbis nucleocapsid protein (molecular weight 30,000 daltons) and the precursor of the two glycoproteins of the virion (molecular weight 100,000 daltons). A larger polypeptide (molecular weight 130,000 daltons) was synthesized in response to ts2 26S RNA, a species of RNA which was isolated from cells infected with the ts2 mutant of Sindbis virus. This large polypeptide is apparently the protein which accumulates in cells infected with the mutant virus and which is thought to be a precursor of all three viral structural proteins.

These results support the hypothesis that 26S RNA (molecular weight 1.6 x 10^6) is the messenger for the three structural proteins of the virion and that the RNA codes for one large polypeptide precursor. The precursor is then cleaved at a specific site to yield the nucleocapsid protein and a second polypeptide which, in infected cells, is cleaved in a series of steps to yield
the two glycoproteins of the virion.

Sindbis 49S RNA (molecular weight 4.5 x 10^6) was translated into 8 or 9 polypeptides ranging from 60,000 to 180,000 daltons in molecular weight. The viral structural proteins, as such, were not synthesized in response to the added 49S RNA.

45. THE MESSENGER RNAs OF SINDBIS VIRUS

Investigators: Daniel T. Simmons, James H. Strauss Jr.

Support: National Science Foundation, Public Health Service

Messenger RNAs from Sindbis-infected cells have been identified by isolation of polysomes from infected cells followed by release of messenger with EDTA. We had previously identified 26S RNA and 33S RNA as messengers by this technique (Biology 1973, No. 206). 26S RNA is the messenger of the three structural proteins of the virion (Biology 1974, No. 44). 33S RNA appears to contain only those base sequences present in 26S RNA and thus could not contain the information for translation into the other virus-specific proteins required during the infection cycle. We have now identified 49S RNA as a messenger in the infected cell. Approximately 90% of messenger RNA is 26S RNA (accounting for most of the 26S RNA inside the infected cell), with the remaining 10% being 49S RNA (33S RNA is but a minor component). Most of the 49S RNA in the infected cells is in the form of nucleocapsids which sediment at 140S and have the potential of maturing into infectious virions; only a minority of the 49S RNA serves as messenger. Thus it is 49S RNA which is translated to form the nonstructural virus-specific proteins during the virus infection cycle.

46. POLY A IN SINDBIS VIRUS SINGLE-STRANDED AND DOUBLE-STRANDED RNAs

Investigator: Teryl K. Frey

Support: National Science Foundation, Public Health Service

Polyadenylic acid (poly A) has been discovered in both virion (49S) RNA and intracellular (26S) RNA of Sindbis virus. This poly A is heterogeneous in size with a mean length of approximately 80 to 100 nucleotides. Both the location of the poly A within the Sindbis RNA molecule, and the poly A content of all Sindbis RNA molecules are uncertain.

Sindbis double-stranded replicative complexes (RFs and Rls) also contain poly A. The poly A in double-stranded Sindbis RNA is the same size as the poly A in Sindbis single-stranded RNA, indicating that poly A is synthesized as a heterogeneous species as part of the transcription process. Poly A is found specifically in RF I and RF III, but little or no poly A is found in RF II. This supports the idea that Sindbis 26S and 49S RNA (which contain poly A) are synthesized from RF III and RF I respectively. The lack of poly A in RF II indicates that little or no RNA with a messenger function is synthesized in this RF.

47. APPEARANCE OF SINDBIS VIRUS GLYCOPROTEINS IN THE CELL SURFACE

Investigator: Charles R. Birdwell

Support: National Institutes of Health, Public Health Service

After the infection of cells with Sindbis virus, newly synthesized viral glycoproteins are inserted into the cell surface. The virus then matures as the viral nucleocapsids bud through regions of the plasma membrane containing the viral envelope glycoproteins. Sindbis is a relatively simple virus,
and is thus a good system for studying the insertion of proteins into the plasma membrane and the mechanism of membrane biosynthesis in general.

I have studied the appearance of the virus glycoproteins in the cell surface by an indirect electron microscope labeling technique. Rabbit anti-Sindbis IgG and goat (anti-rabbit IgG) IgG were prepared. The goat (anti-rabbit IgG) IgG was then covalently coupled to hemocyanin with glutaraldehyde. Hemocyanin is a large, cylindrical protein which is relatively easy to identify in the electron microscope. For a typical experiment, chick embryo fibroblasts were infected with Sindbis virus, and at various times after infection the cells were treated with the rabbit anti-Sindbis IgG, followed by the conjugate of goat (anti-rabbit IgG) IgG and hemocyanin. Cells were then examined by the surface replica technique (Revel and Wolken, 1973). The position of hemocyanin in the cell surface thus indicates the approximate position of newly inserted virus glycoproteins.

When infected cells were prefixed with glutaraldehyde and labeled as described at 37°C, viral glycoproteins were detected at the cell surface as early as 2 hours after infection. The distribution of viral glycoproteins in the cell surface was fairly even, although a slight clustering was observed on some cells. By 3 hours after infection, cells were heavily labeled with hemocyanin. Previous studies have shown that Sindbis virus is not released in large amounts until 3.5 to 4 hours after infection, and is often released in patches (Birdwell et al., 1973); therefore, the insertion of viral glycoproteins into the cell surface precedes viral release by 1 to 1.5 hours. Furthermore, since the viral glycoproteins are inserted evenly into the cell surface but the virus buds in patches, the viral glycoproteins may be rearranged after insertion.

When unfixed, infected cells are labeled as described at 37°C, distinct clusters of hemocyanin are found in the cell surface. Clustering is also observed when cells are labeled at 4°C and allowed to warm to 37°C. This clustering implies that the newly inserted glycoproteins are free to diffuse laterally within the plasma membrane and that the rabbit anti-Sindbis IgG can cross-link these viral glycoproteins after they are inserted into the cell surface.

References:


48. MATURATION OF VESICULAR STOMATITIS VIRUS: ELECTRON MICROSCOPY OF SURFACE REPLICAS OF INFECTED CELLS


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

We had previously found (Biology 1973, No. 208) that Sindbis virus, an enveloped virus which matures by budding through the cell surface, was often released from infected cells through virus-specific "processes" originating at the cell periphery. To determine whether similar processes were found in cells infected by other enveloped viruses, we examined cells infected with an unrelated virus, vesicular stomatitis virus (VSV) using the surface replica technique. Virus-specific processes analogous to those seen in Sindbis-infected cells were seen in about 10% of chick cells infected with VSV. In addition, considerable numbers of VSV particles are released singly from the top and sides of the cells. The cell shown in the electron micrograph (Figure 1) illustrates both types of virus maturation.

In hamster cells infected with VSV, no such processes were seen.

We concluded that in contrast to the case of Sindbis maturation, VSV matures primarily by budding singly from all regions of the cell, and that VSV-specific "processes" represent only a minor mode of virus release.
49. GROWTH CHARACTERISTICS OF \textit{ts}103, A SINDbis TEMPERATURE-SENSITIVE MUTANT DEFICIENT IN MATURATION


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

\textit{ts}103 was isolated from a “small plaque” stock of Sindbis virus after nitrous acid mutagenesis. Although it was originally selected as a temperature-sensitive mutant because of its failure to form plaques on chick monolayers at 39°C, it has been shown to grow quite well up to 37°C. However, at all temperatures the plaques are minute, and the yield for \textit{ts}103 virions is seldom more than 5% of a comparable wild-type infection (with Sindbis HR strain) under the same conditions.

The high cut-off temperature makes unambiguous assignment of this mutant to a complementation group impossible. However, experiments to examine reversion frequency have demonstrated it to be a single mutation. \textit{ts}103 was first examined carefully after it was found that cells infected with it showed greater than normal amounts of hemadsorption; this may be due to the large numbers of abnormal processes extending from the infected-cell periphery (see \textit{Biology} 1974, No. 51).

Subsequent experiments have shown that the virus matures very slowly, lagging several hours behind wild-type infection. The particles produced are more thermolabile than the HR strain, indicating some alteration of the structural proteins or of the mode of assembly of the virions.

In addition many of the virions produced in \textit{ts}103 infection sediment more rapidly than wild-type Sindbis through a sucrose gradient. When these particles are rerun on a second gradient, they again sediment faster, indicating that they are not physical aggregates. Thin sections of pelleted \textit{ts}103 “fast” material indicate that these are particles containing more than one core within a single envelope. As would be expected, such particles show density heterogeneity on isopycnic centrifugation.

We are currently studying these particles in more detail (see \textit{Biology} 1974, No. 50) in order to understand the genetic defect in molecular terms. We hope that these studies will aid in understanding how viruses effect cell surface modification, and the forces which control the “budding” of the enveloped viruses.
50. THE PHYSICAL PROPERTIES OF ts103, A MUTANT OF SINDBIS VIRUS DEFICIENT IN BUDDING

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

Several lines of evidence led us to believe that the defect of ts103 was in the maturation process. These included the growth characteristics outlined in the previous abstract (Biology 1974, No. 49) as well as the visualization of the long, relatively empty, and abnormal processes observed in surface replicas of infected cells with the electron microscope (Biology 1974, No. 51).

We examined the membrane glycoproteins of ts103 virions in some detail. The two species of polypeptides are well resolved on polyacrylamide gels at pH 4.5 in the presence of Triton X-100 and urea. Regardless of the label used (carbohydrate or amino acid) the ts103 glycoproteins co-migrate exactly with the proteins of the HR parent. A slight difference in the ratio of fucose between the ts103 and HR proteins is seen, which may represent a difference of one carbohydrate residue. We also digested the glycoproteins with pronase and examined the resulting glycopeptides on Bio-Gel P-6 columns. Four sizes of carbohydrate chains were resolved, but no differences between those of ts103 and those of HR Sindbis could be detected.

Since the large classes of ts103 (1.3X, 1.7X, 2.2X, etc.) appear to contain multiple cores within a single envelope, we wanted to examine the cores and the RNA inside. We isolated various classes of rapidly sedimenting forms of ts103 and further purified them by isopycnic centrifugation in a shallow gradient of sucrose and D2O. All of the rapidly sedimenting particles exhibited marked density heterogeneity compared to a wild-type virus marker. The lighter particles appear to contain smaller cores while the heavier particles have apparently normal-sized cores. If the ts103 defect is in the core protein, then the core must play a very important role in organizing the cell surface during virus maturation.

51. ELECTRON MICROSCOPIC STUDIES OF CELLS INFECTED WITH TEMPERATURE-SENSITIVE MUTANTS OF SINDBIS VIRUS

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

The surface replica technique (Revel and Wolken, 1973) was used to study the maturation of two temperature-sensitive mutants of Sindbis virus, ts23 and ts103. The mutant ts23 has a defective envelope glycoprotein; the mutant ts103 has some sort of budding defect (see Biology 1973, No. 209).

The maturation of ts23 was identical to that of wild-type Sindbis virus at the permissive temperature, 30°C. At the nonpermissive temperature, 39°C, no ts23 virions were formed. The maturation of ts103, however, was quite different from that of wild-type Sindbis virus. Few ts103 particles were observed budding from the cell surface. Most of the virus was released through a variety of virus-specific processes extending from the cell periphery.

Such processes were also found in wild type-infected cells (Birdwell et al., 1973), but the morphology of the ts103 processes was much different than the wild-type processes. For example, ts103 processes were not evenly packed with virions, and the diameter of many of the particles was 100 to 150 nm; the diameter of a wild-type particle is approximately 70 nm. Many processes were devoid of particles throughout much of their length, with 100 to 150 nm particles at their tips. Particles with 70 nm diameters were sometimes associated with processes that contained mostly 100 to 150 nm...
particles. Thin sections of ts103-infected cells revealed that the 100 to 150 nm particles appeared to contain several viral nucleocapsids; many of these multicored particles appeared to be detached from the cell, although this could represent cross-sections of processes containing several core particles.

When the virions released from ts103-infected cells are purified on a sucrose gradient, most of the infectivity is associated with material which sediments faster than 70 nm particles (E. G. Strauss, personal communication). Thin-section studies have shown that this faster sedimenting material contains predominantly multicored particles, so it would appear that much of the virus released from ts103-infected cells consists of multicored particles.

References:

52. THE DISTRIBUTION OF THE RECEPTOR SITES FOR SINDBIS VIRUS ON THE SURFACE OF CHICK CELLS

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

I have previously used the surface replica technique to study the maturation of Sindbis virus from infected cells (Birdwell et al., 1973). This technique has now been used to study the adsorption of purified Sindbis virus to chick embryo fibroblasts. The adsorption of a virion to the cell surface involves attachment to a specific virus receptor on the cell surface; therefore, in this study the position of Sindbis virions on the cell surface was used to indicate the location of Sindbis virus receptors.

When chick cells were prefixed with glutaraldehyde and adsorbed with purified Sindbis virus at 37°C, there was an even distribution of virus receptors over most of the cell surface. The concentration of receptors on the cell surface varied from cell to cell, and areas of the cell surface with different concentrations of receptors were sometimes found on the same cell. The accompanying electron micrograph (Figure 2) shows two cells which are partially overlapping and which contain different concentrations of adsorbed Sindbis virus. One cell has adsorbed 46 virus particles per square micron of cell surface; the other has adsorbed 96 virions per square micron. We have observed concentrations from 20 to 160 per square micron, and the total number of receptors observed is thus on the order of 10^5 per cell.

When unfixed cells were adsorbed with purified Sindbis virus at 4°C, the virus particles appeared to be clustered on the cell surface. The exact mechanism for the formation of clusters is not known, but this clustering must arise by the movement of the virus particle-virus receptor complex within the plane of the plasma membrane, after the virus particle adsorbs to the cell surface.

Reference:
53. STUDIES ON THE STRUCTURAL PROTEINS OF SINDBIS VIRUS

Investigators:  John R. Bell, Richard F. Uhlmann

Support: National Institutes of Health, Public Health Service

The virion of Sindbis virus contains three proteins; two, of molecular weight about 50,000, which are associated with the lipid envelope, and one, molecular weight 27,000, which is associated with the RNA to form a nucleocapsid. The two envelope proteins can be resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, a strong ionic detergent, but the separation is not great enough or reproducible enough to make this a feasible method for preparatively separating these two proteins.

All three proteins are insoluble without a detergent or other solubilizing agent present. We are investigating alternate methods of solubilizing and separating the three proteins of the virion, in particular the two lipophilic envelope proteins. This presents special challenges due to the general lack of knowledge regarding hydrophobic protein chemistry. Among the techniques being examined are the use of the nonionic detergent Triton X-100 or organic solvents to solubilize the proteins, together with the use of column chromatography, polyacrylamide gel electrophoresis, and salt precipitation to effect separation.

The purification of the three proteins will permit chemical studies to be performed on them, which should provide information on the structure and morphogenesis of the virion. In addition, the techniques we develop may be applicable to other lipophilic proteins such as cellular membrane proteins and envelope proteins of other viruses, as the same problems of solubility and similarity of size often arise in working with these other proteins.

PUBLICATIONS


THE MABEL AND ARNOLD BECKMAN LABORATORIES
OF BEHAVIORAL BIOLOGY

James Olds

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The CNS is a tangled web into which the stimulus message diverges from the sensor and from which the decision message converges on the effector. Between these two, information becomes lost to analysis for some time. This has caused study to focus on sensory and motor mechanisms where messages are relatively localized prior to divergence or after convergence. Mechanisms of learning were ascribed to the raveled knot between. A method aimed to circumvent this was to follow the message in time and space up to a point where learning would happen on the outpost of the undiverged sensory systems, at the frontier of the great lost area. Studies aimed in this direction have systematically moved the frontier in the wrong direction, constricting the undiverged region. This is because newly recognized collaterals of the successive sensory pathways carry the message into new lost areas long before it reaches the terminus of the sensory path. Furthermore the message that reaches the upper stations of the sensory system is not a stable one unmodified by things like "interest" and "training." Instead the lost areas bring these influences to bear on it at every station. The progress from station to station is therefore labile, being amplified or attenuated by training procedures. While the backward movement of the frontiers has increased the raveled area, it has also showed some of the important switching processes of "learning" to occur in the early steps of the sensory system where these changes can be better studied. They show up during the first brief (10 msec) interval after stimulus onset, prior to the time when feedback loops from higher centers have had much time to modify and therefore confuse the picture. In other words, even though the tangled region is now recognized as starting almost at the boundaries of the brain, there is still a time required for information to diverge. The changes in neuronal process caused by a signal during the first 10 msec after it arrives at the sensor are relatively few in number and relatively localized. Studies can be directed at the switching operations that occur within these relatively undiverged message pathways. This has turned out to be an attractive way to simplify some features of the complex mammalian brain and to study some of the switches that are opened or closed in the brain while an animal is being trained. Because the field of stimulus influence was relatively small during the initial 10-msec interval, the number of interacting variables was reduced and some progress has been made.

54. MODIFICATION OF THE EARLY RESPONSES IN THE AUDITORY PATHWAYS DURING PAVLOVIAN CONDITIONING

Investigators: James Olds, Frank B. McCoy, Robert Nienhuis, Robert F. Nickerson, Donald D. Morris, Jill E. McGinnis, Richard K. Iizuka, Lisa Greenstadt

Support: National Institutes of Health, Public Health Service

Unit responses to a soft (20 db) tone were recorded in the cochlear nucleus, the inferior colliculus, the medial geniculate and the auditory cortex, stations 1, 3, 4 and 5 respectively of the ascending auditory pathway. Recordings were made before, during and after training. Several features of the stabilized message in an awake and behaving but untrained animal were observed. Progress was made toward tracking down the way the course of the message was changed by training. Briefly the observations were as follows (see Figure 1).

1) The message moved up the auditory pathway at 2 msec per station. It consisted in an acceleration of the spike rates in the stations through which it passed.

2) In each station there was a brief period during which the response rose to a peak and then retreated to a high plateau where it stayed for at least several more msec.

3) The earliest part of each response was almost unchanged by training.

4) However, at each station the peak or some part of the plateau was raised.

5) The changes were largest in the medial
Figure 1. Here the average spike rate (given in multiples of prestimulus background firing rate) is presented for successive 2-msec time bins before and after presentation of a soft (20 db) tone at the ear. In each auditory station three sets of histograms are presented (one behind the other). The first is during the 60 pseudoconditioning trials that preceded training. The second is the first 60 trials of training. The one in the rear is the second 60 trials of training. In each case responses for all 60 trials are averaged together to get the histogram shown. The curves were gotten by averaging together all those units in a brain area that showed increments caused by training. The actual number (N) for each curve is indicated in the box to its left as well as the larger number from which it was selected (From). A change caused by training appeared in the 10-msec time bin in all cases.
geniculate where there was more than a trebling of one part of the response and the peak was moved to an earlier point.

(6) These changes were next largest in the inferior colliculus where there was almost a doubling and a similar movement of the peak.

(7) They were smaller in the cochlear nucleus where there was almost a doubling of one part of the plateau.

(8) The most surprising feature of the changes was that they were synchronized in time. All started in the same “time bin” (i.e., at 10 msec after the stimulus reached the ear).

(9) While this appeared to indicate a common source of all the changes, the development of changes at different stages of conditioning pointed to separate sources. The collicular change appeared during the first 10 trials of training. The geniculate one not until more than 20 trials of training had passed (see Figure 2).

(10) These data were gotten by averaging together those “units” that showed increments caused by training. There was a significant number of these in stations 3 and 4 and a smaller but substantial number for the cochlear nucleus or the cortex.

Figure 2. Data computed as in Figure 1, but for only the 10-msec time bin. In this case the time points are further broken down. In Figure 1 they were grouped by 60 trial sets. Here they are by 10 trial sets. This shows the immediate change in the colliculus during the first 10 training trials and the 20 trials without change in the geniculate.
A second ground for caution was that there were changes (small ones) in the earliest components and this suggested a change in the tuning of the receptor itself (either by moving the head or the ear muscles). But these changes seemed too small and there were logical reasons against them as the source of the main changes.

The inferences and suppositions based on these data were the following:

1. The sliding relation between the onset of the change and the onset of the response was considered incompatible with the concept of a changed input moving smoothly up an unchanged pathway.

2. The fact that the response in each station began first and then showed the increment caused by training milliseconds later, and the fact that all the increments appeared at once suggested some outside source feeding into the auditory stations.

3. The fact that 2 msec would be required between source and target suggested that the changes should appear in the source at 8 msec after stimulation struck the ear. Because the source response needed to be changed by training at this time, this ruled out the stations of the auditory path (at least those studied) as sources of the change.

4. Because there were different times for the emergence of collicular and geniculate changes in the training series, it seemed there had to be more than one source.

5. Taken together these ideas suggested that reticular formation neurons lying along the boundaries of the auditory pathway might have plastic synaptic relations capable of linking them directly to auditory collaterals and these neurons might project back into the auditory pathway to facilitate and amplify important signals over unimportant ones.

Earlier data from a different experiment suggested that reticular formation neurons in the anterior pons might have the correct plastic responses and latencies for this function but comparison of other data from the two experiments showed that the two kinds of effects would need to be demonstrated in one experiment to make the case clear.

These experiments validate the assumption that progress can be made toward resolving some of the processes of "conditioning" as it occurs in the complex mammalian brain if the problem is limited to considering changes in unit responses occurring during the first 10 msec after auditory stimulation. Furthermore, the separation of unit response changes in colliculus and medial geniculate indicate that the several stages of a Pavlovian training procedure can be used in much the same way as paper chromatography is used by the protein chemist to "pull apart" things that seemed tightly coupled.

55. MIDBRAIN UNIT ACTIVITY DURING CLASSICAL CONDITIONING USING FOOD AND ELECTRICAL BRAIN STIMULATION REWARDS

Investigators: Steven E. Brauth, James Olds

Support: National Institutes of Health, Public Health Service

Many theories of conditioning and associative learning postulate the existence of neuron pools at "intersections" between CS and UCS message pathways in the brain (Hebb, 1949; Kandel and Tauc, 1965). The influences of the two signals must converge in space and time if learning is to occur. New responses might well emerge in brain regions outside the area of intersection, but these would have to be secondary to other new responses which emerged in the intersection field. It seems likely, therefore, that a priori connections with either the CS or the UCS would increase the chances of a neuron participating in the conditioning process.

In this study neurons were monitored in the midbrain reticular formation and ventral tegmentum during a conditioning experiment. Many units in these regions show unconditioned responses to auditory and visual stimuli. Moreover, Kornblith and Olds (1973) have shown that cells in the midbrain reticular formation evidence enhanced responding to an auditory tone CS after it has been paired with a food reward. Nevertheless, food reward is a complex stimulus whose neural mechanisms are not understood well enough to allow identification of those cells driven by the reinforcement. Rewarding electrical stimulation of the medial forebrain bundle, however, does drive neurons throughout the midbrain and ventral forebrain which can be studied in a freely-moving animal (Ito and Olds, 1971). For these reasons medial forebrain bundle stimulation was used as the UCS in the present experiment, and an auditory tone was used as the CS+. A food
reward condition was also employed in order to assess the extent to which the brain stimulation reward produced unique effects.

Results indicate that only those cells possessing prior responses to the CS+ change response as a result of conditioning. Neurons not possessing prior responses to the CS+ do not develop new responses. Those cells which are driven by both the auditory tone CS+ and the brain shock UCS are significantly more likely to change response than those driven by the CS alone. In addition, neurons driven by both CS+ and brain stimulus UCS in the reticular formation show response changes during the first 10 conditioning trials before response changes appear in other midbrain units studied in this experiment. These effects imply that intersection of reward pathways and auditory arousal pathways plays a primary role in the appetitive conditioning response.

References:

56. UNIT RESPONSES IN THE MEDIAL FOREBRAIN BUNDLE ELICITED BY REWARDING STIMULATION OF THE POSTERIOR LATERAL HYPOTHALAMUS

Investigators: Marianne E. Olds, Roy E. Parrish
Support: National Institutes of Health, Public Health Service

Until recently very little was known of the neurophysiological events that take place during self-stimulation behavior. The reasons for this were, on the one hand, the complexity of input and output connections to the main anatomical substrate of self-stimulation behavior, the medial forebrain bundle, and on the other hand the absence until recently of methods for recording extracellular activity in the freely behaving animal. Yet information about the neuronal events related to self-stimulation behavior might help resolve the paradoxical observation that almost anywhere in the medial forebrain bundle electrical stimulation is reinforcing although the inputs to and connections of this pathway are so complex. The present study was undertaken therefore to continue previous investigations of the type of neuronal activity that correlates with self-stimulation. It has been shown previously by Ito that excitatory and inhibitory neuronal effects were elicited by rewarding brain stimuli. The excitatory effects occurred mainly in the preoptic region, the inhibitory effects mainly in the hypothalamic region, slightly anterior to the site of stimulation. The main emphasis in the present study was on relating the properties of these neuronal effects to properties of self-stimulation behavior observed when a continuing series of current intensities was applied. For this purpose, the unit activity elicited by the reinforcing electric train was analyzed by a computer of average transients which enabled the correlation of post-stimulus histograms with the various rates of self-stimulation behavior. Our results suggest the existence of a diffusely distributed excitatory influence throughout the medial forebrain bundle which is partially or completely suppressed in a circumscribed region by an inhibitory influence originating near the site of stimulation. The latencies and the magnitude of the excitatory effects elicited by rewarding stimulation varied as a function of the intensity of stimulation. This was not the case for the inhibitory effects which reached peak magnitude at the lowest intensity applied, regardless of whether or not self-stimulation behavior was produced at the same intensity. It appears at this point that the excitatory effects produced by the rewarding stimulation, because they are so diffusely produced throughout the medial forebrain bundle, are consistent with the anatomical and pharmacological evidence implicating the long ascending fibers originating in the mesencephalon as crucial elements of the reinforcing circuit. To what extent the inhibitory influence plays a similar role, and to what extent moving the site of stimulation closer to where the cell bodies of these long ascending fibers lie, will influence the interaction between the excitatory and inhibitory neuronal events taking place during self-stimulation behavior is at present being studied.
57. EFFECT OF INTRAVENTRICULAR NOREPINEPHRINE ON NEURON ACTIVITY IN THE MEDIAL FOREBRAIN BUNDLE DURING SELF-STIMULATION BEHAVIOR

Investigators: Marianne E. Olds, Roy E. Parrish

Support: National Institutes of Health, Public Health Service

The convergence of evidence relating the presence of the catecholamines in the medial forebrain bundle to the positively reinforcing regions has resulted in the hypothesis that one, or possibly both, of the amines are transmitters in this functional system, or that at least they modulate neuronal activity in it. At the neuronal level, with the use of the iontophoretic method, much evidence has accrued to suggest that the action of norepinephrine on neuronal activity is inhibitory. The present study is an attempt to obtain evidence correlating the action of the monoamines on self-stimulation behavior with their action on neuronal activity in the medial forebrain bundle during this behavior. For this purpose cumulative doses of 10, 20, 30 and 40 µg of each substance were introduced into the lateral ventricle of the rat while he was depressing a lever to obtain an electrical stimulus applied to the posterior lateral hypothalamic region. The effects of each dose on behavior and on neuronal activity were determined after each 10 µg. Four substances—l-norepinephrine, d-norepinephrine, dopamine, and serotonin—were tested. It was found that all four facilitated self-stimulation behavior, but to different degrees and at different doses. L-Norepinephrine was the most effective of the substances and caused an increase in the rate of behavioral responding at each of the doses tested; d-norepinephrine was the least effective of the four compounds. The effects of dopamine and serotonin were facilitatory in some animals but not in others. At the time when these effects on behavior occurred, excitatory neuronal activity elicited by the electrical stimuli during self-stimulation behavior was suppressed by l-norepinephrine, and inhibitory neuronal activity elicited by similar stimuli was potentiated by l-norepinephrine. Similar effects on neuronal activity were not observed with the other transmitters or with d-norepinephrine. This study is part of a larger effort aimed at answering the question whether a hypothalamic adrenergic neuronal system is involved in the regulation of positive reinforcement behavior in the rat.

58. EFFECTS OF CHLORDIAZEPoxide, DIAZepam, PHENOBarBITAL, AND CHLOORPROMAZINE ON CONDITIONED EMOTIONAL BEHAVIOR AND CONDITIONED NEURONAL ACTIVITY

Investigators: Marianne E. Olds, Mamoru Umemoto, Michael Gardner

Support: National Institutes of Health, Public Health Service

The anti-anxiety action of the benzodiazepines is reflected in their effect on conflict behavior. In tests designed to produce conflict the subject is working for a food reward. At randomly-spaced intervals a light stimulus (or some other stimulus) is introduced, and this is followed immediately by an aversive stimulus (in our tests, stimulation of the central gray region). As a result, the lever-pressing behavior of the animal for the food reward becomes suppressed during the presentation of the light, the conditioned stimulus. The benzodiazepines' effect on this behavior at nonsedative doses is to release suppressed behavior. The mechanism of this release is not known. It could be a behaviorally stimulant action, which is often observed after the administration of low doses of the minor tranquilizers or the barbiturates, or it could be some special action on central pain mechanisms. We have investigated these possibilities with two main aims in mind. One was to compare the anti-anxiety effect of the benzodiazepines with the effect of the barbiturates, and the other was to correlate the action on behavior with the action on neuronal activity in the amygdala, the hippocampus, and the hypothalamus, regions that probably regulate the expression of emotional behavior. Our results to date show that chlorpromazine was the only drug that was completely ineffective in conflict situations. The minor tranquilizers and the barbiturates disinhibited behavior suppressed by painful stimuli, but at different doses and to a different degree. Chlordiazepoxide was the most effective of the compounds tested. Generally, the effective compounds reduced the spontaneous rate
of discharge of neurons in the limbic and hypothalamic regions. Before the administration of drugs, a correlate of suppressed behavior was a change in the rate of discharge during the presentation of the conditioned stimulus when the subjects were conditioned. This could be either an acceleration or a deceleration in the rate of firing compared to the spontaneous rate during the period immediately preceding the conditioned stimulus. Diazepam and phenobarbital abolished these conflict-conditioned changes in the rate of firing at the time when they also disinhibited behavior. Chlordiazepoxide, which was more effective than diazepam on behavior, was not as effective as diazepam on the conditioned neuronal activity. On the other hand, chlordiazepoxide was the only compound that was effective on behavior and that simultaneously reduced the spontaneous rate of firing and disrupted the conflict-conditioned neuronal rate in the amygdala. The fact that the amygdala has been implicated in the expression of emotion to a greater degree than the other regions studied may be significant in understanding the mode of action of those drugs. The findings are being pursued in several directions: (1) tests with central stimulants to determine the extent to which their action on conflict behavior is comparable to the action of the minor tranquilizers and the barbiturates tested at doses that also cause behavioral stimulation; (2) tests with the anti-anxiety compounds on escape behavior maintained by electrical stimulation of the central gray region of the midbrain in order to determine whether, at the doses where these compounds are effective in conflict situations, they also modify the threshold for brain applied painful stimuli; and (3) the modification of central levels of transmitter substances to see the effects of such manipulation on the action of the anti-anxiety drugs. Eventually, it should be possible to predict the anti-anxiety effect of a compound in conflict tests from its action on neuronal activity; this will be possible when the mode of action of the presently effective drugs becomes known.


Investigators: Marianne E. Olds, Eugene Akutagawa, Roy E. Parrish

Support: National Institutes of Health, Public Health Service

It is at present well established that 6-hydroxydopamine (6-OHDA) injected into the ventricle is taken up by noradrenergic and dopaminergic terminals, and that after a suitable interval it causes the degeneration of these terminals. This compound has been used as a pharmacological tool to cause selective chemical lesions in the central nervous system. In the present study we have used this method to investigate the extent to which self-stimulation behavior obtained in several different regions of the diencephalon and mesencephalon is dependent on the integrity of the noradrenergic and/or the dopaminergic pathways. Our subjects were albino rats with electrodes implanted in three different anatomical regions. In the first series, the probes were placed in the posterior lateral hypothalamus, the midbrain raphe nucleus, and the nucleus ceruleus. In addition, each subject had a cannula permanently implanted for subsequent injection into the lateral ventricle. The animals were trained to self-stimulation for electrical reward at each of the three sites. The intensities ranged from zero to threshold and maximal level, producing optimal rates of responding. Training resulted in characteristic patterns of responding for the brain reward at each site where stimulation was applied. 6-Hydroxydopamine was infused into the lateral ventricle, and tests were made first daily and then weekly to determine the immediate and long-run effects of such infusion on the rate of self-stimulation tested sequentially in the three regions where electrodes were placed. It was apparent that differential effects were produced. Self-stimulation in the nucleus ceruleus and in the hypothalamus was abolished at the time when self-stimulation behavior in the raphe nucleus was not even affected. Furthermore, replacement therapy with intraventricular injection of amphetamine or l-norepinephrine was considerably more effective on reinstating self-stimulation behavior in the posterior lateral hypothalamus than in the raphe nucleus or in the substantia nigra. On
the other hand, when serotonin was infused into the lateral ventricle, self-stimulation behavior in the raphe nucleus was reinstated. Further testing is aimed at determining whether dopamine will reinstate self-stimulation behavior in the substantia nigra to a greater extent than it does in the hypothalamus. At present the results suggest that more than one of the monoamines is implicated in the regulation of self-stimulation behavior. Future tests along these lines will be aimed at circumscribing the chemical sympathectomy of 6-OHDA either to the noradrenergic terminals or to the dopaminergic terminals with the use of the tricyclic compounds because of their action on amine uptake, and additionally, to use 5,6-hydroxytryptamine to cause degeneration of serotonergic nerve endings.

60. VARIATIONS IN THE LEVELS OF BRAIN MONOAMINES AND IN TURNOVER OF THESE SUBSTANCES AS A FUNCTION OF SELF-STIMULATION BEHAVIOR

Support: National Institutes of Health, Public Health Service

There is good evidence to suggest that the monoamines have an important role in the expression of emotional behavior. One possible model for exploring the existence of such a relationship is the utilization of self-stimulation behavior, since the rewarding stimuli can be applied to a number of regions, each rich in one or more types of monoaminergic terminals. Previously we have shown that after a period of self-stimulation the levels of norepinephrine are reduced in the hypothalamus, but not in the rest of the brain. We have also shown that such reduction was not correlated with stress, a factor that often intrudes in the maintenance of steady responding for direct stimulation of the brain. At the present time, these studies point in two directions. The first has to do with the question of utilization of norepinephrine as measured by the turnover rate of $^3$H-norepinephrine infused into the cerebrospinal fluid. In this study small tissue samples from a variety of regions are assayed for labeled norepinephrine after a period of self-stimulation. The second study is concerned with the possible correlation between a fall in the rates of self-stimulation after lesions of the nucleus ceruleus with 6-OHDA and the levels of norepinephrine, dopamine, and serotonin in the brain. Evidence from these experiments will help to determine the validity of the view that the monoamines are utilized during self-stimulation behavior.

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61. TIME COURSE OF LEARNING AND MEMORY

Investigator: Alvin J. Hill Jr.
Support: National Institutes of Health, Public Health Service

Recent studies using mice have indicated that performance in appetitive tasks may be enhanced during inter-session intervals following partial training. The existence of such “incubation” effects in rats as well as in mice would have interesting implications for current theories of learning and memory consolidation. In particular, it would be of interest to see whether interventions (such as electroconvulsive shock, antibiotics and anesthetics) which modify consolidation of well-learned tasks have similar effects on incubation of partially-learned tasks.

The possibility of such effects in rats is being investigated, using both behavioral and electrical brain recording indicators of learning. Rats are trained briefly to associate one of two different tones with a food reward and are then tested after intervals ranging from 1 to 12 hours. Appropriate feeding and start-time schedules are maintained in order to minimize motivational and activity changes. Brain regions being investigated include hippocampus, midbrain reticular formation, amygdala, medial geniculate nucleus and auditory cortex.
PUBLICATIONS


In many ways, the mammalian visual pathway is an ideal model for the study of brain function. Single neurons can be studied at each of a number of well-defined hierarchical levels of the pathway with patterns of stimulation which are at the same time precisely controlled yet complex and interesting enough to be relevant to a human's own realm of experience. At the cortical level, neurons require quite sophisticated patterns of stimulation in order to respond vigorously and the parameters which may have to be specified include the orientation, direction, velocity, length, width and binocular parallax of a moving contour. The origins of this remarkable selectivity lie in the complex wiring of excitatory and inhibitory connections which have been revealed by structural studies with both light and electron microscope.

The most fundamental question one can ask about the complicated circuitry of the cortex is how it gets wired up in the first place so as to perform such fine visual discriminations. Amazingly, even this question is beginning to be answered. There have now been many tantalizing glimpses of the choreography of the mammalian visual system's development: a long dance with many involved steps unfolding according to genetic program and continually modulated by the subtle chords of environmental music.

One example of a step in the genetic program of the visual pathway is provided by the recent discovery that the optic nerve fibers of albinos (including the Siamese cat) go astray at the optic chiasm. In cats and other predators this mutation is literally atavistic since it produces a brain which has connections appropriate for evolutionary forebears with panoramic vision, and quite inappropriate for the needs of an animal with binocular vision.

Of more interest to us are the environmental effects upon the developing circuitry in view of the exciting new field of investigation which has sprung up in the last few years. This field concerns the effects of early visual environment on the immature cortex. A large number of studies in kittens now indicates that the complex properties of cortical neurons are dependent upon the nature of early visual stimulation. Some specialized properties can even be set up in reference to an environment which does not "naturally" occur, such as a planetarium, which contains only "stars" and which lacks the usual extended contours of our familiar environment. After rearing in such an environment, kittens have visual cortices lacking in the normal neurons selective for moving contours and are instead responsive only to patterns of moving points of light.

We are not reluctant to admit that such studies, on the way in which cortical neurons are modified by early visual experience, are relevant to the general problem of the way in which our own brains are changed by experience.

We have spent this year trying to set up optimal lab conditions for studying the structure, function and development of the feline visual pathway. The specific nature of some of our projects is indicated by the following reports and by our publications. If the work has a central theme it might be the following: relatively rigid, genetically preprogrammed, excitatory connections provide information transfer between levels in the hierarchy of organization; sophisticated analysis, fine tuning and environmental modifiability are provided within a given level by the local connections of the recently evolved, diverse microneurons, many of which are inhibitory.

62. A NEW CLASS OF NEURONS WHICH DETECT COMPLEMENTARITY

Investigators: John D. Pettigrew, Gary Blasdel*

Support: Spencer Foundation

A new class of cortical neurons, called complementary cells, are best understood by examining the range of stimuli which fail to make them fire. In contrast to the well-described classes of
cortical neurons which respond over an exquisitely narrow range of stimuli, complementary cells respond over a rather wide range with an exquisitely narrow range of unresponsiveness which is easy to miss (see Figure 1).

The existence of the class first became apparent from the study of the cortex of kittens reared with experience confined to vertical contours. As expected from previous studies, most neurons of these kittens responded to a narrow range of orientations about vertical. However there were some neurons which had preferences for horizontal contours. Further study of this unexpected group revealed very broad preferences which appeared to complement the narrow preferences of the vertically tuned majority of expected neurons.

Complementary cells have now been found in normally reared cats in small numbers (<5%). For example, in a particular “column” of cortex where most cells have sharp preferences for horizontal stimuli, one neuron (the complementary cell) will have a wide range of responsiveness (to a variety of shapes and sizes and orientations) but fails to respond if a vertical target is very precisely positioned on the receptive field.

Complementarity suggests an inhibitory interaction between “opposing” groups of neurons and we are currently testing this suggestion with inhibitory antagonists.

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Figure 1. Complementarity illustrated by two neurons recorded from the visual cortex of a kitten reared with experience only of vertical lines. The density of line segments indicates the vigor of the response evoked from the neuron by lines of the same orientation used during an experimental test of the neuron’s properties. U40 has properties like the majority of neurons recorded from the kitten—it responds only to a narrow range about the orientation used for rearing (i.e., vertical). U10 is a complementary cell which has preferences at right angles to the early environment’s orientation but which responds to a very wide range which nearly exactly complements that of U10. (Graphics by Ken Marton.)
63. ADAPTIVE SIGNIFICANCE OF ENVIRONMENTAL MODIFICATION OF THE CORTEX

Investigators: John D. Pettigrew, David P. Walker*
Support: Spencer Foundation

Much of the literature on the effects of specific rearing procedures on the visual system is couched in the language of “deprivation.” This language reflects a bias in that it suggests that the experimenter already knows not only what optimal brain development looks like but also what sort of environment will produce such optimal brain development. Of course it is quite clear that rearing in the dark is visual deprivation, but what about rearing in an all-vertical world? One cannot be sure until all the evidence is in, but there is a strong possibility that the kitten reared in an environment consisting entirely of vertical contours shows a superior performance on vertical tasks. In other words it may be possible to produce “supernormality” by appropriate rearing.

Many rearing procedures produce neuronal changes which are not subnormal—just different from normal. In some cases, neuronal properties can be produced which are never seen in normal cortex but which would nevertheless have considerable usefulness for the animal if he were to continue to live in the odd environment used for rearing.

The first examples are “dot-detecting” cells which are not seen in normal cortex, but which form the majority of the cell population of kittens reared in a planetarium-like environment of salt and pepper appearance and lacking the usual edges (Pettigrew and Freeman, 1973; Van Sluyters and Blakemore, 1973). The brains of these animals are in no way “sick” since they have the usual high density of neurons and synapses and since the cells fire very vigorously and have normal patterns of activity in the presence of their required stimulus, which happens to be a small moving spot instead of the usual edge.

We are looking for further examples of neurons which illustrate how adaptive the cortex can be when faced with a new environment. It appears possible to produce binocular cells which have quite different properties in the two eyes if the early visual input is made to differ binocularly. This can be achieved with a Dove prism placed in front of one eye so as to rotate that eye’s field of view. Subsequent recording shows single neurons which have different preferred directions in each eye.

References:

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64. PHARMACOLOGICAL ANTAGONISM OF INHIBITION AT THE SINGLE UNIT LEVEL IN THE VISUAL CORTEX

Investigator: Jerry D. Daniels
Support: Spencer Foundation

Using antagonists of suspected inhibitory neurotransmitters, we have sought to determine the role of synaptic inhibition in forming the complex trigger features of cortical neurons. With good evidence at hand that GABA may be an inhibitory transmitter of some stellate cells in the cortex, and with the proven potency of bicuculline, a convulsant alkaloid, as a specific antagonist of GABA, we examined the altered response properties of classes of cortical neurons after intravenous administration of bicuculline (0.2 mg/kg). Complex cells showed the greatest effect, increasing their evoked and spontaneous activity, losing some orientation and direction specificity and developing ON-OFF responses. Hypercomplex cells were seen to lose their inhibitory flanks. Simple cells showed the least effect; in many cases their responses were depressed by the drug. A cell’s response could return to control within an hour after injection of the drug. Using strychnine (0.5 mg/kg), a drug thought to antagonize some non-GABA forms of inhibition, we saw simple cells excited, lending support to the idea that there may be a second strychnine-sensitive inhibitory pathway from complex cells back to
some simple cells. Of great aid in carefully studying how responses changed over time was a computer-controlled stimulus presentation system, which automatically gave precisely the same stimuli both before and after the drug. Intravenous administration of drugs has the advantage that a precisely controlled dose can be delivered to all cells involved in the formation of the response of the cell under study, but it does require us to check non-local effects of the drug to be sure the site of action is in the cortex. With the help of Professor H. Wayland (Division of Engineering and Applied Science, Caltech) we have shown that the doses of bicuculline and strychnine we use to influence cortical cells do not affect the blood pressure of cats; and LGN cells, which are the afferent fibers to the cortex, are little affected by the doses of the drugs which affect the cortical cells themselves.

Reference:
Pettigrew, J. D. and Daniels, J. D. (1973) Science 182: 81.

65. ULTRASTRUCTURAL DISSECTION OF THE PRIMARY VISUAL CORTEX

Investigator: Rona Pettigrew
Support: Edwin H. Schneider Fund
Spencer Foundation

There are a number of well-defined categories of both neuronal cell type and synaptic terminal which can be recognized in ultrastructural studies of the visual cortex. Cell bodies can be identified by shape of soma, the shape of neurites, the position within the cortical system of layering and other similar criteria. Similarly synaptic terminals can be categorized on the basis of vesicle shape, symmetry of synaptic thickening and the type of postsynaptic cell. Since the presynaptic terminal and the postsynaptic cell can be classified readily, and since in many cases such classification can be related to a terminal's excitatory or inhibitory function, it becomes of crucial importance to ascertain the cells of origin of the different synaptic terminals. This is not feasible with serial-sectioning techniques because axons are so fine and usually travel at least several millimeters from cell of origin to destination. Combined Golgi-EM techniques allow one to make a ready identification of a particular synaptic terminal's cell of origin but interpretation is very difficult because of the disturbance of morphology produced in the terminal by the Golgi process.

The approach to this problem used here is based on the observation (Heuser and Reese, 1973), that terminals which are physiologically active at the time of fixation will show a depletion of synaptic vesicles. Since a high degree of spontaneous activity is a characteristic of some terminals within the cortex, the corollary observation should also hold true, viz., terminals which normally show a high degree of spontaneous activity, and which are made inactive during fixation, will show a higher vesicle density than normal.

Cats are perfused for electron microscopy during the peak of some physiological action whose time course has been worked out during single neuron studies on the cortex. The manipulations include (1) elimination of spontaneous activity in the LGN input fibers to cortex by increased intraocular pressure, (2) increased activity of the complex cell population by intravenous bicuculline, and (3) increased activity of the simple cell population by intravenous strychnine.

After these manipulations many specific terminals show changes in vesicle density when compared to normal. For example, after (1), the presumed LGN inputs can be recognized as a specific population of large terminals with pale mitochondria which synapse with spines in layer IV and which have a very high density of vesicles compared to normal. Observations made after (2) and (3) give strong clues as to which terminals belong to simple and complex cells and suggest that some simple cells have inhibitory-type endings.

Reference:
Evidence has been found suggesting that the increasing selectivity of a single neuron's response to a trigger feature following visual experience may be in part due to development of inhibition (Pettigrew, 1974). γ-Aminobutyric acid (GABA) appears to be an important inhibitory transmitter in the visual cortex (Iverson et al., 1971) and antagonism of its effects induces marked changes in the responses of many visual cortical neurons (Pettigrew and Daniels, 1973). Glutamic acid decarboxylase (GAD) is the synthetic enzyme that produces GABA and developmental changes in the levels of this enzyme can be followed using an assay that measures the quantity of $^{14}$CO$_2$ evolved from saturating labeled glutamate (Roberts and Simonsen, 1963).

GAD levels will be followed in a variety of developmental paradigms. It has already been shown that GAD levels in the superior colliculus and visual cortex of the adult rat are as much as 4 times that found in the 6-day-old rat (R. Pettigrew, unpublished results). With kittens, it will be possible to vary the visual experience of the two hemispheres independently by presenting different stimuli in each of the hemisfields while the animal is paralyzed. One hemisphere can be used as a control for the other. The effects of no visual experience, limited experience, and abnormal experience on GAD levels will be studied at ages 3 to 8 weeks. Further, it will be possible to slice a frozen "plug" of cortex and compare GAD levels in the various cortical layers. Because different cell types are found in the different layers, it will be possible to partially distinguish between GAD levels in the various kinds of cortical cells.

References:

Pettigrew, J. D. and Daniels, J. D. (1973) Science 182: 81-83.

*Undergraduate, California Institute of Technology.

PUBLICATIONS


In the past year, our group has continued to work toward analyzing the simple and genetically alterable nervous system of the small soil nematode *Caenorhabditis elegans*. Because the sensory parts of this system are particularly amenable to study, we have isolated and genetically characterized sensory-defective mutants, both chemotaxis-defective and thermotaxis-defective, and we are currently examining these for structural defects by the serial-section electron microscopical techniques which we have previously used to characterize the wild-type anterior sensory anatomy. These same techniques are also being applied now to the tail ganglion which, like the anterior nerve ring, has both sensory input and motor output, but is internally much simpler and therefore more tractable.

Developmental variations can be observed in the behavior of *Caenorhabditis elegans*, particularly in association with each of the four molts. To study these variations more thoroughly, we have designed and built a sizing counter which has proven very useful for following normal growth and development. In addition we have obtained mutants conditionally defective in the molting-associated process of dauerlarva formation, and we hope to use these in studying the mechanisms, presumably hormonal, by which molting and its associated behaviors are controlled.

Finally, because we recognize the importance of biochemical and electrophysiological approaches, we continue to examine neurotransmitter-associated enzymes in *Caenorhabditis elegans* and the electrophysiology of muscle activity in its much larger but homologous relative, *Ascaris lumbricoides*.

67. CHEMOTAXIS-DEFECTIVE MUTANTS OF *CAENORHABDITIS ELEGANS*

*Investigators:* David B. Dusenbery, Richard L. Russell  
*Support:* National Institutes of Health, Public Health Service  
Sloan Fund for Basic Research

The 17 chemotaxis-defective mutants which we isolated by countercurrent separation (see *Biology* 1973, No. 242) have been characterized further. Using a modified version of the tracking assay described by Ward (1973), we have been able to determine reliably whether individual nematodes are normal or chemotaxis-defective (98 to 99% reliability in double-blind tests). This assay has made possible a genetic analysis of the available mutants, which has shown (1) that all 17 are single-gene mutations, as judged by a 3:1 segregation ratio of normals: defectives among the progeny of heterozygous hermaphrodites, (2) that all 17 are autosomal and recessive, and (3) that they define at least 5 complementation groups. In three of the complementation groups, repeated, independent mutations have been obtained in the first set of 17 mutants, suggesting that the number of genes involved may be small. For at least one mutant the penetrance of the mutant character is incomplete; interestingly, the penetrance is temperature-dependent, being very low at 16°C and very high at 25°C. Initial behavioral classification of these mutants by countercurrent separation (see *Biology* 1973, Nos. 241 and 242) suggests that mutants of each complementation group may have distinct behavioral phenotypes. Further classification by this method and by thermotaxis (see *Biology* 1974, No. 68) is planned in the hope of establishing a pathway of information flow for chemotaxis.

*Reference:*
68. THERMOTAXIS OF CAENORHABDITIS ELEGANS

Investigator: Edward M. Hedgecock*
Support: National Institutes of Health, Public Health Service
Sloan Fund for Basic Research

The thermal behavior of *C. elegans* provides a simple paradigm with elements reminiscent of memory and learning in vertebrates. When introduced to a thermal gradient, *C. elegans*, like plant parasitic nematodes (Croll, 1967), migrates to the temperature at which it was raised over a range of growth temperatures from 16 to 25 degrees. Behavioral acclimatization proceeds with a time course of several hours when animals are shifted to a new growth temperature. Dauerlarvae, a special developmental stage induced by starvation (see Biology 1973, No. 246), disperse from the growth temperature, exhibiting a minimum where the adult accumulation profile is at a maximum. The thermal response of normal *C. elegans* adults is a true taxis as evidenced by the ability of nematodes to follow isothermal lines at the ecritic temperature on the gradient and by measurements indicating that *C. elegans* becomes more active as it acclimates to new temperatures.

About twenty thermotactic mutants, including mistactic and atactic phenotypes, have been obtained to date. Dauerlarvae with "adult" thermal behavior and slow-acclimatization mutants are being sought. Two mistactic mutants which have been characterized, T2 and T8, display cryophilic and thermophilic "overshoots," respectively, over a range of growth temperatures.

In addition the chemotaxis-defective strains isolated in this laboratory (see Biology 1974, No. 67) fall into two classes, thermotactic and atactic, suggesting a branched behavioral pathway.

Additional behavioral mutants, selected in other ways, are being examined in the hope of revealing the mechanism of orientation in thermotaxis.

Reference:

*Student Assistant.

69. THE NERVE RING OF CAENORHABDITIS ELEGANS: SENSORY INPUT AND MOTOR OUTPUT

Investigators: Randle W. Ware, David T. Clark, Kathryn D. Crossland*, Richard L. Russell
Support: National Institutes of Health, Public Health Service
Sloan Fund for Basic Research

We have continued our analysis of the anterior sensory anatomy of *C. elegans* (see Biology 1973, Nos. 243 and 244) in greater detail, and have also characterized the anterior muscle cells which receive output from the central nerve ring. To our previous description of the anterior sensory endings (each of which is composed of ciliated neuronal dendrites plus associated "cap" and "pocket" cells) we can add the following facts: (1) that the papillae have fine holes at their tips through which one of the two ciliated dendrites projects to come in direct contact with the external environment; (2) that the two additional sensory endings found on each dorsal and ventral lip are distinct in type, one corresponding quite closely to the type of "tubular body" observed in insect campaniform mechanoreceptors, the other without clear precedent in other systems; (3) that the amphid, like the other sensory endings, is surrounded by a "cap" and a "pocket" cell; (4) that the amphid contains, in addition to its 10 major cilia and its 4 sheet-like neuronal processes, an additional, highly branched ending, with an internal basal body, embedded in the cytoplasm of the amphidial pocket cell; (5) that there are 4 additional ciliated endings (2 in each lateral lip) which end in convoluted structures that are presumably sensory but of unknown type; (6) that there are 10 additional dendritic endings which are not ciliated and end without obvious specializations near the tips of the lips.

By movie reconstruction of electron microscope serial sections, the posteriorly directed processes from these endings have been traced backward to their cell bodies in the area of the nerve ring, in two independent animals. In this way cell bodies previously identified by purely morphological
criteria have been linked to sensory endings of a given type, and morphologically homologous cells have always been found to be associated with identical endings. The reproducibility of cell body position from animal to animal, and from one symmetrical position to another in a given animal, is high but not absolute. The cells whose processes constitute an anteriorly migrating bundle are only loosely grouped and do not correspond to typical, neuropil-containing invertebrate ganglia; instead their synaptic contacts are made within the central nerve ring itself.

A total of 32 muscle cells receives synaptic input in the nerve ring; the anterior 16 receive only this input, whereas the posterior 16 receive input from more posterior sources as well. To receive the nerve ring input, arms from the muscle cells of a given quadrant of the animal push radially together through the nerve ring, and upon reaching the central pharynx, migrate around it to reach their morphologically distinct areas of synaptic input, the muscle plates. There are four such plates, two medial and two lateral, and within each quadrant arms from the most medially placed muscle cells go to the medial plate while those from the lateral cells go to the lateral plate. At the medial plates, apparent gap junctions are often seen between arms from homologous muscle cells on either side of the midline. The structure of the neuropil in the area of the muscle plates is sufficiently reproducible to conclude that a given muscle cell always receives input from the same neurons. Although many of these neurons are not yet identified further, we do know that at least one sensory neuron (whose ciliated ending is one of the two in the papillae) makes direct synaptic contact with a muscle cell, thereby establishing itself as an evolutionarily primitive sensorimotor cell.

*Student Assistant.

**70. STRUCTURAL ANALYSIS OF THE TAIL OF C. ELEGANS**

*Investigator: David H. Hall*

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

The tail of the nematode, *C. elegans*, is a simple structure, capable of participating in several simple behaviors. The male's tail is more specialized than that of the hermaphrodite, and it achieves more complex behavior, notably in mating. In each case, the total number of cells is very small, and the behaviors may be controlled by a single ganglion near the end of the ventral nerve cord.

Examination of Feulgen-stained tails has indicated that the number of cell bodies and their relative positions are extremely uniform from one individual to another. The development of individual males and hermaphrodites has been followed closely from hatching through adulthood by direct microscopic observation. These preliminary studies indicate that the male tail begins to show specialization just prior to the third larval molt.

Serial reconstruction of the tail is now under way. Thin sections (~500-1200 Å) are viewed by transmission electron microscopy, following the methods developed by Randle Ware (see Biology 1972, No. 16). Special emphasis is currently being given to the sensory-motor organization in the tail of the adult nematode (both hermaphrodite and male). The sensory structures and the muscles are highly reproducible from individual to individual, and the sensory endings show strong structural homologies with those already studied in the head (see Biology 1973, No. 243). The overall complexity of the tail ganglion is somewhat less than that of the anterior nerve ring, and should be small enough to permit a complete three-dimensional reconstruction, including all synaptic contacts between cells. The structural changes associated with male tail development are of particular interest, and will be examined in the near future.
71. SCANNING ELECTRON MICROSCOPY OF CAENORHABDITIS ELEGANS

Investigator: Carl D. Johnson
Support: National Institutes of Health, Public Health Service
Sloan Fund for Basic Research

The external morphology of *C. elegans* has been examined by scanning electron microscopy. Nematodes are prepared for SEM by (1) either heat killing (50°C, 1 min) or anesthetizing the animals with 1% propylene phenoxyethol (20 min), (2) fixation with glutaraldehyde and/or OsO₄, (3) dehydration with increasing concentrations of ethanol and (4) critical point drying from freon. They are then mounted, usually in silver paste, on a metal stud and coated with gold.

External features of adult hermaphrodites include cuticular annuli, vulva, anus, excretory pore and external sensory structures (papillae, amphids, phasmids, deirids). Particular attention has been devoted to the anterior tip of the head, with its attendant sensory structures, and to the copulatory apparatus of the male tail. Correlations have been made between external structures and internal structure seen from three-dimensional reconstruction of serial section transmission electron micrographs. The resolution afforded by the scanning electron microscope has permitted a much more extensive characterization of some morphological mutants (see *Biology* 1972, No. 12) than was heretofore possible by light microscopy.

72. AN ELECTRONIC COUNTER FOR CAENORHABDITIS ELEGANS

Investigators: W. Louis Byerly, Randall C. Cassada, Richard L. Russell
Support: National Institutes of Health, Public Health Service
Sloan Fund for Basic Research

A machine has been developed which can count *C. elegans* according to size. The machine uses the same basic principle as the Coulter counter, but differs in a number of essential ways to handle the problems of counting nematodes. The sensitive element of the counter is a cylinder (1 mm in length and 200 µ in diameter) situated between two hollow stainless steel electrodes. An ionic solution (0.35% Na₂HPO₄, 0.15% KH₂PO₄, 0.2% NaCl) flows into the sensitive volume through one electrode and out through the other with a mean velocity of 2 to 3 meters per second. Nematodes carried by the stream are detected and their size measured as they pass between the electrodes due to the increased resistance they offer to the flow of a 56 khz alternating current. The size of the signal produced is roughly proportional to the worm’s volume, but may vary by a factor of 2 according to the worm’s orientation. To minimize this variation, a number of features which tend to align the worms (including a double sheath flow) have been built into the flow system. The signals caused by passing worms are rectified to pulses, operated on mathematically and then fed to a 16-channel pulse height analyzer. The mathematical operation is that of taking the 1/3 power of the signal in order to give a signal roughly proportional to worm length. The 16 channels on the pulse height analyzer can be used to count worms over the whole range of sizes or over any subsection (down to 1/16 of the total).

The worm counter can count 10,000 worms per minute with less than 2% loss of counts due to coincidence. The resolution of the machine depends on the type of sample being counted. The resolution is best for eggs (which are nearly spherical). It is better for stiff worms (heat killed or paralyzed by a drug) than for active worms. An active nematode 250 µ long can be measured with an accuracy of 25 µ (standard deviation from the mean); an active adult nematode 1400 µ long can be measured with an accuracy of 50 µ. These are upper limits on the errors; it has not yet been possible to obtain a synchronous population sufficiently homogeneous in size to better test the machine’s resolution. Repeated measurements of a reference population of glass beads give mean values which vary by less
than 1% (standard deviation) over a several month period. Runs of identically prepared worm samples also vary by less than 1%.

The worm counter is being used in three ways. The first use is the obvious one of rapidly and accurately determining the number and size distribution of certain populations obtained in the lab, either from behavioral fractionation or from different methods of culture. The second type of use is to study the development of synchronous populations with time. By using the average of a population of several hundred worms, very small changes in nematode size can be detected. This approach has been used to study the worm’s growth throughout its life span, with special interest paid to the rate of growth through molts. The worm counter signal (w.c.s.) shows that the wild-type worm’s growth is greatest in the period from 20 to 80 hours after hatching. There is a sharp increase in w.c.s. at the point where egg production starts (50 hours after hatching) and an actual drop in w.c.s. at the end of egg production (around 100 hours after hatching). If w.c.s. is plotted against the optically-measured lengths of synchronous populations of worms, the relation is seen to be quite linear. However, the resolution is sufficient to show structure in the relationship which corresponds to the changes in the worm’s axial ratio at various stages of development. For instance, at each molt, the worms get fatter just before the molt, and longer and thinner just after the molt. This change is such that a population of worms killed by heat in the middle of the 4th molt show a bimodal distribution on the worm counter. Most of this work has been done with nematodes grown on agar plates. Worms grown in liquid culture show different growth curves, increasing in w.c.s. faster and having a much higher length to volume ratio. It will be of considerable interest to repeat these same studies with developmental mutants. The third type of use for the counter is to measure whole populations containing worms of many sizes. From such populations the rate of growth and rate of egg-laying can be inferred. Such studies done on the young worms that appear on a plate with a known number of egg-laying worms have produced detailed information on the egg-laying rate of worms as a function of age. For the wild-type worm the egg-laying rate is found to be sharply peaked, reaching a maximum of about 9 eggs/worm/hour at about 80 hours after hatching. Thus, growth and egg-laying for any mutant strain of C. elegans can be measured as functions of temperature or drug treatment by running only a small number of samples through the counter.

73. FURTHER STUDIES ON THE DAUERLARVA OF CAENORHABDITIS ELEGANS

Investigators: Randall C. Cassada, Richard L. Russell
Support: National Institutes of Health, Public Health Service
Sloan Fund for Basic Research

When conditions are unsuitable for maturation, juveniles of C. elegans can enter a morphologically recognizable, inactive, nongrowing state specialized for survival, called the dauer larva (see Biology 1973, No. 246). Less than 1 in 10^4 animals enter the dauer larva state under good growth conditions, so it is not an obligatory part of the life cycle. We have obtained various kinds of evidence that dauer larvae are arrested at a molt, always the second of the four molts.

Normal animals feed essentially continuously except for about an hour just before each molt, during which time feeding (pharyngeal pumping) and general locomotion are suppressed. Just before each molt the old cuticle separates and remains briefly as an outer sheath. Dauer larvae maintain similar inactivity indefinitely and have a loose outer sheath. This sheath is lost after a day or two and is not required to maintain the dauer larva state. The size of dauer larvae is variable (400 to 600 µ length) but when food is restored and they resume feeding they always show two feeding and molting cycles, as would be expected if they were initially arrested at the second molt. The first post-dauer molt in which the special dauer cuticle is shed involves a transverse split in the cuticle near the head and a longitudinal split apparently always along the left lateral line.

Mutants have been selected which conditionally (temperature-sensitively) enter the dauer larva state spontaneously, even when food is abundant (see Biology 1973, No. 246). One such mutant, an autosomal recessive, has a temperature-sensitive
period just after the first molt. If animals are at the restrictive temperature (25°C) during this part of their development, they all arrest as dauerlarvae; less than 1 in $10^4$ reaches adulthood at 25°C. At the permissive temperature (16°C) less than 1% arrest as dauerlarvae. The defect in dauerlarva formation in this mutant does not directly affect recovery from the dauer state since dauerlarvae formed by starvation at 16°C recover normally at 25°C. Other unlinked autosomal recessive mutants have earlier temperature-sensitive periods, during embryogenesis, and still other mutants appear to be salt-conditional, forming dauerlarvae in the presence of food at high salt concentrations but not at low. Some of these mutants have secondary phenotypes which may be related to the mechanism of dauerlarva induction, including slow movement, characteristic clear regions in the head, defecation behavior defects, or other locomotion defects. Other mutants have been selected for defective recovery from the dauerlarva state at 25°C.

The arrest at a molt strongly suggests that regulation of dauerlarva formation and recovery is related to the molting process. It is hoped that the dauer mutants will be useful in elucidating the mechanisms, presumably hormonal, which regulate molting in this simple animal.

74. A SEARCH FOR CHOLINESTERASE GENETIC VARIANTS IN CAENORHABDITIS ELEGANS

Investigator: Carl D. Johnson
Support: National Institutes of Health, Public Health Service
Sloan Fund for Basic Research

Three separable forms of cholinesterase activity have been found in sonicates of C. elegans. Two approaches to the genetic exploration of these forms have been devised. The first involves the isolation of mutants resistant to cholinesterase inhibition. We have chosen the drug 2-methyl-2-(methylthio)-propionaldehyde-0-(methyl carbamoyl)-oxime (Temik, Aldicarb) for initial isolations as (1) it has a pronounced effect on the animals leading to hypercontracted paralysis and eventual death, and (2) resistant mutants are rare. Mutants have been selected by placing the F2 or F3 of mutagenized adults onto plates containing 0.1 mg/ml of Temik. Resistant mutants are retrieved, cloned on drug-free plates, and retested on plates containing the drug. Resistant mutants are characterized by survival and growth. Some of them have improved motion. The mutants will be examined for alterations in the inhibition of the separable forms of cholinesterase in vitro.

A second approach has been to look for variations in biochemical characteristics of the cholinesterase forms among isolates from natural populations. To isolate strains of C. elegans from soil samples, an initial screening of soil nematodes for those morphologically and behaviorally similar to the known strains of C. elegans is followed by cloning individuals on plates. Those clones which grow rapidly are tested for their ability to cross with males of the N2 strain of C. elegans. In this way more than 35 strains of C. elegans have been isolated from various locations in and around Southern California. These isolates will now be examined for polymorphism, with particular attention being paid to searching for variations in biochemical characteristics of the separable forms of cholinesterase.

75. ELECTROPHYSIOLOGY OF ASCARIS LUMBRICOIDES

Investigator: David A. Weisblat*
Support: National Institutes of Health, Public Health Service
Sloan Fund for Basic Research

The cells in C. elegans are too small to be easily impaled with microelectrodes. Therefore, electrophysiological studies are being carried out on C. elegans' large, parasitic relative, the nematode...
Ascaris lumbricoides, in the hope that results thus obtained will be of use in understanding C. elegans behavior. Use of Ascaris also permits studies on the cellular effects of pharmacological agents that are active on C. elegans (see Biology 1973, No. 250).

Both Ascaris and C. elegans lie on their sides and wiggle dorsoventrally when placed on a flat surface. This motion is regulated by separate dorsal and ventral muscles, which receive innervation from dorsal and ventral nerve cords. A. O. W. Stretton (personal communication) has shown that in Ascaris motor neuron cell bodies are found in the ventral nerve cord, and that the dorsal cord is formed by branches of commissures exiting to right and left from the ventral cord in repeating patterns along the length of the animal. The muscles in each half of the animal send out processes to pick up innervation from the appropriate nerve cord. These processes contain swellings from which the electrical activity of the muscle can be recorded.

The pioneering observations of Jarman (1959), and del Castillo and co-workers (1967) have been repeated, and experiments are under way to examine the relationship between the myogenic activity of electrically coupled muscle cells, and the nervous control of muscle contraction in Ascaris.

Stretton's anatomical work suggests that there may be distinct muscle fields along the length of the dorsal and ventral sides, corresponding to the repeating patterns of commissures. This is being examined by measuring the synchrony of the myogenic activity of widely separated muscle cells in the absence of neural input (transmission blocked by solutions of high Mg\(^{2+}\), low Ca\(^{2+}\) concentration). Preliminary results indicate that myogenic waves travel at least several centimeters along the length of the worm in either direction, and that there are no functionally separable groups of muscles within the dorsal and ventral fields.

In preparations where muscles are still under the control of the nerve ring, there appears to be coordinated activity between the dorsal and ventral halves of the worm. This coordination is affected by various treatments:

1. Isolating the dorsal muscles from their nerve supply leads to a state in which the ventral side's activity is unchanged, while the dorsal side now undergoes rhythmic, presumably myogenic, oscillations of constant frequency and amplitude.
2. Treatment with 1 mM La(NO\(_3\))\(_3\) silences all electrical activity and stops visible muscular contractions. Washing out the La(NO\(_3\))\(_3\) solution leads to partial restoration of activity.
3. Replacement of the bathing fluid with one containing elevated Mg\(^{2+}\) and reduced Ca\(^{2+}\) causes both dorsal and ventral halves to enter a state in which they undergo slow, presumably myogenic, oscillations of variable amplitude and frequency. In this state, dorsal and ventral sides no longer show coordinated activity, but nearby cells within each side still fire synchronously. Replacement with normal bathing fluid restores original activity.
4. Preliminary observations indicate that there may be distinct consequences depending upon whether the incision to expose the muscle bellies is made along the left or the right lateral line. We hope that such observations will allow us to draw conclusions about the function of the left as opposed to right exiting commissures which join the ventral and dorsal cords.

References:


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PUBLICATIONS


NORMAN W. CHURCH LABORATORY
FOR CHEMICAL BIOLOGY

Giuseppe Attardi
Seymour Benzer
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Jerome Vinograd
In the past year, we have made significant progress in the analysis of the expression of the mitochondrial genome and of mitochondriogenesis in animal cells.

Our continuing investigation of the nature of the primary gene products of mitochondrial DNA in HeLa cells has led to the identification of 8 discrete poly(A)-containing RNA components, which presumably represent messenger RNA species. Together with the previously identified ribosomal and 4S RNA species, these poly(A)-containing RNA components, if distinct in sequence, would account for about 70% of the single-strand informational content of HeLa mitochondrial DNA.

A considerable effort has also been made towards the characterization of the products of mitochondrial protein synthesis, which are probably all or in part coded for by the mitochondrial genome. Ten discrete protein components have so far been identified, the size range of which correlates fairly well with that of the poly(A)-containing RNA components coded for by mitochondrial DNA, suggesting that these may be the templates for at least some of the mitochondrially synthesized proteins.

The problem of whether the growth and division of mitochondria during the cell cycle is correlated with the time pattern of mitochondrial DNA and RNA synthesis has been investigated by a morphometric analysis of electron micrographs of synchronized HeLa cells. The results indicate that, in contrast to mitochondrial DNA replication and transcription, which are restricted to, or greatly accelerated, in the S phase and especially the G2 phase, growth and division of mitochondria occur in each cell throughout the cell cycle.

Along a different line of investigation, the possibility that the symmetrical transcription demonstrated for mitochondrial DNA, SV40 DNA, and polyoma virus DNA are particular cases of a more widespread mechanism of transcription in eukaryotic cells has been tested by examining heterogeneous nuclear RNA (HnRNA) synthesis in HeLa cells and in differentiating duck erythrocytes. No evidence for the synthesis of high molecular weight complementary HnRNA molecules was detected in either cell type.

76. IDENTIFICATION AND PARTIAL CHARACTERIZATION OF MULTIPLE DISCRETE POLYADENYLIC ACID-CONTAINING RNA COMPONENTS CODED FOR BY HeLa CELL MITOCHONDRIAL DNA

Investigators: Deanna K. Ojala, Giuseppe Attardi
Support: National Institutes of Health, Public Health Service

Poly(A)-containing RNA isolated from the components of a HeLa cell mitochondrial lysate which sediment in the polysome region of a sucrose gradient has been analyzed for the presence of discrete species. Eight distinct components have been identified by polyacrylamide gel electrophoresis after formaldehyde treatment. These components, which are highly reproducible in their occurrence and relative amounts under widely varying conditions of isolation, have been characterized as to their sedimentation behavior under denaturing conditions, poly(A) content and homology to separated strands of mitochondrial DNA (mit-DNA).

One of the discrete components was previously shown to have a sedimentation constant of about 7S in the native state and a molecular weight of about 9.0 x 10^4 daltons, as estimated from its
sedimentation rate in formaldehyde. The molecular weights of the other seven components, as derived from sedimentation data, range between 2.6 and 5.3 x 10^5 daltons.

The 7S RNA is homologous to the L mit-DNA strand, while the other seven components are homologous to the H strand. Together with the two mitochondrial rRNA species and with mitochondrial 4S RNA, the eight poly(A)-containing RNA components, if distinct in sequence, would account for about 70% of the single-strand informational content of HeLa mit-DNA.

77. ATTEMPTS TO CONSTRUCT A PHYSICAL MAP OF HeLa MITOCHONDRIAL DNA

Investigators: Deanna K. Ojala, Sue Murakami*, Giuseppe Attardi
Support: National Institutes of Health, Public Health Service

Analysis of the transcription products of HeLa mitochondrial DNA has revealed the occurrence in the mitochondria of several discrete poly(A)-containing RNA components (Ojala and Attardi, 1974). In order to map the location of the genes from which these components are transcribed and the position of other important functional sites on the genome, we have begun the isolation and ordering of fragments produced by several bacterial restriction enzymes.

The enzymes being used in this study, either singly or in combination, are Eco RI, from E. coli, Hae from Hemophilus aegyptius, Hind III from Hemophilus influenzae, and Hpa I and Hpa II from Hemophilus parainfluenzae. Electrophoresis through polyacrylamide or agarose gels is used to resolve the fragments.

Reference:

*Student Assistant.

78. MITOCHONDRIAL tRNAs AND tRNA GENES OF HeLa CELLS

Investigators: Dennis C. Lynch, Giuseppe Attardi
Support: California Division of the American Cancer Society National Institutes of Health, Public Health Service

A detailed study of mitochondrial (mit) tRNAs from HeLa cells has been undertaken. Mit-4S RNA has been tested for amino acid incorporation with a crude preparation of aminoacyl-tRNA synthetases obtained from the mitochondrial fraction. A similar cytoplasmic system has been developed, and cross-charging between cytoplasmic and mitochondrial species examined. In this fashion it has been possible to demonstrate separate cytoplasmic and mitochondrial activities for at least 10 different aminoacyl-tRNA synthetases.

The aminoacyl-tRNA products have been separated from the enzymes and examined for the ability to hybridize to separated strands of mit-DNA at 37°C in the presence of formamide. This technique should reveal the aminoacyl specificity of the tRNA genes on mit-DNA. A number of mit-tRNA species has been observed to hybridize so far, including all those where a separate mitochondrial enzyme activity has been detected. To date, no hybridization with cytoplasmic tRNA, whether charged with mitochondrial or cytoplasmic enzyme, has been observed.

Previous work in this area has revealed an apparently limited number of 4S genes on mit-DNA (Aloni and Attardi, 1971; Wu et al., 1972) and an atypical utilization of amino acids by the mitochondrial protein-synthesizing system (Costantino and Attardi, 1973). Results obtained to date in this project seem to indicate no connection
between the extent of utilization and the apparent presence of a tRNA gene for a given amino acid. Further, the results of the current project are verging on a conflict with previous data regarding the number of 4S sites on mit-DNA. Efforts are continuing to obtain a definitive solution of the problem.

**79. INITIATION SITES FOR MITOCHONDRIAL DNA TRANSCRIPTION IN HeLa CELLS**

*Investigators:* Paul G. Young, Giuseppe Attardi  
*Support:* North Atlantic Treaty Organization  
National Institutes of Health, Public Health Service

Work is progressing towards the identification of the transcription initiation sites on HeLa cell mitochondrial DNA. Initial attempts utilizing the mitochondrial RNA polymerase in vitro were unsuccessful (see Biology 1973, No. 79). A second approach aimed at specifically labeling the 5'-termini with $\gamma^{32}$P-ATP in intact mitochondria incubated in vitro failed primarily due to the rapid hydrolysis of the label. Methods in use at present include long-term labeling of cells with $^{32}$P-orthophosphate and pulse-labeling of intact mitochondria in vitro with the same precursor under conditions supporting oxidative phosphorylation. The 5'-termini have been tentatively identified and shown to specifically hybridize to mitochondrial DNA. Efforts are now directed at providing an unambiguous identification of these nucleotides and determining the number of initiation sites on each DNA strand.

**80. SEARCH FOR AN RNase III IN HeLa CELL MITOCHONDRIA**

*Investigators:* Paul G. Young, Giuseppe Attardi  
*Support:* North Atlantic Treaty Organization  
National Institutes of Health, Public Health Service

HeLa cell mitochondrial DNA has been shown to be symmetrically transcribed. This accounts for the presence in these cells of complementary RNA transcripts which tend to base pair and form, *in vivo* and/or during extraction, duplex structures (Aloni and Attardi, 1971). These findings suggest the existence in mitochondria of an RNase activity specific for double-stranded RNA (dsRNA). Such an enzyme has been demonstrated in *E. coli* and is designated RNase III (Robertson et al., 1968).

A dsRNA substrate was produced by extensive RNase A digestion of the self-annealed heavier components (> 40S) of mitochondrial RNA derived from cells uniformly labeled with $^3$H-adenine. The dsRNA had a specific activity of about 60,000 cpm/µg. Following fractionation on Sephadex G-100, the dsRNA eluted in the excluded volume was recovered and analyzed on sucrose gradients. This dsRNA had a mean S-value of about 8S and was completely resistant to RNase A at salt concentrations of > 1X SSC.

Using this substrate, a search for RNase III activity was initiated, based on loss of TCA precipitability or on changes in the sedimentation properties of the substrate in sucrose gradients. No RNase activity against this substrate has yet been demonstrated in HeLa cell fractions under a variety of incubation conditions.

**References:**

81. RELATIONSHIP BETWEEN MITOCHONDRIAL DNA TRANSCRIPTION AND REPLICATION IN HeLa CELLS

Investigators: Daniele S. Carré, Giuseppe Attardi

Support: World Health Organization
National Institutes of Health, Public Health Service
Ministère de l'Éducation Nationale (France)

A great acceleration of mitochondrial DNA (mit-DNA) transcription during the S and G2 phases of the cell cycle, that is, at the time when replication of mitochondrial DNA occurs, has been previously reported (Pica-Mattoccia and Attardi, 1971).

In order to determine if the synthesis of RNA precedes, accompanies or follows the replication of individual DNA molecules, the replicating DNA has been labeled with short pulses of $^3$H-thymidine followed by a chase with unlabeled thymidine. The transcription complexes have been isolated by a procedure derived from that of Aloni and Attardi (1972), on the basis of their sedimentation velocity and/or equilibrium density in CsCl in the presence of ethidium bromide.

The distribution of mit-DNA among different forms in a nonsynchronized population of $^{14}$C-thymidine uniformly-labeled cells has been investigated. The flow of $^3$H-thymidine radioactivity among these mit-DNA forms in the pulse-chase experiments is at present being studied.

References:

82. MITOCHONDRIAL DNA TRANSCRIPTION AND NUCLEAR CONTROL

Investigators: Charles E. Novitski, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service

Mitochondrial RNA (mit-RNA) synthesis is greatly accelerated in the S phase and reaches a peak in the G2 phase of the cell cycle in HeLa cells (Pica-Mattoccia and Attardi, 1971) and mouse L cells (Bosmann, 1971). Furthermore, the reported in vivo inhibition of mit-RNA synthesis by cycloheximide (Storrie and Attardi, 1972) suggests that mit-RNA synthesis requires concomitant protein synthesis on cytoplasmic ribosomes. Two approaches have been taken to understand the mechanism of the temporal control of this mitochondrial function, using in vitro labeling of isolated HeLa mitochondria.

One proposed approach is the study of the compensatory effect of added normal cytoplasm on mitochondria affected in their RNA-synthesizing capacity by in vivo cycloheximide treatment. An effective method of washing mitochondria free of high specific activity $^3$H-UTP used in the incubation has been developed. We find that in vivo cycloheximide treatment for up to 8 hours does not dramatically affect the rate of incorporation of $^3$H-UTP, the uridine nucleotide pool size, or the UTP specific activity in the isolated mitochondria.

A second approach is to examine the changes in the RNA-synthesizing activity of the isolated mitochondria and in the controlling activity of the cytoplasm during the cell cycle, using synchronized cells. In testing methods for obtaining large numbers of mitotic cells, selective detachment was chosen over the Ludox density gradient fractionation technique because there was inadequate enrichment of mitotic cells using the latter method. We find that Ludox density is most easily monitored on linear organic solvent density gradient columns rather than on the exponential gradient column proposed by Miller and Gasek (1960).

References:
83. ANALYSIS OF RNA SYNTHESIS BY AN ISOLATED RAT BRAIN SYNAPTOSOMAL FRACTION

Investigators: James M. England, Giuseppe Attardi

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

Previous results had shown that most, if not all, of the RNA synthesized by an isolated rat brain synaptosomal fraction is mitochondrial RNA (Biology 1972, No. 149).

Electron microscope autoradiography has been used to localize the sites of $^3$H-uridine incorporation. This autoradiographic analysis has shown that about half of the mitochondrial RNA synthetic activity within the synaptosomal fraction is localized in presynaptic neural endings. These results indicate that mitochondrial RNA synthesis occurs in neurons, i.e. nondividing cells which are blocked in the G1 phase of the cell cycle.

84. IDENTIFICATION AND PARTIAL CHARACTERIZATION OF DISCRETE PRODUCTS OF MITOCHONDRIAL PROTEIN SYNTHESIS IN HeLa CELLS

Investigators: Paolo E. Costantino, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service
Damon Runyon Memorial Fund for Cancer Research

The proteins synthesized in vivo by HeLa cell mitochondria in the presence of emetine, an inhibitor of cytoplasmic protein synthesis, have been characterized as to their electrophoretic mobility, solubility properties in organic solvents and kinetics of labeling with $^3$H-isoleucine.

Ten distinct protein components in the molecular weight range from about 11,000 to 42,000 have been identified. Control experiments involving direct sodium dodecyl sulfate lysis of whole cells, or the use of the protein inhibitor phenylmethylsulfonylfluoride during cell homogenization and fractionation, have excluded enzymic degradation during extraction as playing a significant role in producing the observed electrophoretic pattern of mitochondrial protein products.

A selective solubilization of mitochondrial protein products, representing a 10- to 20-fold purification with respect to the cytoplasmically synthesized proteins, has been obtained by a neutral chloroform-methanol mixture. At least six of the discrete products larger than 20,000 daltons were found to be extracted to an appreciable extent by neutral chloroform-methanol.

A fairly coordinate labeling of the discrete mitochondrial protein products has been observed during 1-hour exposure of the cells to $^3$H-isoleucine in the presence of emetine, without any clear evidence of interconversion of components.

85. METABOLISM OF THE PRODUCTS OF MITOCHONDRIAL PROTEIN SYNTHESIS IN HeLa CELLS

Investigators: Paolo E. Costantino, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service
Damon Runyon Memorial Fund for Cancer Research

The metabolic stability of the protein components synthesized by HeLa cell mitochondria and the possible precursor to product relationship between them are under investigation. For these purposes, HeLa cells are pulse-labeled for different lengths of time with $^3$H-isoleucine in the presence of emetine or cycloheximide (inhibitors of cytoplasmic protein synthesis), and then chased with an excess of cold isoleucine, in either the presence or absence of the above inhibitors.

Additional insight into the metabolic fate of the mitochondrially synthesized proteins and into the
relationship between mitochondrial and cytoplasmic protein synthesis in HeLa cells can hopefully be obtained by means of long-term exposure of the cells to emetine (cycloheximide) and/or chloramphenicol (a mitochondrial protein synthesis inhibitor), coupled with pulse- or long-term-labeling of the mitochondrial products.

86. MITOCHONDRIAL PROTEIN AND RNA SYNTHESIS IN A MONKEY KIDNEY CELL LINE (TC-7) ENUCLEATED WITH CYTOCHALASIN B

Investigators: James M. England, Paolo E. Costantino, Giuseppe Attardi

Support: Dernham Fellowship of the American Cancer Society
National Institutes of Health, Public Health Service
Damon Runyon Memorial Fund for Cancer Research

The rates of cytoplasmic soluble protein synthesis, mitochondrial protein synthesis and mitochondrial RNA synthesis were examined in a monkey kidney cell line (TC-7) which had been enucleated with cytochalasin B. It was found that, following enucleation, there is a relatively rapid decline in the rate of cytoplasmic soluble protein synthesis with a half-life of about 5 hours; 14 hours following enucleation, the rate of synthesis is reduced to about 15% of the original level. The remaining 15% of the synthetic activity declines with a half-life of about 24 hours.

The rate of emetine-resistant and chloramphenicol-sensitive \(^{3}\)H-isoleucine incorporation (due to mitochondrial protein synthesis) consistently shows a slight (30 to 50%) transient stimulation about 5 hours after enucleation, returning within 2 or 3 hours to the original level and declining thereafter very slowly. This stimulation cannot be duplicated in nucleated cells which are not centrifuged, but allowed to recover from cytochalasin B treatment. The rate of \(^{3}\)H-isoleucine incorporation 24 hours after enucleation is still about 80% of the original level.

No significant change in the uptake of \(^{3}\)H-isoleucine into the mitochondrial fraction was noted during the incubation of the enucleated cells.

SDS polyacrylamide gel electrophoresis analysis of the products of mitochondrial protein synthesis labeled during a 1-hour \(^{3}\)H-isoleucine pulse showed that the electrophoretic profile of these products in enucleated cells (TC-7) resembles closely that of the proteins synthesized by mitochondria of intact HeLa cells. Two hours following enucleation, there is, however, a decrease by about 30 to 50% in the labeling of a protein component of \(~\)20,000 molecular weight, relative to that of the other components; this pattern is maintained, essentially unmodified, up to at least 24 hours following enucleation.

In contrast to mitochondrial protein synthesis, the rate of mitochondrial RNA synthesis by enucleated cells, as followed by \(^{3}\)H-uridine incorporation, declines fairly rapidly, so that by 24 hours following enucleation there is no detectable incorporation of the precursor into mitochondrial RNA species.

87. MITOCHONDRIAL GROWTH AND DIVISION DURING THE CELL CYCLE IN HeLa CELLS

Investigators: James W. Posakony*, James M. England, Giuseppe Attardi

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

The growth and division of mitochondria during the cell cycle in HeLa cells was investigated (see Biology 1973, No. 85) by a morphometric analysis of electron micrographs of synchronized cells. Synchronous populations of HeLa F-315 cells (85 to 90% pure) were obtained by the method of selective detachment. Samples for morphometric analysis were withdrawn, at various times, from the synchronous populations, pulse-labeled with radioactive thymidine, and prepared for electron microscopy. A technique involving correlation of electron microscopy of thin sections with autoradiography of thick sections allowed the recognition within the “G1” and “G2” samples of
contaminating S phase cells (synthesizing nuclear DNA), and within the "S" sample of contaminating non-DNA-synthesizing cells. Electron micrographs were taken of approximately 30 cells from each time point; measurements were made on the cellular, nuclear, and mitochondrial profiles using a Hewlett-Packard Digitizer-Calculator apparatus. The mean area and outer membrane contour length of the mitochondrial profiles were found to remain constant during the cell cycle; furthermore, a mathematical elaboration (in collaboration with Dr. Joel N. Franklin, Division of Engineering and Applied Science) of the data for the area of the mitochondrial and cytoplasmic profiles yielded the result that the mitochondrial number and total volume per unit of cytoplasmic volume also remain constant. Thus, it appears that in HeLa cells there is no fixed temporal relationship between the growth and division of mitochondria and the events of the cell cycle. In addition, the distributions of axis length ratios of the mitochondrial profiles were compared with the theoretical distributions expected from random sectioning of various 3-dimensional shapes. The data were found to be consistent with a generalized ellipsoidal shape for the intact mitochondrial organelles.

88. THE FATE OF MITOCHONDRIAL DNA IN MOUSE CELL HETEROKARYONS

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

A derivative of mouse L fibroblasts, LM(TK-)Cl 1D, which is unable to incorporate thymidine or 5-bromodeoxyuridine (BudR) into nuclear DNA, has retained its ability to incorporate these precursors into mitochondrial DNA (mit-DNA) at a substantial rate (Attardi and Attardi, 1972; Clayton and Teplitz, 1972). Cells grown in the absence of BudR contain mit-DNA with a buoyant density of about 1.69 g/cm³; in the presence of 30 µg/ml BudR, cells contain mit-DNA ranging in buoyant density from 1.73 to 1.78 g/cm³ (34 to 79% substitution of BudR for thymidine).

In order to look for possible recombination between mit-DNAs, cells grown in the absence of BudR and labeled for 24 hours with high levels of ³H-thymidine were fused, using inactivated Sendai virus, with cells grown in the presence of BudR. Large heterokaryons were allowed to recover from fusion for varying periods of time, after which mit-DNA was isolated. Purified mit-DNA was centrifuged both on neutral and alkaline CsCl density gradients and examined for the presence of heavier than normal, ³H-thymidine-labeled mit-DNA recombinant forms. As yet, no recombinant forms have been identified; however, further experiments are in progress.

References:

89. THE USE OF CHLORAMPHENICOL-RESISTANT HUMAN CELLS IN HUMAN-MOUSE CELL HYBRIDIZATION

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

A chloramphenicol-resistant variant, CAP-23, isolated from the human cell line WI-18-VA2 (transformed by SV40), grows with a normal generation time of about 30 hours in 200 µg/ml chloramphenicol (CAP). Protein synthesis in mitochondria isolated from this variant is also resistant to high levels of the drug. Electron microscopy reveals no detectable differences in mitochondrial morphology of parental and resistant cells grown in the presence of the drug. A derivative of CAP-23 was further selected for resistance to 6-thioguanine (10 µg/ml). These cells are unable to survive in HAT (hypoxanthine-aminopterin-thymidine) medium (used as a selection agent in cell
fusion studies), indicating that the resistance to 6-thioguanine results from a deficiency of the enzyme hypoxanthine-guanine phosphoribosyl transferase.

The fusion of human with mouse cells usually results in a progressive loss of human chromosomes and a total loss of detectable human mitochondrial DNA (Attardi and Attardi, 1972). The chloramphenicol-thioguanine-resistant human cells described above have been fused, using inactivated Sendai virus, with 3T3, a mouse cell line deficient in thymidine kinase. We are now in the process of attempting to isolate and characterize mouse-human hybrids, selected in chloramphenicol-HAT medium: these must retain their CAP resistance and, therefore, at least some of their human chromosomes and/or mitochondrial DNA coding for the mitochondrial components responsible for maintaining the resistant phenotype.

Reference:

90. PHOTOSENSITIVITY CONFERRED BY BudR UPON A THYMOIDINE KINASE-DEFICIENT MOUSE CELL LINE WITH PERSISTENT MITOCHONDRIAL ENZYME ACTIVITY

Investigators: Giuseppe Attardi, Benneta Keeley, Catherina Tu
Support: National Institutes of Health, Public Health Service

The LM(TK)Cl 1D cell line, a thymidine kinase-deficient derivative of mouse L cells with persistent mitochondrial enzyme activity (Attardi and Attardi, 1972; Clayton and Teplitz, 1972), can incorporate thymidine and 5-bromodeoxyuridine (BudR) into mitochondrial DNA (mit-DNA) at a substantial rate, in the absence of any significant incorporation of these precursors into nuclear DNA. Furthermore, these cells can be grown indefinitely in the dark in the presence of 30 µg/ml BudR. For these properties, LM(TK)Cl 1D cells represent a valuable tool to investigate the possible photosensitizing properties of BudR which depend on its incorporation into the organelle DNA, in the absence of nuclear effects.

LM(TK)Cl 1D cells, which had been grown for at least 10 days in the presence of 30 µg/ml BudR, were irradiated for different lengths of time, in the presence of the halogenated precursor, with visible light, under the conditions described by Puck and Kao (1967). A progressive loss of cloning efficiency, with a half-life of about 9 hours, was observed. No such loss of viability occurred in cells grown in the absence of BudR. Furthermore, control experiments showed that the photosensitivity of Cl 1D cells was due to their previous exposure to the halogenated precursor, and not merely to the presence of BudR in the medium during irradiation.

From the possible residual extramitochondrial thymidine kinase activity in Cl 1D cells, compared to that of another L cell derivative, A9 (as estimated from the relative incorporation of 3H-thymidine into total cell DNA in the two cell lines), and from the photosensitivity of A9 cells grown for 2 or 3 generations in the presence of 30 µg/ml BudR, it can be calculated that the photosensitivity of Cl 1D cells is an order of magnitude higher than expected from any residual incorporation of BudR into mit-DNA, if this were random. These results point to BudR-substituted mit-DNA as a likely killing target of visible light irradiation.

References:
91. SELECTIVE IN VIVO DAMAGE BY VISIBLE LIGHT OF BudR-CONTAINING MITOCHONDRIAL DNA IN A CYTOPLASMIC THYMIDINE KINASE-DEFICIENT DERIVATIVE OF MOUSE L CELLS

Investigators: Bernard Croizat, Giuseppe Attardi
Support: Gosney Fund
Centre National de la Recherche Scientifique
National Institutes of Health, Public Health Service

The persistence of mitochondrial thymidine kinase activity in the LM(TK)Cl 1D cell line, a derivative of mouse L fibroblasts which has no detectable extramitochondrial thymidine kinase activity, offers the possibility of a selective incorporation of thymidine or 5-bromodeoxyuridine (BudR) into mitochondrial DNA (mit-DNA). This selective incorporation of BudR was used to selectively damage mit-DNA by visible light irradiation.

LM(TK)Cl 1D cells can be grown indefinitely, in the dark, in the presence of 30 µg/ml BudR, without any effect on the growth rate. When these cells were exposed to visible light, they exhibited a progressive loss of cloning efficiency with a half-life of about 9 hours (see Biology 1974, No. 90). An analysis of the preexisting mit-DNA in these cells, uniformly labeled with 3H-deoxycytidine and subsequently exposed to visible light, showed a progressive decrease, with an increasing length of irradiation, in the amount of closed and open circular mit-DNA; this became about 40% of the control after 65 hours of irradiation. Likewise, an analysis of the incorporation of 3H-deoxycytidine into mit-DNA during a 3-hour pulse following irradiation showed a progressive decline of the mit-DNA labeling, which became about 30% of the control after 48 hours of irradiation. No effect of irradiation on the size of the extracted nuclear DNA or on its labeling with 3H-deoxycytidine was observed.

These data are compatible with the idea that the loss of cell viability depends on the progressive destruction of mit-DNA.

92. MITOCHONDRIAL THYMIDINE KINASE ACTIVITY IN A THYMIDINE KINASE-DEFICIENT DERIVATIVE OF MOUSE L CELLS

Investigators: Robert A. Gelfand, Sue Murakami*
Support: National Institutes of Health, Public Health Service

In a derivative of mouse L cells, LM(TK)Cl 1D, deficient in the enzyme thymidine kinase (Kit et al., 1963), it has been demonstrated that 5-bromodeoxyuridine and 3H-thymidine are incorporated into mitochondrial DNA (Attardi and Attardi, 1972; Clayton and Teplitz, 1972), suggesting the existence of mitochondrial thymidine kinase activity. We have been developing techniques to assay thymidine kinase directly in order to investigate the properties and site of synthesis of the mitochondrial enzyme.

The assay is a modification of the method of Berk et al. (1972) in which tritiated thymidine and thymidine kinase are incubated in the presence of ATP. Reaction products are separated by thin-layer chromatography.

We have demonstrated, using this assay, that there is thymidine kinase activity in isolated mitochondrial fractions of LM(TK)Cl 1D cells. In an attempt to discover whether this thymidine kinase activity depends on mitochondrial protein synthesis, we have grown cultures of LM(TK)Cl 1D cells in the presence of chloramphenicol and assayed the isolated mitochondrial fractions from these cultures for thymidine kinase activity. Two days incubation with chloramphenicol has no effect on thymidine kinase activity.

References:

*Student Assistant.
93. USE OF A DNA PROBE FOR MAPPING BY ELECTRON MICROSCOPY THE RIBOSOMAL SEQUENCES IN RIBOSOMAL RNA PRECURSORS FROM AVIAN CELLS

Investigators: William I. Murphy, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service

In a previous report we have described the isolation of nuclear DNA sequences complementary to ribosomal RNA (rRNA) from duck erythrocytes (Biology 1973, No. 91). We have used this purified ribosomal DNA (rDNA) as a probe for mapping by electron microscopy the ribosomal RNA sequences in the ribosomal RNA precursor from duck embryo fibroblasts. When the purified rDNA was annealed with a mixture of 18S and 28S rRNA and the hybrids spread for electron microscopy, they appeared as two distinct populations with a number-average length of $0.62 \pm 0.13 \mu m$ and $1.37 \pm 0.18 \mu m$, respectively. Likewise, hybrids between the rRNA precursor isolated from duck embryo fibroblasts and the rDNA appeared as structures containing two duplex regions of lengths $0.60 \pm 0.11 \mu m$ and $1.38 \pm 0.15 \mu m$, separated from each other by a single-stranded region appearing as a large bush. The obvious interpretation is that these duplex regions correspond to the 18S and 28S RNA portions of the rRNA precursor molecule, while the internal bush is a portion of the precursor not conserved in the processing of the parent molecule.

94. FAILURE TO DETECT HIGH MOLECULAR WEIGHT COMPLEMENTARY TRANSCRIPTS IN THE NUCLEAR RNA FROM ANIMAL CELLS

Investigators: William I. Murphy, Giuseppe Attardi

Support: National Institutes of Health, Public Health Service

The HnRNA sedimenting faster than 50S, isolated from either HeLa cells or immature duck erythrocytes labeled for different times with $[5^\text{3H}]$uridine, was examined for the presence of complementary transcripts capable of forming RNase-resistant duplexes. After extensive self-annealing of the HnRNA, carried out under conditions such that complementary RNA sequences present a few times or possibly even once in the RNA population would have formed hybrids, no evidence was found for the existence of symmetrical transcripts in either cell system. In these experiments, the sensitivity of detection was such that even 1 to 2% of complementary transcripts in the HnRNA labeled after a 2-minute $[5^\text{3H}]$uridine pulse in HeLa cells, or after a 15-minute $[5^\text{3H}]$uridine pulse in duck erythrocytes, would have been detected. However, 2 to 3% of the purified duck HnRNA and 4 to 5% of the HeLa HnRNA did form RNase-resistant RNA duplexes. These duplexes resulted from base-pairing of complementary regions within the HnRNA molecule, as judged from the lack of concentration dependence and from the kinetics of their formation. The weight-average length of the RNase-resistant fragments from the duck HnRNA was found to be approximately 100 nucleotide pairs.

After annealing the duck HnRNA with an excess of 10S hemoglobin mRNA, 2% of the HnRNA was found to form RNase-resistant hybrids with the 10S mRNA in excess of the duplexes expected from intramolecular homology. The RNase-resistant complexes formed between the 10S mRNA and HnRNA had about the same size range as the intramolecular duplexes.

The failure to detect any intermolecular hybridization in the pulse-labeled HnRNA from either actively growing or highly differentiated, nondividing cells, strongly suggests that the mechanism for the synthesis of HnRNA in animal cells does not involve the production of high molecular weight complementary transcripts.
95. SIZE DISTRIBUTION OF MESSENGER RNA FROM FREE POLYSOMES OF HeLa CELLS

Investigators: William I. Murphy, Giuseppe Attardi
Support: National Institutes of Health, Public Health Service

The sedimentation properties of pulse-labeled and long-term-labeled mRNA from HeLa cell free polysomes, selected for poly(A) content by two successive passages through oligo(dT)-cellulose columns, were analyzed under native and denatured conditions. The sedimentation profile of the mRNA on both sodium dodecyl SO₄-sucrose gradients and formaldehyde-sucrose gradients showed a broad distribution of components with estimated molecular weights ranging from 2 x 10^5 to 5.5 x 10^6 daltons and a weight-average molecular weight of 8.5 x 10^5 daltons.

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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 lends itself readily to manipulation of its genetic material, and also has a broad repertoire of behavior.

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. Given a mutant defect, a first step in the analysis of a behavioral mutant is 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 other organ whose malfunction influences behavior. 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. By determining which part of the fly must be mutant in order to produce the mutant behavior, the responsible focus can be identified. Presynaptic and postsynaptic phenomena can thus be distinguished by genetic methods. In a certain mutant showing abnormal muscle function, for example, it can be shown that this defect is not neurogenic, since a mosaic fly with genetically mutant nervous system and genetically normal muscles does not show the defect. Using a histochemical method which makes it possible to section a mosaic fly and score the internal tissues for mutant and normal cells, a behavioral focus can be assigned to a specific area of the nervous system.

This methodology is being applied to developmental problems such as pattern formation in the eye, the development and maintenance of the nervous and muscular systems, and behavioral phenomena ranging from relatively simple responses such as phototaxis to the more complex phenomena of sexual courtship and learning.

96. MUTATIONS AFFECTING THE SEXUAL COURTSHIP PATHWAY

Investigators: Jeffrey C. Hall, Seymour Benzer
Support: National Institutes of Health, Public Health Service
National Science Foundation

Drosophila courtship represents a sequence of fixed action patterns in which each step causes an alteration of the state of the nervous system such that subsequent steps progressively become more likely to occur. Mutants may be obtained in which the transition from one state to the next is blocked. Several of these have been found by screening mutants that are male-sterile, in spite of having motile sperm. A series of such male steriles was obtained from D. L. Lindsley (University of California, San Diego) and analyzed for courtship behavior yielding three X-linked courtship-defective mutants that have been named celibate, coitus interruptus, and longshot. In addition, the autosomal mutant stuck discovered by C. Beckman, and fruity found by K. Gill, have been studied.

The celibate mutant (cel, X-chromosome, recessive, map position 36) readily follows females and performs wing vibration, but rarely attempts copulation, and never achieves it.

In this respect, it is similar to fruity (fru, Gill, 1963, third chromosome, recessive, map position 62). However, fruity exhibits an additional defect in that fru males will court and are courted by other males (fru or fru") far more frequently than normal. This abnormal behavior can lead to the formation of chains of courting males.

The coitus interruptus mutant (coi, X-chromosome, map position 21) performs properly all the courtship actions up to copulation, but
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copulation is terminated after a mean duration of only 11 minutes instead of the normal 20 minutes. Thus, it usually does not transmit sperm (normal males tend to ejaculate during the last half of the copulation period, see review by Fowler, 1973). When, as happens with a probability of about 0.1, sperm is transferred, viable progeny are indeed produced. This mutant may be defective in the signal from the central nervous system which acts to terminate copulation.

The longshot mutant (ls, recessive, X-linked) on the other hand, has an unusually long copulation duration (mean time = 26 to 28 minutes). Nevertheless, it often fails to transfer sperm. This might fail to provide a feedback signal that normally controls the end of copulation.

The autosomal stuck mutant (sk, Beckman, 1970) is one in which there is a considerable probability (around 0.5) that the mated pair will have difficulty in disengaging after copulation. After the normal 20-minute period, the male attempts to pull away, sometimes succeeding after some minutes or hours, but often not succeeding even in several days, leading to death.

Further analysis of the anatomy and physiology of these mutants may lead to identification of specific defects in neural function that delineate the normal pathway of events.

References:

97. MOSAIC ANALYSIS OF SEXUAL COURTSHIP IN DROSOPHILA

Investigators: Jeffrey C. Hall, Seymour Benzer

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

Sex mosaics (gynandromorphs) can be used to map the controlling centers for the various steps involved in sexual behavior. The behavior of gynandromorphs in the presence of normal females was observed, recording the occurrence or absence of each male-like courtship action in several hundred mosaic flies. In separate tests, each gynandromorph was tested for its ability to provoke courtship by normal males. It was found that the first steps in male courtship (orientation toward the female, wing vibration) map to the brain area. However, the next step (attempted copulation) can be dissociated from the first two. Thus, some mosaics court females with great vigor, yet never attempt copulation. In order for a gynandromorph to be courted by normal males, it appears that part of the abdomen must be female.

About 100 of the mosaics were scored for their distribution of genetically male and female tissues, not only on the cuticle of the fly, but also within the nervous system, utilizing a histochemical enzyme marker such that male tissues lack activity for acid phosphatase (Kankel and Hall, *Biology* 1973, No. 264). Preliminary indications are that genetically male tissue in a dorsal part of one-half of the brain is necessary, and usually sufficient to trigger following of a female, and the wing vibration step. For the mosaic to attempt copulation, male tissue in the brain is necessary, but not sufficient; the thoracic ganglion must also have some male tissue. To provoke courtship by normal males, some female tissue on the abdominal cuticle is generally required.

References:
Patterson, J. T. and Stone, W. S. (1938) *University of Texas Publ.* No. 3825, pp. 5-67.
98. LOCALIZING SEXUAL RECEPTIVITY IN DROSOPHILA FEMALES

Investigator: William A. Harris
Support: Gordon Ross Medical Foundation
National Science Foundation

Sex mosaics with female genitalia were produced by the loss of the unstable ring X-chromosome In(1)w^C. Two hundred and sixty-four of these gynanders were tested to find out which parts of the fly have to be female in order for female receptivity to occur. Receptivity to male courtship was tested by putting a gynander in a vial with 4 to 5 males for at least five days. Normal females are virtually all fertile in this test. Among gynanders with male heads, only 2% were found to be fertile even though they all had female genitalia. This result is consistent with the observations of L. V. Morgan (1929) and indicates that embryonically there is a site near the head region which must be responsible for female receptivity. From preliminary fate mapping, this seems to be a focus in or near the brain. While over 90% of the gynanders had functional ovaries, only 60% laid eggs. Mosaic analysis revealed a focus for egg-laying behavior in a region of the map near the posterior portion of the thoracic ganglion. Among the gynanders with female heads, about 28% did not produce viable offspring, including those which did not lay any eggs; about 10% of the gynanders that had female heads and also the capacity to lay eggs, nevertheless did not mate. This indicates that there may be an additional focus, near to but separable from the egg-laying focus, which is important in female receptivity.

References:

99. EXPERIMENTS ON LEARNING IN DROSOPHILA: EFFECTS OF ANESTHESIA AND SCREENING FOR LEARNING MUTANTS

Investigators: Duncan Byers, William G. Quinn Jr.
Support: National Science Foundation
Gordon Ross Medical Foundation
National Institutes of Health, Public Health Service

We are seeking mutant flies specifically unable to learn in the avoidance conditioning paradigm developed in this laboratory (Quinn et al., 1974). Ethyl methane sulfonate mutagenesis of sperm in males is followed by two generations of single-male mating to attached-X females to produce stocks in which males have identical, mutagenized X-chromosomes (Lewis and Bacher, 1968; Benzer, 1967). These populations are then screened for learning ability, and several lines with deficient performance have been obtained.

During the screening procedure, anesthesia is employed to separate the attached-X females from males. Ether, CO₂, or immobilization at 4°C have been found to decrease learning capability for several days. On the other hand, flies immobilized under nitrogen regain normal learning capacity within 10 minutes.

References:
100. THE EFFECT OF HOMOEOTIC MUTATIONS ON THE NERVOUS SYSTEM OF DROSOPHILA

Investigator:  Ilian Deak
Support:  Muscular Dystrophy Association of America
National Science Foundation

A homoeotic mutation causes the replacement of one organ by another. The most studied examples are ones causing transformation of cuticular structures. The spineless-aristapedia mutation results in replacement of the distal antennal structures by distal leg structures (tarsi) (Balkaschina, 1929). In normal flies the tarsi bear chemoreceptors that are used for tasting; stimulation of these receptors with a sugar solution produces a feeding response, i.e. extension of the proboscis (Minnich, 1926). Experiments on the mutant spineless-aristapedia, show that stimulation of antennal tarsus also elicits proboscis extension. This indicates that the sensory neurons present in the homoeotic antennal tarsus produce the same central response as leg neurons, i.e. they assume a function appropriate to the structure of the surrounding cuticle and not to their position. Histological examination shows that there is a large nerve bundle in the antennal tarsus. To determine where the tarsal axons terminate, an attempt is being made to develop a neural degeneration stain for Drosophila. This would provide a possible model system for studying neurospecificity in Drosophila.

References:

101. CELL PROLIFERATION AND PATTERN FORMATION IN THE DEVELOPING EYE OF DROSOPHILA

Investigator:  Donald F. Ready
Support:  National Science Foundation
National Institutes of Health, Public Health Service

The compound eye of Drosophila is a highly regular repeated pattern. Morphological evidence of formation of this pattern first appears in the eye disc during the third larval instar. The posterior of the disc first shows well developed cell clusters which are precursors of the ommatidia. Anteriorly, the clusters become less distinct until all obvious pattern is lost. This occurs at a furrow which runs across the disc perpendicular to the postero-anterior axis. Just posterior to the furrow, mitotic figures are seen. Anterior to the furrow lies another area of mitotic activity.

The fate of cells in these mitotically active areas has been followed by labeling dividing cells with $^3$H-thymidine injected into the larva. Discs prepared for autoradiography shortly after $^3$H-thymidine injection show label restricted to areas directly posterior and anterior to the furrow. Several hours after the injection, the furrow has moved anteriorly, increasing the extent of patterned area. Autoradiography of discs at this time shows the posterior band of label to be concentrated within the region of distinct clusters. Labeled cells remain anterior to the furrow. However, the advance of the furrow has shifted some of these cells to a position immediately posterior to the furrow.

Previous work (Biology 1973, No. 267) indicated that retinular cells 1, 6 and 7 are formed in the posterior mitotic wave along with other ommatidial cells. Preliminary results show labeling of the remaining retinular cells in a more anterior band.

Tentatively, cell proliferation might be related to pattern formation in two steps. At or slightly ahead of the furrow, “preclusters” are assembled from cells produced in the large, anterior mitotic region. A second wave of division, from which retinular cells 2, 3, 4, 5 and 8 are excluded, then occurs in the “prepatterned” area posterior to the furrow completing the ommatidial clusters.
102. HEREDITARY RETINAL DEGENERATION IN DROSOPHILA

**Investigator:** William A. Harris

**Support:** Gordon Ross Medical Foundation
National Science Foundation

Two X-linked mutant genes, *rdg* I and *rdg* II, cause degeneration of the photoreceptors in *Drosophila* (Hotta and Benzer, 1970). Mosaic analysis puts the defect of both loci into the eye itself; histology of mosaicism within the eye shows that the photoreceptor cells rather than the pigmented cells are responsible (Hotta and Benzer, 1970). Degeneration of *rdg* II mutants was found to be enhanced by light; the photoreceptors of *rdg* II flies raised in complete darkness do not degenerate. On their first exposure to light, these flies have a normal ERG and optomotor response. Over the course of about one day in light the optomotor response diminishes and the ERG becomes defective. Within three days the degeneration becomes visible by the deep pseudopupil technique (Franceschini, 1972). This degeneration is not reversible by putting the flies back into the dark. The *rdg* I mutants degenerate even in darkness.

One allele of *rdg* II has been studied in detail. In this mutant, there is specific degeneration of only the outer six receptor cells (#1-6) of each ommatidium, leaving the inner two (#7 and #8) intact. In the degenerated state the mutant lacks the optomotor response but retains normal phototaxis. Thus 7 and 8 are sufficient for the phototactic response, and 1-6 are necessary for a normal optomotor response. A double mutant of this allele and one that prevents the formation of the 7th rhabdomere (LY3) leaves only photoreceptor 8 in each ommatidium. These flies are also phototactic.

**References:**

103. STUDIES ON SLUGGISH MUTANTS OF DROSOPHILA

**Investigators:** Alain Ghysen, Seymour Benzer

**Support:** Fonds National de la Recherche Scientifique (Belgium)
European Molecular Biology Organization
National Science Foundation

*Drosophila* displays a strong positive phototactic response when agitated. Various mutants do not show this response. From among mutants previously isolated in this laboratory, 15 have been found which have no evident morphological defects, and normal vision (as judged by their ERG and optomotor ability), yet do not show the normal phototactic response when agitated. Complementation experiments indicate that the 15 mutants represent 13 different genes on the X-chromosome.

Measurements have been made of the movement of flies in a constant, quiet environment (a long, narrow glass tube) after an initial mechanical jolt. Normal flies initially walk at an average velocity of about 80 cm/min, this rate decreasing to half within 1 minute and reaching a level of about 4 cm/min within 15 minutes. Typically, when mutant flies are agitated by being jolted to one end of a tube, they will begin to move toward a light placed at the other end, but soon abort this movement. This suggests that their defective behavior is caused by an inability to maintain the proper excited state. The sluggish mutants exhibit various initial velocities, ranging down to as low as 5% of normal.

A mosaic mapping analysis (Hotta and Benzer, 1972) has been performed on 9 of the mutants to determine the controlling behavioral foci. For 6 of the mutants, the focus maps to the brain. In the
three other mutants the foci map in the thoracic ganglion region. More precise localization of the foci will be obtained by the use of histochemical genetic markers allowing the detection of internal mosaicism. These mutants should be useful in analyzing the mechanism producing general excitation in the Drosophila nervous system.

References:

104. DEVELOPMENT OF MUSCLE IN DROSOPHILA

Investigator: Han Deak
Support: Muscular Dystrophy Association of America
National Science Foundation

Two mutants of Drosophila in which the wings are held vertically were described by Hotta and Benzer (1972); in one of these the indirect flight muscles form and subsequently degenerate, while in the second these muscles do not form normally. The development of these muscles in normal and mutant flies is being investigated using histochemical techniques.

A rapid histochemical technique has been developed for differentially staining degenerating flight muscles. This permits one to identify degenerating and normal muscles in genetically mosaic flies. These techniques are being used to construct a detailed fate map of the flight musculature and to analyze the clonal relationship between muscle fibers.

Several Drosophila mutants are available in which the nervous system functions normally at low temperature while at high temperatures the flies become paralyzed (Suzuki et al., 1971; Hall et al., 1972). It is possible to obtain mosaic flies in which such mutant nerves innervate genetically normal muscles. By shifting the temperatures up or down at suitable times, the activity of the nerves can be manipulated (Siddiqi and Benzer, 1974). Thus the influence of the nervous system on the development and maintenance of muscles can be studied. One can thus distinguish between the various models now invoked to explain the requirement by muscles of functional innervation.

References:

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We are prejudiced but we believe the study of the chromosomal programs which control the development of the forms of living creatures to be one of the most fascinating problems in all of biology. At the same time it is undoubtedly one of the most difficult areas in which to obtain definitive molecular information. Not surprisingly, several research groups within the Division of Biology at Caltech have approached this problem in diverse ways. Our approach places emphasis on the study of the cell surface recognition properties which cells display as they sort out and construct living organisms. The surfaces of differentiated cells display a remarkable diversity of potential for recognizing the cells and/or the environment which surrounds them. Biological studies dating back more than one century make it clear that many types of cells do indeed recognize each other in most precise ways; yet very little except descriptive biology is known of the nature of the developmental events which cause specific cell surface molecules to appear as a cell is committed to a particular developmental pathway. We do know that the potential of a given cell type becomes increasingly narrow as each successive determinative event unfolds during organogenesis. We also know that these changes are reflected in the surface display of molecules or antigens exposed on cells. Nevertheless, we can only express curiosity concerning more explicit questions: How, for example, do nerve cells recognize other cells during the process of wiring up the nervous system? How do pigment cells “know” how to migrate and where to stop as they move to form the elaborate patterns seen in the feathers of birds, the wings of insects or the stripes on a zebra? How do individual antibody cells become committed during development so that they have the ability to express on their surface one, and only one, out of hundreds of thousands of different possible antibodies? Evolutionary and theoretical considerations led us to believe that the answers to questions of this type may reflect certain common mechanisms of development. Results already obtained from studies in the immune system suggest that certain long-standing dogmas commonly accepted by developmental biologists now have to be reevaluated (see Biology 1973). These studies have led us to the suspicion that the genetic translocational mechanisms uncovered in the immune system are not unique but may reflect an earlier evolution of other highly specific molecules expressed on differentiated cell surfaces. According to this hypothesis, such molecules may play a role in cellular recognition processes involved in the shaping of organisms during embryological development.

We have undertaken a study of specific cell surface molecules. Our program includes the following areas:

1. A continued study of developmental events occurring during the early formation of the immune system.
2. A study of the photoreceptor membrane protein, rhodopsin, as a prototype prior to the study of more complex membrane recognition molecules.
3. The study of molecules expressed on chemically-induced and/or ultraviolet-induced tumors. Cells from such tumors reveal a particularly intriguing diversity of antigens on their surfaces. These antigens, referred to as individually specific or “unique” tumor antigens, may turn out to represent abnormal molecules resulting from the action of carcinogens on the genetic mechanism of particular cell types. Alternatively it seems possible that they may represent normal molecules which are expressed appropriately during periods of embryological development and which play a role in cell surface specificity in the embryo.
4. Tool making. At this time the principal limitation to our program of study discussed above lies in the available methods for the assay, isolation and definitive analysis of such molecules.

Antibody molecules, known to be the receptors exposed on the surface of two different types of lymphocytes referred to as B cells and T cells, can be isolated in amounts more than sufficient for molecular studies and the results of such studies have proven to be extremely valuable in leading to insights concerning genetic mechanisms controlling development in the immune system. Other molecules which play a role in the specificity of surfaces of cells in the developing embryo or on tumors are present in amounts which are far too
small to permit studies by methods currently available. Accordingly, we have undertaken a program aimed at developing methods which will permit the definitive analysis of other molecules important in the specificity of cell surfaces. Our progress in this program of development of tools is discussed in several abstracts.

General References:

105. STUDY OF THE DEVELOPMENT OF THE IMMUNOGLOBULIN-PRODUCING SYSTEMS IN CHICK EMBRYOS

Investigators: Henry V. Huang, William J. Dreyer
Support: National Institutes of Health, Public Health Service
       Danforth Fellowship

In the study of development one often finds evidence of precisely programmed sequential events which occur during embryogenesis. Particular types of stem cells are formed at prescribed times. They in turn divide and in an orderly way generate specific types of daughter cells. Virtually nothing is known of the nature of the chromosomal events which lead to the programming of such highly ordered sequential events in development. Studies of the nature of antibody molecules have revealed evidence of extraordinary genetic translocational events which occur during the production of committed antibody cells. It is unclear at this time whether these translocational events represent a special adaptation of the immune system or whether they reflect more general developmental processes which occur in other systems. The embryological development of the immune system does resemble other developing systems in many respects. Hence, one wonders whether the genetic translocational events known to occur as a part of the process of commitment of individual cells to production of specific antibody molecules might occur in an orderly manner. Alternatively, the genetic mechanisms involved might generate committed cells in a completely random fashion in the case of the immune system. We have initiated a research program which may help to illuminate this subject.

During embryogenesis in birds, the precursors of cells which are to make specific immunoglobulins are generated in a small organ called the bursa of Fabricius. Such cells migrate from the bursa to seed various lymphoid tissues where immunoglobulin production occurs. Bursectomy produces animals partially or completely deficient in the production of immunoglobulins, depending on the exact stage in development at which it is performed. Little is known, however, of the nature of the antibody molecules made when bursectomy is performed at a time which leads to partial deficiencies in the production of immunoglobulins. By examining the immunoglobulins made from birds bursectomized at different periods of embryological development, we hope to elucidate the pattern of developmental programming of this system. We have chemically bursectomized chicks which are F1 hybrids of two highly inbred lines provided by Hans Abplanalp of The University of California, Davis. Work is in progress to assay these chickens for ability to respond to antigenic stimulation and to compare the immunoglobulins made in each chicken with those of the others. If the birds which are seriously restricted in their abilities to produce antibody molecules show similar or identical antibodies in different birds, it will suggest a sequential and ordered process of development. Alternatively if no relationships can be found in such birds, a random process will appear more likely.

Reference:
106. DEVELOPMENT OF METHODS FOR THE STUDY OF THE STRUCTURE OF MEMBRANE RECEPTORS

Investigators: Katherine A. Woodin, William J. Dreyer

Support: National Institutes of Health, Public Health Service
         Chromatonix, Inc.

Molecular level studies of recognition molecules found on cell membranes require the development of methods and skills beyond those which have proven successful in studies of conventional water soluble molecules. We have used the membrane receptor, rhodopsin, as a relatively simple model. This glycoprotein accounts for over 90% of the disc membranes in rod outer segments and hence is readily available in nearly pure form (Papermaster and Dreyer, 1974); it is apparently subject to control by a protein kinase which phosphorylates the protein only after it is triggered by light (Kühn et al., 1973); furthermore, dark adapted and/or bleached rhodopsin can be fractionated and separated with high resolution by means of isoelectric focusing in the presence of neutral dissociating agents (Huang et al., 1973). We have also learned a great deal about the primary amino sequence of this molecule but the sequence information is not yet complete.

Our primary emphasis at this time is in fractionating large fragments of the protein (cleaved by chemical and/or enzymatic methods). We have developed a method for preparative gel electrophoresis of these fragments with subsequent removal of SDS detergent and recovery of pure peptides. This is accomplished (in the case of peptides too small for dialysis) by means of small cartridges containing special anion and cation exchange resins. Peptides thus obtained are sequenced in a new design solid phase peptide/protein sequencer also under development.

The methods discussed are now working so well with the model system that we have begun exploratory studies aimed at isolating the unique tumor antigens referred to in the introduction to our group.

References:

107. APPLICATION OF NEW IMMUNOLATEX SPHERES IN STUDIES OF SURFACE ANTIGENS ON LYMPHOCYTES BY SCANNING ELECTRON MICROSCOPY

Investigators: Robert S. Molday, William J. Dreyer, Alan Rembaum*, Shiao-Pin S. Yen*

Support: The President's Fund
         American Cancer Society
         National Institutes of Health, Public Health Service

New immunochemical reagents consisting of antibodies bound to small latex spheres were used as visual markers for the detection and localization of cell surface antigens by scanning electron microscopy (SEM). Cross-linked latex spheres of various sizes from 300 Å to 3400 Å in diameter were synthesized by aqueous emulsion copolymerization of methacrylate derivatives containing hydroxyl and carboxyl functional groups. Proteins and other molecules containing primary amino groups were covalently bonded to the acrylic spheres under a variety of mild conditions by the aqueous carbodiimide, cyanogen bromide, and glutaraldehyde methods. For use in the indirect immunochemical labeling technique, goat antibodies directed against rabbit immunoglobulins were bonded to the spheres. These immunolatex reagents were shown to bind only to cells (red blood and lymphocytes) which had previously been sensitized with rabbit antibodies against cell surface antigens. Mouse spleen lymphocytes with exposed immunoglobulins on their surface (B-cells) were labeled with these spheres and distinguished from unlabeled or
T-lymphocytes by SEM (Figure 1). The distribution of Ig receptors on lymphocytes was also studied using the spheres as visual markers. When lymphocytes were fixed with glutaraldehyde and subsequently labeled with the immunolatex reagents, a random distribution was observed by SEM; a patchy distribution was observed when unfixed lymphocytes were used. These results are consistent with studies using ferritin-labeled antibodies (De Petris and Raff, 1973) and support the view that Ig receptors on lymphocyte undergo translational diffusion. In addition to serving as visual markers for SEM, these latex spheres tagged with fluorescent or radioactive molecules have applications as highly sensitive markers for fluorescent microscopy and as reagents for quantitative studies of cell surface antigens and other receptors.

References:

*Jet Propulsion Laboratory.
A new method for the isolation of plasma membranes is currently being developed. This method uses new immunolatex spheres (Biology 1974, No. 107) with an affinity for specific cell surface molecules as a membrane density perturbant. The binding of these latex reagents to membrane fragments can result in an increase in membrane density, and the immunolatex membrane complex can readily be resolved from unlabeled membranes by equilibrium density centrifugation.

In a preliminary study, goat antibodies specific for rabbit immunoglobulins were bonded to tritiated latex spheres. These immunochemical reagents were shown to specifically bind to erythrocyte ghosts pretreated with rabbit antibodies against erythrocytes, as determined by radioactivity measurements. Erythrocyte membranes labeled with the latex spheres and centrifuged on a linear sucrose gradient were found to have a significantly higher density than unlabeled erythrocyte membranes. A comprehensive study is currently being carried out to determine the effect of various parameters (latex sphere density, size, number of spheres per unit area of membrane, and membrane vesicle size) on membrane density perturbation. Application of this method to the purification of plasma membranes of more complex cells is also being initiated.

*Undergraduate, California Institute of Technology.

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Our laboratory is interested in studying multigene systems; that is, tandemly-linked gene families whose products exhibit sequence homology and overlapping functions. Multigene systems fall into two general categories: those whose members are identical or nearly identical (e.g., histone genes) and those whose members differ significantly (antibody V genes). Our studies are carried out primarily through the chemical analysis of the protein products of these families.

The antibody genes are the most thoroughly studied nonidentical (informational) multigene system. They are comprised of three families of genes, λ, κ and H, which are unlinked in the mammalian genome. Each antibody polypeptide is coded by two distinct genes, a variable (V) and a constant (C) gene. Accordingly, these genes or their products must be joined during the differentiation of the antibody-producing cells. Each antibody family generally has a large number of V genes and a much more limited number of C genes. The antibody V genes have two unusual evolutionary features: they evolve together in a “coincidental” or species-specific fashion and the size of the gene family can change rapidly during evolution. One major effort is directed at understanding the nature of the evolutionary mechanism whereby these unusual features are generated. In this regard we are examining in a comparative fashion antibody gene products of recent divergence (e.g., inbred strains of rodents—see abstracts 109 to 113) and ancient divergence (e.g., avians vs. mammals—see abstracts 116 and 117). A second major effort is directed at understanding expression and control in this multigene system through a study of the immunoglobulins produced by human biclonal myeloma patients (abstracts 114 and 115). A third effort is directed at understanding the structure-function relationships of antibody molecules as well as gaining insight into the genetic mechanism whereby antibody diversity is produced (abstracts 109 to 112). One of the most fruitful studies in this regard has been the comparative analysis of myeloma proteins with binding activity for the same hapten (abstracts 109 and 110). Finally, this past year we have initiated studies into the equally intriguing evolution of C genes (abstracts 113 and 118). It should be stressed that the antibody system is the only multigene system in which individual gene products can be obtained in large quantities for chemical analysis.

We are also studying a second multigene system—the H-2 gene complex of the mouse (with sufficient DNA for perhaps 1000 genes) which codes for a variety of cell surface molecules (K, Ir, Ss and D) (abstracts 119 and 120). This complex can be divided into four regions, and at least three of these regions (K, Ir, D) code for cell surface molecules. We have developed techniques for the isolation of these molecules as well as microsequencing techniques for the analysis of picomole quantities of these polypeptides. We are interested in the nature and extent of diversity which exists in the polypeptides coded by this extremely polymorphic region as well as in the homologies these molecules will exhibit for each other. We are also interested as to whether these gene complexes will employ strategies similar to those seen for antibodies (e.g., V and C regions).

We are scanning chromosomal proteins from Drosophila in order to determine whether microheterogeneity exists in a series of known multigene families, i.e. the histones (abstract 121). The presence of microheterogeneity will have important implications concerning the mechanism whereby histone genes of a given class are maintained identical or very similar. We are also screening nonhistone chromosomal proteins in Drosophila to search for new multigene families (abstract 122).
A dream of the chemist interested in protein evolution is to be able to study fossil proteins. We have taken a step in this direction through the study of matrix proteins in the clam (abstract 123). The implied existence of a repeating structural subunit in these proteins finds its counterpart in other structural proteins and has interesting implications for the mineralization process as well.

Multigene systems are an exciting new area in modern genetics. We are interested in questions such as the extent to which multigene systems are employed in eukaryotes, whether they share common evolutionary and control mechanisms, and the roles they might play.

**109. INVESTIGATION OF THE GENETICS OF IMMUNOGLOBULIN SPECIFICITIES BY SEQUENCE ANALYSIS**

*Investigator:* Paul A. Barstad  
*Support:* National Institutes of Health, Public Health Service  
National Science Foundation

Myeloma proteins with known antigen-binding specificities have been studied by amino acid sequence analysis of heavy and light chains for the first 40 residues. Myeloma proteins which react with three haptens have been examined to date: (1) phosphorylcholine which includes immunoglobulins derived from tumors T15, H8, S107, M603, and M167; (2) α-1,3 dextran—tumors J558 and M104E; and (3) levan—tumors 5476, J606, and 3082. For the immunoglobulins reacting with phosphorylcholine, the amino acid sequence of the heavy chains is highly conserved, whereas the sequences of the light chains appear less highly conserved. For the other two specificities studied here as well as for a fourth specificity, α-1→6 galactan, taken from the literature, both the heavy and light chain sequences appear highly conserved for immunoglobulins of each specificity.

This work was carried out in collaboration with Drs. S. Rudikoff and M. Potter, of the National Cancer Institute, National Institutes of Health, Bethesda, Maryland, Dr. M. Cohn of Salk Institute for Biological Studies, San Diego, California, and M. Weigert of the Institute for Cancer Research in Philadelphia.

**110. SURVEY OF DIVERSITY IN MOUSE MYELOMA HEAVY CHAIN**

*Investigator:* Paul A. Barstad  
*Support:* National Institutes of Health, Public Health Service  
National Science Foundation

The N-terminal 20 residues of 13 heavy chains derived from the BALB/c myeloma proteins were determined and compared with 15 other sequences from the literature. Many of the chains in this survey were from molecules of known antigen-binding specificity. These sequences revealed several interesting points concerning myeloma diversity: (1) the heavy chains in this limited study must be coded for by at least 8 germ line genes; (2) the profile of diversity of these proteins is much different than that reported for the normal pool heavy chains; (3) the amino acid sequence of the heavy chains outside, as well as within, the hypervariable regions correlates with the specificity of the molecules from which the heavy chains were derived; and (4) several unblocked heavy chains had little homology to the human V_HIII subgroup.

These studies were done in collaboration with Dr. M. Weigert of the Institute for Cancer Research, Philadelphia, Pennsylvania and Dr. M. Cohn, The Salk Institute, San Diego, California.
111. EVOLUTION OF NORMAL IMMUNOGLOBULINS IN MICE

Investigators: Elwyn Loh, Rene Gandolfi*

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

The germ line theory must explain how parallel ("coincidental") evolution occurs in multiple germ line V genes. If one assumes that expansion and contraction events (e.g., homologous but unequal crossing-over) are responsible for that parallel evolution, one can ask about the frequency of these evolutionary events. Inbred strains of mice, which probably shared common ancestors several hundred years ago, provide a system to study the divergence of the antibody multigene family. By comparing normal antibodies from several strains we hope to study the rate of this evolution.

We are continuing to explore techniques of preparing large amounts of purified normal light and heavy chains. These techniques include the induction of ascites tumors in order to raise large amounts of ascites fluid, the elution of purified proteins from acrylamide gels, and affinity chromatography.

We are running the normal antibody chains on the automatic protein sequencer to obtain amino acid profiles of the N-terminal residues. Preliminary data indicate that the normal BALB/c and C57B1/6J pools closely resemble the combined data of myeloma proteins, but that both quantitative and qualitative differences do occur.

*Undergraduate, California Institute of Technology.

112. AMINO TERMINAL STUDIES OF MYELOMA PROTEINS OF THE NZB STRAIN OF MICE

Investigator: Elwyn Loh

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

Myeloma tumors of immunoglobulin-producing cells are experimentally inducible in only two strains of inbred mice, BALB/c and NZB. Extensive sequence studies of BALB/c myeloma tumors have revealed important facets of immunoglobulin genetics, structure, and evolution. We have begun amino acid sequence studies on the NZB myeloma proteins which provide a different way to view antibody evolution and genetics.

Presently we are conducting a survey of the amino terminal 40 residues of both the light and heavy chains from 25 NZB myeloma proteins. As with BALB/c myelomas, most NZB immunoglobulin chains appear to have an unblocked N-terminus.

These studies are in collaboration with Dr. Martin Weigert of Cancer Research Institute, Philadelphia, Pennsylvania.

113. RAT LIGHT CHAIN ALLOTYPES: STRUCTURAL CORRELATES AND EVIDENCE FOR MULTIPLE CONSTANT GENES

Investigator: Elwyn Loh

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

A light chain genetic marker in the rat has been defined by serological techniques. Each inbred strain of rat studied has one of two allelic forms of a light chain which is inherited as a codominant gene.

We have studied the amino acid sequence differences of the C regions for the two light chain allotypes. Light chains from pooled immunoglobulins of the two inbred strains which differ in allotype, LE (Lewis) and DA (Dark Agouti), were
prepared by conventional techniques. Peptides were isolated by fingerprinting and the amino acid compositions of peptides from nearly the entire C region of both allotypes were compared. Relevant peptides were sequenced by the dansyl Edman technique.

From comparisons of the C regions of the normal light chains of the two strains and the C region of a myeloma light chain (S211) of the same allotype as the LE chains (A. Querinjean, personal communication), we have made the following observations:

(1) Unlike the human InV marker in which only one substitution was found between two alleles, the rat allotypes differ by at least seven substitutions and one size difference. Two possible explanations for the multiple substitutions are (a) a single gene exists which has accumulated the differences by an unknown mechanism or (b) multiple genes exist for the C region and the expression of individual genes is controlled by another gene which has two forms, one expressing the LE allotype and one the DA allotype. A model for the multiple substitutions in duplicated genes exists in the hemoglobin system. We favor this latter interpretation.

(2) The pooled rat light chain of the LE strain and the S211 C region differ at two positions even though they share the same allotype. Furthermore, Querinjean has found that other myeloma chains share the LE pool sequence (personal communication). This suggests that at least two genes code for C regions in rats of the LE allotype.

These studies were in collaboration with Dr. George Gutman, Pathology Department, Stanford University, Stanford, California.

114. BICLONAL MYELOMA SCREENING

Investigator: Carlos R. Sledge
Support: American Cancer Society
National Institutes of Health, Public Health Service

About 1% of patients with multiple myeloma (a cancer of antibody-producing cells) have two (or more) homogeneous immunoglobulins in their serum rather than just a single protein. Since myeloma strikes about 1/20,000 individuals, it appears that having one myeloma protein predisposes the patient toward the production of a second. These "biclonal" proteins generally appear to be synthesized by two distinct populations of cells which probably were derived from a common clonal origin. Accordingly, the biclonal proteins appear to have been expressed at different stages of the differentiation of a single lymphocyte and hence yield interesting clues about the differentiation pathways of these cells (see Biology 1974, No. 115).

In order to study these various pathways we have set up a screening program to increase our library of biclonal proteins. The biclonal proteins can readily be detected by serum electrophoresis in agar. To date we have received over 50 samples from myeloma patients and have detected two biclonal proteins.

These studies are being carried out in collaboration with Drs. Daryl Fair and Robert Krueger of the Mayo Clinic, Rochester, Minnesota, and Dr. Stebbing Chonder at the University of Southern California Medical School, Los Angeles, California.

115. BICLONAL MYELOMA PROTEINS: A SHARING OF VARIABLE REGIONS BETWEEN GAMMA AND ALPHA HEAVY CHAINS

Investigators: Carlos R. Sledge, Bruce D. Black
Support: American Cancer Society
National Institutes of Health, Public Health Service

The variable regions of two immunoglobulins (IgA and IgG) derived from a patient with biclonal myeloma were studied. The light chains appeared to be identical by the following criteria: fingerprints, basic acrylamide gel electrophoresis, isoelectric focusing, and amino acid analysis. The
light chains were also identical throughout their N-terminal 40 residues. The heavy chains were identical for their N-terminal 45 residues. Serologic studies on these intact molecules also suggest that they are very similar if not identical to one another. A similar observation has been made for a biclonal patient with IgG and IgM proteins.

If the V regions of the light and heavy chains are identical, a joining mechanism for V and C genes must be postulated which permits the same V\(_H\) region to be associated with two distinct C\(_H\) genes (C\(_\alpha\) and C\(_\gamma\)).

This work was done in collaboration with Drs. Daryl Fair and Robert Krueger of the Mayo Clinic, Rochester, Minnesota.

116. PIGEON LIGHT CHAINS HAVE RESTRICTED HETEROGENEITY

Investigators: Carol Nottenburg*, Elwyn Loh

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

Chemical studies on normal pigeon light chains suggest that they are markedly restricted in their chemical heterogeneity when compared with their mammalian counterparts. Initially, quantitative amino acid sequence analyses revealed only five positions of heterogeneity in the N-terminal 24 residues. This observation was supported by the presence of only 4 major and 2 to 3 minor bands by isoelectric focusing.

Intriguing questions arise concerning this restricted heterogeneity—do normal serum light chains represent only a small subset of the V\(_L\) library pigeons can actually express, or is the V\(_L\) library itself markedly restricted? If the latter supposition is correct it would be interesting to know the nature and distribution of the heterogeneity that does exist as well as something about the corresponding degree of heavy chain heterogeneity.

*Undergraduate, California Institute of Technology.

117. STRUCTURAL ANALYSIS OF CHICKEN ANTIBODIES

Investigators: Stephen Beverley*, Bruce D. Black

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

Antibodies were raised against phosphorylcholine in individual chickens. These antibodies were purified by affinity chromatography. An analysis of the light chains derived from these antibodies by polyacrylamide gel electrophoresis demonstrated that some preparations had markedly limited heterogeneity when compared against normal light chains, whereas other antibody preparations were more heterogeneous. The N-terminal regions of these antibody light chains were examined by quantitative amino acid sequence analysis. This analysis affords the first opportunity to compare similar antibodies (antiphosphorylcholine) from two distinct families of vertebrates (mouse and birds—see Biology 1974, No. 109).

*Undergraduate, California Institute of Technology
118. STRUCTURAL STUDIES ON RABBIT LIGHT CHAIN ALLOTYPES

Investigator: Vincent R. Farnsworth
Support: National Institutes of Health, Public Health Service
National Science Foundation

Rabbit light chains can be serologically divided into four classes (b4, b5, b6 and b9) which appear to segregate in a Mendelian fashion in family studies. The location of these serologic markers has not been determined, however various studies have demonstrated amino acid sequence differences in the V as well as the C regions. Preliminary comparative studies of the C_L sequences of b4 and b9 chains suggest that these regions differ by 20% or more in amino acid sequence. Accordingly, these chains are quite distinct from those coded by a single structural gene with two allelic forms which differ by just one or two amino acid residues (e.g., the normal hemoglobin β gene and the sickle cell β gene). This observation raises the possibility that the apparent Mendelian behavior of the rabbit C_L regions may be explained by a special control mechanism operating in an allelic fashion on one of four (or more) duplicated C_L genes (i.e., the b4, b5, b6, and b9 C_L genes). Studies are now under way to determine the complete amino acid sequence of the b4, b5 and b9 C_L regions.

These studies were carried out in collaboration with Dr. Scott Rodkey, Biology Department, Kansas State University, Manhattan, Kansas.

119. CHEMISTRY OF HISTOCOMPATIBILITY ANTIGENS

Investigators: Jack Silver, Carol H. Sibley, Paul R. Morand
Support: National Science Foundation
National Institutes of Health, Public Health Service

All vertebrate species examined to date have been shown to have a complex genetic locus which represents the major barrier against organ and tissue transplants. In mice this genetic region (called the H-2 locus) controls the expression of several kinds of cell surface molecules and a number of immunologically important reactions. Mice differing solely at the H-2 locus have been used to prepare antisera directed against the products of the H-2 locus.

By indirect immunoprecipitation with specific anti-H-2 sera we have isolated the H-2 alloantigens, which are glycoproteins, from the surfaces of mouse spleen cells. The molecules were labeled by culturing the spleen cells in the presence of tritiated amino acids. Following solubilization of the cell membrane with nonionic detergent, the soluble H-2 glycoproteins were complexed with the specific antisera, precipitated with rabbit anti-mouse gamma globulin, purified by SDS polyacrylamide gel electrophoresis, and eluted from the gel. Tritium-labeled H-2 alloantigens were then subjected to amino acid sequence analysis, in order to determine the chemical basis of their antigenicity.

A thorough chemical knowledge of the H-2 alloantigens is important for devising methods of circumventing the normal graft rejection process. Chemical characterization of the H-2 alloantigens may also help to elucidate their function.
120. CHEMISTRY OF THE Ia ANTIGENS

Investigators: Jack Silver, Carol H. Sibley, Paul R. Morand

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

The major histocompatibility locus in mice is a complex genetic region which controls a number of biologically important traits in addition to graft rejection (see Biology 1974, No. 119). The region can be divided by biological and serological tests into at least four regions: two controlling the H-2 alloantigens, one controlling the level of a serum protein, and a fourth, the immune response (Ir) region, controlling the level of response to synthetic polypeptide antigens, and the synthesis of additional serologically detectable cell surface antigens. These antigens, whose function and tissue distribution are still poorly defined, are called Ir-associated (Ia) alloantigens. Since inbred strains of mice differing from one another at this Ir region have been developed, specific antisera have been prepared which permit the isolation of these Ia antigens. Employing the methods used for the isolation of the H-2 alloantigens (described in Biology 1974, No. 119), we have isolated and begun the chemical characterization of the Ia alloantigens.

Cell surface molecules were radioactively labeled with \( ^{125}\text{I} \), the membranes solubilized with non-ionic detergent and the Ia antigens specifically complexed with mouse antisera directed against them. These complexes were then precipitated with rabbit anti-mouse gamma globulin and the complexes analyzed by SDS polyacrylamide gel electrophoresis. There are at least four groups of Ia antigens on the surfaces of lymphocytes which may be distinguished by molecular weight. We are presently examining the distribution of these molecules and their degree of diversity. Since the Ia alloantigens may be important in the development of the immune response, studies on their chemical structure are also being initiated.

These studies are being carried out in collaboration with Dr. David Sachs, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

121. MICROHETEROGENEITY AMONG HISTONES

Investigator: Robert M. Goodfleish

Support: National Science Foundation

On the basis of RNA-DNA hybridization studies in sea urchins and Drosophila, histones are thought to be coded by several hundred genes. We might expect mutations within these genes to result in a heterogeneous population of histones. Yet each of the five normally occurring histones appears to be a homogeneous protein when characterized by conventional chemical techniques. This suggests the intriguing possibility that eukaryotes have a mechanism capable of suppressing or correcting mutations in certain important multiple gene systems. Callan (1967) has suggested a "master-slave" correction mechanism whereby many "slave" genes are corrected for mutations against a single "master" gene, resulting in multiple and identical genes. In order to test this hypothesis, we will examine purified histones using extremely sensitive chemical techniques for evidence of microheterogeneity. The presence of heterogeneity, even at very low levels, would suggest that a master-slave mechanism does not operate within eukaryotes.

These studies were carried out in collaboration with Dr. Sarah C. R. Elgin, Biology Department, Harvard University, Cambridge, Massachusetts.

Reference:
122. PARTIAL AMINO ACID SEQUENCING OF NONHISTONE CHROMOSOMAL PROTEINS

Investigator: Robert M. Goodfliesh
Support: National Science Foundation

Nonhistone chromosomal proteins (NHC proteins) comprise 100 or more diverse proteins which are associated with the DNA of most eukaryotic cells. These proteins are presumably important in, among other things, the regulation of genes and the maintenance of chromosomal structure. The amino acid sequence of NHC proteins may reveal important facets of their genetics and evolution. For example, have genes coding for NHC proteins of different molecular weights evolved from a common ancestral gene? Are sequences of essential NHC proteins conserved in divergent species? Can we find NHC proteins with closely related but distinct sequences that may be involved in regulating similar genes?

Sequencing of NHC proteins is complicated by two intrinsic problems: (1) the purification of individual NHC proteins, particularly those with biological importance, from the many different proteins associated with DNA; and (2) technical difficulties in sequencing small quantities of proteins. We are actively investigating approaches to both of these problems, the resolution of which is important for studying many important cellular proteins including NHC proteins.

These studies were carried out in collaboration with Dr. Sarah C. R. Elgin, Biology Department, Harvard University, Cambridge, Massachusetts.

123. INVESTIGATION OF THE SHELL ORGANIC MATRIX OF THE OYSTER CRASSOSTREA VIRGINICA

Investigator: Stephen Weiner*
Support: National Science Foundation

The organic material in shells present in close association with the “inorganic phase” is possibly involved in the mineralization process, the mechanism of which is as yet unknown. Furthermore, this organic material is present to some degree in the fossil record. An attempt is being made to characterize this material, especially the protein components, in order to learn more about the mechanism of mineralization, and if possible to compare living and fossil proteins.

The organic matrix comprises 1 to 2% by weight of the shell. The major high molecular weight components are protein and polysaccharide, which are covalently bound. The glycoprotein composes about 30% by weight of the matrix. The protein component is dominated by only three amino acids (Asp, Gly, Ser) in approximately equal proportions. An infrared spectrum indicates the presence of chondroitin sulfate among the polysaccharide components. There are an as yet undetermined number of glycoproteins present in this material. There are two shell layers present which are texturally distinct and contain different Mg contents. They also differ with respect to their amino acid compositions.

Hydrolysis of the organic matrix with dilute HCl and acetic acid cleaves on the N and C sides of Asp and Asn residues. Analysis of the products of this cleavage indicates that almost all the Asp and Asn residues are separated by either Gly and Ser or by di- and tripeptides of Gly and Ser. The carbohydrate appears to be covalently bound to the Ser (and possibly to Asn) associated with the Gly-Ser peptides and not the Ser bounded on both sides by Asp or Asn residues.

An attempt is now being made to isolate individual proteins in order to determine the primary structure of these glycoproteins.

The general sequence as indicated by the products of the partial acid hydrolysis suggests that the Asp residues present as every third residue might provide a regularly spaced charge necessary to hold Ca\(^{++}\) ions in a position such as to induce nucleation of the CaCO\(_3\).

*Division of Geological and Planetary Sciences, California Institute of Technology.
PUBLICATIONS


Research in psychobiology encompasses a spectrum of experimental interests concerned directly and indirectly with the neural bases of higher central nervous functions. Recent projects have been focused around problems in five general areas: (a) hemispheric specialization and functional organization in human commissurotomy, hemispherectomy and other neurosurgical patients; (b) mechanisms and pathways for visual processing and interhemispheric transfer and recall of visual information in cats and monkeys; (c) neural and behavioral plasticity after neonate brain surgery in kittens; (d) cerebral structures mediating memory consolidation, learning and motivation in brain-operated, newly hatched chicks; (e) embryonic plasticity and left-right specificity in the growth and developmental organization of visual and tactile nerve pathways in lower vertebrates. These different approaches are all concerned with various aspects of brain organization: its developmental patterning, its modification by experience, or its operational design and integrative principles.

124. A TEST FOR LANGUAGE DOMINANCE: EVOKED POTENTIALS FROM COMMISSUROTOMY AND NORMAL SUBJECTS

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

Evoked potentials were recorded from homologous sites (F7 and F8) over right and left frontal cortex in a search for electrophysiological correlates of speech processes that might be used to determine hemispheric lateralization of language. Tests were conducted on five commissurotomy patients (L.B., C.C., N.G., N.W. and R.Y.) and six normal individuals. All individuals were right handed as determined by the Harris Tests of Lateral Dominance. Brain activity was recorded as the subject was presented a word (white on black) or control flash (white area approximately equal to that of the sum of the area of the letters in a word) back-projected on a screen at a position 3° from a central fixation point for a duration of 100 msec. As the subject fixated a dot in the center of the screen a series of 40 stimuli was flashed in each of three experimental and four control conditions. In the experimental trials where the stimuli were words, the subject was instructed to immediately report the word aloud after each presentation.

Results show that evoked potentials obtained by computer averaging from the onset of the stimulus were distinctly larger from the left hemisphere of all commissurotomy patients and five of the six normal individuals when the word reported was in the right visual field. In addition, potentials obtained by averaging backwards from the response were larger from the left hemisphere of the normal subjects when two words, one in each field, were reported, and of all individuals when the word reported was in the right visual field.
125. DEFICITS IN 2-DIMENSIONAL TACTILE FORM PERCEPTION FOLLOWING COMMISSUROTOMY

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

Past studies of patients with unilateral brain lesions and with callosal section yielded mixed or conflicting evidence about asymmetry of higher order somatosensory function. In particular it was not known whether laterality effects on 2-dimensional tactile form perception were responsible for apparent dominance on more complex somesthetic tasks following commissurotomy.

Consequently, a tactile perception test of 2-dimensional geometric shapes was administered to complete and partial commissurotomy patients. The stimuli were palpated by one hand out of vision and the choice array was presented in either free vision or lateralized continuously to one visual field.

The results indicate first a general bilateral deficit in the complete commissurotomy patients relative to the partially sectioned subjects. Secondly, there occurred no consistent laterality effect for maximum deficit with half the patients exhibiting left and half right hand superiority. Rather, superiority on this test was exhibited for the hand ipsilateral to the hemisphere in which predominance of extracallosal damage is indicated by seizure patterns and EEG records. As expected there was a severe, though not complete, anomia for stimuli lateralized to the right hemisphere independent of modality (tactual and visual).

When tactile stimuli were palpated by one hand and the choice array restricted to the contralateral visual field, there occurred a general but asymmetric decrement (without complete failure) in performance, with the right visual field-left hand combination consistently surpassing the left visual field-right hand combination.

It follows that consistent laterality effects between subjects following commissurotomy on higher cognitive tests using somesthetically lateralized stimuli cannot be attributed to simple asymmetry in 2-dimensional tactile form perception. Bilateral deficit of completely vs. partially sectioned patients further suggests interhemispheric interaction by normal subjects on this task. Evidence is also adduced for substantial and perhaps asymmetric noncallosal compensatory mechanisms for integrating behavior following commissurotomy.

126. PHONETIC DISCRIMINATION BY THE RIGHT HEMISPHERE

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

As part of a continuing study of auditory language comprehension in the disconnected right hemispheres, binaural and dichotic tests for discriminating phonetic features of English consonants were administered to selected commissurotomy and hemispherectomy patients. The binaural test measured discrimination between consonant sounds differing in one, two or three of the following distinctive features: place of articulation, voicing, stop and nasality. A prerecorded tape presents single monosyllabic consonant-vowel-consonant (CVC) or CV words in quiet or with realistic distracting background noise, and the response consists of pointing to a plate lateralized to one visual field at a time (Biology 1972, No. 99) which depicts the target word as well as three distractor words differing from the target in one, two or three phonetic features.

Mean right hemisphere score on the Quiet subtest was about equal to that of a 5:5 (5 years and 5 months old) child and on the Noise subtest to that of a 4:7 child. In general the presence or absence of a given phonetic feature makes more difference for normals than for the right hemisphere which resembles children more than adults in this respect. With one minor exception the right hemisphere, just like normals, found plosives easier to discriminate than continuants and the presence of nasality or voicing harder to recognize than its absence, both in quiet and during noise. An analysis of right hemisphere confusions reveals the following hierarchy of competence (in order of decreasing
errors) for distinguishing words that differ in one consonant feature: nasality, voicing, stopping, and place of articulation. Further, the right hemisphere finds it only slightly harder to distinguish words that differ in one feature from words differing in two consonant features.

127. DEGREE OF SUPPRESSION OF THE IPSILATERAL PATHWAY IN DICHOTIC LISTENING IS A FUNCTION OF HEMISPHERIC SPECIALIZATION ON THE TASK

Investigators: Eran Zaidel
Support: National Institutes of Health, Public Health Service

It has been previously shown on normal subjects (Shankweiler and Studdert-Kennedy, 1967) that dichotic listening to synthetic consonant syllables yields a large and stable right ear advantage and that the effect is greater for consonant-vowel (CV) pairs differing in two articulatory features than for pairs differing on one. The effect is usually interpreted to reflect left hemisphere specialization for the processing of stop consonants on the assumption that the ipsilateral auditory pathways are largely suppressed during dichotic listening.

A tape containing dichotic pairs of nonsense synthetic stop consonants (CV syllables with C = p,b,d,g,t,k and V = a) was administered monaurally and dichotically to six commissurotomy patients who were required to say aloud what they heard. The CV pairs displayed contrasts in one or two phonetic features: place of articulation (labial, alveolar, velar) and voicing (present or absent).

A small right ear advantage (right ear = 63% correct, left ear = 56% correct) emerged even in the monaural condition, showing directly the functional dominance of the crossed (right ear-left hemisphere) over the uncrossed (left ear-left hemisphere) auditory projection system.

In the dichotic condition a small decrement in right ear score relative to monaural presentation (right ear = 50% correct) was accompanied by a massive left ear suppression (left ear = 14% correct). This verifies the occurrence of ipsilateral suppression in dichotic listening since in the commissurotomy patients callosal effects are excluded. Furthermore, left ear suppression was found to be three times greater in pairs which differ in both the place and voicing features (7% correct) than in pairs that differ in one feature alone (20% correct for voice or place). In view of Shankweiler and Studdert-Kennedy’s results it is suggested that the extent of suppression of the ipsilateral input in dichotic listening is proportional to the relative degree of specialization of the corresponding hemisphere for the stimuli and task in question.

Reference:

128. IS THE RIGHT HEMISPHERE MORE ARTISTIC THAN THE LEFT?

Investigators: Eran Zaidel
Support: National Institutes of Health, Public Health Service

Recent speculations about a special role of the right hemisphere in creativity and art have been put to a direct test by administering separately to left and right hemispheres of two commissurotomy patients a figure preference test which purports to measure aesthetic perception and judgment. The Graves Design Judgment Test (1948) was administered in continuously laterialized visual presentations and preferences were indicated by the hand ipsilateral to the stimulated visual field. The test “has been designed to measure certain components of aptitude for the appreciation and production of art structure ... by evaluating the degree to which a subject perceives and responds to the basic principles of aesthetic order—unity, dominance, variety, balance, continuity, symmetry, proportion and rhythm.” Each item consists of a pair or a triad of abstract designs of which only one was organized in accordance with all the principles mentioned. The test was constructed and normalized by reference to intersubjective agreement among art teachers and to preference by
Both hemispheres of both patients scored relatively low (e.g., below the first percentile of art, design and architecture students in Pratt Institute in New York) but the right hemisphere was equal to the left in one patient (at 42% correct) and inferior to the left in the second patient (33% vs. 46% correct). Thus the hypothesis that the right hemisphere is more artistic than the left was not supported on this measure.

Reference:

129. LATERALITY EFFECTS ON RAVEN’S COLORED PROGRESSIVE MATRICES ARE MODALITY-NONSPECIFIC

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

D. Zaidel and Sperry (1973) administered a modified unitactile-bivisual form of Raven’s Colored Progressive Matrices (RCPM) Test to a group of commissurotomy patients and found both hemispheres to perform above chance with the right hemisphere slightly superior to the left. It was not known whether the results are modality-specific to somesthesis or whether they will also generalize to purely visual presentations. It was also possible that apparent right hemisphere superiority was at least partially due to lateral asymmetry in simple 2-dimensional tactile form perception.

The RCPM was therefore readministered to two of the original commissurotomy patients in the usual manner except that the patterns with missing parts as well as the answers were lateralized separately to each visual field. This administration permitted unrestricted visual scanning of the stimuli and the subjects pointed to the answer with the hand ipsilateral to the stimulated visual field.

The results showed some bilateral increase in performance on the visual relative to the tactile form of the test, but the pattern of right-left differences remained unchanged with the right hemisphere again being slightly superior. It is concluded that the dominance pattern in RCPM is modality-nonspecific and that it reflects a central cognitive ability independent of tactile form perception (see also Biology 1974, No. 125).

Reference:

130. DYSPRAXIA, DYSGRAPHIA, AND DYSOCOPIA: LONG-TERM EFFECTS IN COMMISSUROTOMY PATIENTS

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

Left-sided dysgraphia (inability to write) and dyspraxia (inability to carry out spoken commands), and right-hand dyscopia (inability to copy visual patterns) have been observed to follow forebrain commissurotomy and appear particularly marked shortly after surgery. However, these symptoms tend to subside with recovery of bilateral motor control during the first year or so and have been presumed to be largely transient. The extent to which these impairments might still be detected many years after surgery was investigated in conjunction with other motor studies by administration of a series of conventional clinical apraxia tests to 6 long-term patients. The results revealed persistence in all but one of the patients of definite symptoms of dysgraphia, dyspraxia, and dyscopia even in cases with minimal extracallosal damage and more than 10 years of recovery. The results seem to be attributable to interhemispheric interference effects involving hemispheric dominance as much as direct functional failure.
131. DEFECTS IN STEREOPSIS FOLLOWING COMMISSUROTOMY AND HEMISPHERECTOMY

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

Preliminary checks for stereopsis were made in six commissurotomy and two hemispherectomy (one left and one right) patients by using two tests of binocular disparity: Julesz random dot patterns and the Keystone Navy Stereopsis Test. The Julesz patterns were first viewed in color through red-green filters and later in black and white in a stereoscope. The stereoscope was also used for the Keystone card. In each test subjects had to report verbally or to draw the figure appearing in relief.

The two hemispherectomy cases failed to perceive depth on all these tests. The commissurotomy cases failed, for the most part, on the colored Julesz stereograms, but generally not on the black and white version or on the Keystone test. None of the patients tested were color-blind as verified by the Ishihara Color-Blindness Test. The results are taken to involve a disturbance in balance between ipsilateral and contralateral optic neural systems and direct functional deficits near the vertical midline.

132. OBSERVATIONS ON RETINAL RIVALRY IN COMMISSUROTOMY AND HEMISPHERECTOMY PATIENTS

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

In conjunction with other tests, six patients with surgical section of the forebrain commissures and two cases with left and right hemispherectomy were tested for retinal rivalry. Through a standard stereoscope they viewed simultaneously two different images, one in each eye, both occupying the same apparent position in space. The subjects were asked to describe their perceptions verbally for 10 different pairs of such images. No time limits were imposed and the trials were carried out under fluorescent lighting.

Whereas normal control subjects were able to describe consistently both left and right images, the surgical patients showed suppression of the image from one eye. This occurred on every trial in two patients (one hemispherectomy, one commissurotomy) and on two to four trials in the remaining five patients. In the commissurotomy subjects it was the image of the left eye that was suppressed while the hemispherectomy cases suppressed the eye contralateral to the remaining hemisphere. When asked to close one eye and then the other, all patients were always able to report the exposed image correctly with either eye.

133. LEARNING ABILITY OF SPLIT-BRAIN MONKEYS

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

Whether the capacity of animals to learn is impaired by cutting the forebrain commissures is unclear; the existing contradictory reports have been based largely on poorly controlled experiments with cats. Therefore we decided to carefully compare the learning ability of initially naive split-brain, partially split-brain, and normal monkeys on identical series of visual pattern discriminations taught monocularly at various times after surgery.

For the first few weeks after surgery, the split-brain monkeys took about 15 times as many trials as the normal monkeys to learn discriminations. After about two months this ratio dropped to about 7; after one year it was less than
The split-chiasm monkeys initially took about 3 times the normal number of trials, indicating that the hemianopia or lateralization of visual input contributes to, but does not explain, the split-brain deficit. After one year the learning rates of the split-chiasm monkeys were indistinguishable from those of the normals. Monkeys split except for the posterior end of the corpus callosum (splenium intact) learned as easily as split-chiasm monkeys, which demonstrates that nonspecific effects of the operation are not the cause of the split-brain deficit.

The most probable explanation for the large, initial split-brain deficits in visual learning is the partial denervation of neurons in the visual cortical areas interconnected by the splenium. The compensatory changes occurring in the succeeding weeks would then reflect recovery of more normal neural function, possibly associated with axonal sprouting and the reestablishment of a normal complement of synaptic contacts. That denervation of visual rather than inferotemporal cortex is specifically responsible for this initial learning deficit is suggested by the preliminary finding that monkeys split except for the anterior commissure, which like the splenium allows interocular transfer, learn at rates similar to split-brain rather than to splenium-intact monkeys.

The residual long-term deficits in learning, if proven reliable, are presumably attributable to the cerebral disconnection per se, which prevents the trained hemisphere from utilizing neural circuitry in the opposite visual cortex. Since these circuits are not primarily concerned with the visual hemifield involved in the training procedure, their function may be considered as a generalized one, in keeping with Lashley’s principle of Mass Action.

134. BILATERAL MEMORIES IN SPLIT-BRAIN MONKEYS

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

It has been suggested recently that the establishment of bilateral memories in the brain is the exception rather than the rule. The corpus callosum, then, would normally allow either side of the brain access to unilateral memories. This idea is contrary to prevailing opinion, although critical tests are not available.

Most of the monkeys that learned problems in the preceding experiment (Biology 1974, No. 133) were subsequently completely split and each hemisphere tested for retention. All problems were taught monocularly to the normal, split-chiasm, and splenium-intact monkeys. After split-brain surgery, the two hemispheres performed nearly equivalently, although not perfectly. Whether memories were present in the two hemispheres depends on interpretation. While split-chiasm and splenium-intact subjects took far fewer trials to reach criterion after being split than they originally took to learn the problems, those monkeys trained as normal subjects often took longer to “remember” the problems than they took to initially learn them. This can be explained at least partly by the findings reported in the preceding abstract: (1) the normals originally learned in fewer trials than the operates, so that, relatively speaking, it is harder for them to perform better after surgery, and (2) the normals turned split are performing after more extensive surgery than the partial splits turned split, which might have transient effects. When the relearning by the three groups of monkeys is compared to split-brain controls which only learned the problems postoperatively, all groups show significant memories. Therefore it seems that bilateral memories are present in all cases, although the memories may be stronger in animals trained with the optic chiasm already split.
HEMISPHERIC SPECIALIZATION IN MONKEYS

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

Previous reports (Biology 1972, No. 115; Biology 1973, No. 42) have indicated a possible specialization of the left cerebral hemisphere of monkeys for perceiving spatial relationships. Evidence for a similar advantage in the perception of facial characteristics, a strongly lateralized function in human subjects, has been less convincing. We are now studying the abilities of each hemisphere to discriminate photographs of monkeys in a paradigm that emphasizes the formation of concepts. Many photographs of the same monkey are taken, intermingled with similar photographs of another monkey, and presented sequentially. The subject's task is to respond to all photos of one monkey and ignore the photos of the other monkey. Such concurrent discriminations are very difficult if all stimuli must be learned individually, but rather easy if positive stimuli can be ascribed to one class and negative stimuli to another. To date four split-brain monkeys have learned two or three such discriminations with each hemisphere. The median trials-to-criterion was 130, which is surprisingly quick and indicates that a single concept was used for coding the stimuli. No significant trends, however, have yet emerged with respect to a dominant hemisphere for this task. Several other classes of stimuli will also be investigated with this technique, such as orientation, perspective cues to depth (with S. B. Tieman), and the concept of number (with E. Zaidel).

HEMISPHERIC SPECIALIZATION IN CATS

Investigators: Carol K. Peck*, Charles R. Hamilton
Support: National Institutes of Health, Public Health Service

There have been several inconclusive reports of cerebral dominance in cats. According to previous investigators, about half of all split-brain cats tested have a dominant right hemisphere and half have a dominant left hemisphere. This raises the possibility that surgical trauma might have produced the apparent hemispheric superiority. If it were possible to predict prior to surgery which hemisphere should be dominant, or to find differential superiorities for different classes of stimuli, this objection would not hold. We are pursuing the question by taking advantage of a possible relationship between paw preference and cerebral dominance, i.e. the hemisphere ipsilateral to the preferred paw has been reported to be superior in the retention of a preoperatively learned visual task. We are testing cats on visual discriminations which differ in orientation, since similar tasks have revealed hemispheric superiority in monkeys (Biology 1973, No. 42). In the one cat tested to date, the hemisphere ipsilateral to the preferred paw required only about half as many trials to learn discriminations between oriented lines as were required by the contralateral hemisphere. Other visual tasks did not reveal such a hemispheric advantage. This technique therefore seems promising, and further cats will be tested with the same method.

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INTEROCULAR TRANSFER OF BRIGHTNESS DISCRIMINATIONS BY SPLIT-BRAIN CATS

Investigators: Carol K. Peck*, Charles R. Hamilton, Melinda Gold**
Support: National Institutes of Health, Public Health Service

Previous investigators have reported complete interocular transfer of simple, suprathreshold brightness discriminations in chiasm-callosum sectioned cats. This transfer has been ascribed to
midbrain commissures but the evidence is inconclusive because the anterior commissure was intact in those cats that transferred. Because monkeys show interocular transfer of visual problems via the anterior commissure, we decided to investigate the possible role of the anterior commissure in such interhemispheric communication. Surprisingly, we have been unable to replicate the original reports of complete transfer in the chiasm-callosum sectioned cat. Furthermore, the amount of transfer is also low although apparently greater than zero in four cats with additional section of the anterior commissure. These data cannot be interpreted clearly until we show good transfer in surgical controls, but they appear to cast some doubt on the importance of midbrain pathways for interhemispheric communication in split-brain cats.

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138. DISCRIMINATION OF MOVEMENT BY SPLIT-BRAIN MONKEYS

Investigators: Suzannah Bliss Tieman*, Charles R. Hamilton

Support: National Institutes of Health, Public Health Service

Split-brain monkeys do not transfer pattern discriminations interocularily, in keeping with the forebrain localization of pattern perception. The midbrain roof contains neurons highly sensitive to movement, and some behavioral data point to this area as a place for movement perception. If so, then interocular transfer of discriminations of direction of movement should occur through the midbrain commissures (Biology 1972, No. 117; Biology 1973, No. 41).

This has now been tested in monkeys with the optic chiasm and forebrain commissures sectioned, and in control monkeys with (1) additional section of the midbrain commissures, or (2) just the optic chiasm split. To date one split-chiasm control transfers perfectly and one midbrain split does not transfer at all, as expected. Eleven forebrain-split monkeys have shown an average savings score based on trials to criterion (1st - 2nd
\[\frac{1st}{} - \frac{2nd}{1st} \times 100\]) of 20%, indicating partial transfer. Statistical evaluation of this result, which is complicated by the additive effects of cerebral dominance, await the completion of the balanced design, but certainly the amount of transfer is not large, indicating that cerebral mechanisms are predominant.

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139. INTERHEMISPHERIC COMPARISON BY SPLIT-BRAIN MONKEYS

Investigators: Charles R. Hamilton, Suzannah Bliss Tieman*

Support: National Institutes of Health, Public Health Service

Split-brain monkeys have been reported to learn to match one of two stimuli presented to one hemisphere to a sample stimulus presented to the other hemisphere. It has been suggested that these matches are made via the midbrain commissures. In our experience, it is difficult and sometimes impossible to train split-brain animals to perform these interhemispheric matching-to-sample tasks. Furthermore, in cases where such performance is established, we have found that additional section of the midbrain commissures has no effect, demonstrating that the performance does not depend on midbrain pathways. Finally, we have now shown that, in some cases, the matches are not based on visual comparisons. For example, if a monkey can perform matches with blue and yellow stimuli, and also with patterned stimuli such as a plus and circle, then it may sometimes immediately “match” a blue stimulus to a plus—clearly not a visual match. This behavior can be explained if one hemisphere makes some differential response to the sample stimuli which can be observed by the other hemisphere. Whenever these responses are the same for two stimuli, such as blue and plus, then
"matches" occur. Such responses could be overt, e.g. postural changes, or covert, e.g. neural or hormonal changes; they could be intentional or not. This kind of mechanism can also account for the variability found in learning interhemispheric matching-to-sample tasks since differential responses must first be made by one side and then decoded by the other, an unpredictable process at best.

140. FUNCTIONAL PLASTICITY IN SPLIT-BRAIN CATS

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

Behavioral studies on adult cats with surgical section of the corpus callosum and optic chiasm have shown a loss of ability to transfer information from one hemisphere to the other. Pattern discrimination problems learned through one eye by the corresponding hemisphere cannot be performed when presented to the other eye, but must be relearned. Intercocular transfer of pattern discriminations does not improve with either practice or time if the surgery is done on adult cats.

Young animals, however, can often compensate for lesions that produce chronic impairment in the adult. Accordingly, in the present experiment the corpus callosum was surgically sectioned in a series of kittens in the first week after birth. About a year later the optic chiasm was also sectioned and behavioral testing started. Although histology is not yet available the initial results indicate essentially normal transfer of pattern discrimination problems in these cats. After completion of the testing, a series of further surgeries will be undertaken to section the anterior and other commissures in an attempt to localize the fiber tracts compensating for the sectioned corpus callosum.

141. EFFECTS OF UNILATERAL CYCLOHEXIMIDE ON INTERHEMISPHERIC TRANSFER OF MEMORY IN CHICKS

Investigator: Karen F. Greif
Support: National Institutes of Health, Public Health Service

Cycloheximide, a protein synthesis inhibitor, has been demonstrated to prevent long-term memory consolidation for one-trial passive avoidance in baby chicks (Mark and Watts, 1971). This task has further been shown to transfer interocularly after monocular training (Cherkin, 1970). A study is presently under way to determine if unilateral inhibition of protein synthesis by cycloheximide prevents interhemispheric transfer of the avoidance task.

A technique has been developed to lateralize drugs to one hemisphere by surgically implanting a small piece of drug-impregnated filter paper on the surface of the forebrain. One hour after surgery the chick is trained monocularly to withhold pecking at a shiny metal lure by coating the bead with methyl anthranilate, an aversant liquid. Transfer is tested at 20 minutes (short-term memory) or at 24 hours (long-term memory) by occluding the trained eye and testing the naive eye.

Preliminary results indicate that transfer occurs during short-term memory, but that long-term memory fails to develop, as evidenced by retention deficits at 24 hours but not at 20 minutes if the treated hemisphere is initially trained. This amnesia is not due to drug diffusion from the treated hemisphere; training and testing the untreated hemisphere results in no loss of memory. Transfer is successful at 20 minutes and at 24 hours if the untreated hemisphere is initially trained and the treated hemisphere tested.

These data suggest that while the untrained hemisphere can draw on the short-term memory trace in the treated and trained hemisphere, a long-term memory cannot be established in the absence of consolidation on the trained side. However, the treated hemisphere can draw on the long-term memory store of the untreated and
trained hemisphere. Further experiments are needed to determine whether bilateral memory is established after the effect of cycloheximide wears off, allowing the treated hemisphere to establish new long-term memory traces.

References:

142. BEHAVIORAL MEASURES OF FUNCTIONAL RECOVERY OF COMPRESSED RETINOTECTAL PROJECTIONS IN GOLDFISH

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

After ablation of the caudal half of the optic tectum in juvenile goldfish, it has been shown by electrophysiological mapping techniques that the entire visual field is compressed onto the remaining rostral tectum (Gaze and Sharma, 1970; Yoon, 1971; Meyer, 1974). The present experiment sought by means of conditioned responses to determine whether the axon terminals in the tectum from which electrophysiological recordings are made were functional behaviorally.

In a Pavlovian conditioning paradigm, red and green lights were paired with an electrical shock while the animal's respiratory rate was monitored. Depression of respiratory rate during presentation of the positive stimulus indicated learning of the discrimination task. The goldfish were immobilized, and lights mounted on a perimeter were presented without shock to various locations in the visual field during testing sessions.

Behavioral mapping 8 to 14 months after excision of the caudal tectum shows gradual recovery of vision to the entire visual field in 3 of the 4 fish tested so far. These results strongly indicate that the reorganized projection from the retina involves functional connections with tectal cells.

References:

143. FURTHER INVESTIGATIONS ON THE PROBLEM OF LEFT-RIGHT SPECIFICITY OF CUTANEOUS NERVES

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

The studies of Franzisket (1959) on selective cutaneous reinnervation in frog were highly suggestive of the existence of left-right cytochemical differentiation of cutaneous nerves. Attempts to support Franzisket's behavioral results by electrophysiological methods were unsuccessful (Biology 1972, No. 126). The intent of the present experiments was to test further for the existence of lateral specificity employing both behavioral and electrophysiological techniques.

The regenerated cutaneous fields of the medial rami of the dorsal cutaneous nerves were mapped after a variety of surgical manipulations including reciprocal crossing of the second dorsal cutaneous nerves, deflection of nerves of opposite laterality into a common denervated cutaneous field, and 180° rotation of an innervated skin patch. Behaviorally reactive zones were plotted on an enlarged drawing of each frog and were compared to receptive fields mapped electrophysiologically from the same individual. The terminal fields of the regenerated nerves were shown electrically to overlap extensively with those of nerves from the opposite side of the body. Ipsilateral and contralateral wipe responses were intermixed in the regions of dual innervation from the two sides of the body in all cases except for those in group 2 where left and right nerves competitively reinnervated a denervated skin patch. In this group, the regions eliciting ipsilateral and contralateral responses were clearly
demarcated although electrophysiology showed that the two nerves overlapped extensively. Behavioral testing after removal of one or the other nerve, however, showed that each nerve was capable of mediating a wipe response in the whole of the electrically active region. The extent of the reflexogenous zones of the regenerated nerves was almost as large as that mapped electrophysiologically, in all cases. The results of the present experiments confirm those reported earlier (Biology 1972, No. 126) and fail to provide any evidence for the existence of cytochemical differentiation between cutaneous nerves from the left and right sides of the body.

Reference:

144. INTEGUMENTAL SPECIFICATION OF SENSORY NERVES

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

The question of whether neurons in the peripheral nervous system acquire functional specificity from motor and sensory end organs remains unsettled. Evidence of the past several years has cast doubt on the existence of myotypic specification of motor neurons by their muscles in that the recovery of muscular coordination after motor nerve section in lower vertebrates is now shown to be a result of selective reinnervation. In view of the above, experimenters (Sklar and Hunt, 1974) are questioning also the presence of integumental specification of cutaneous nerves. The present experiment is a further attempt to investigate the problem using techniques similar to those of Miner (1956).

The tip of the right hindlimb bud of *Rana pipiens* tadpoles was severed and transplanted to the right side of the dorsal trunk region in early larval stages (III-VI). *R. pipiens* were selected because of the good cutaneous localizing responses they exhibit after metamorphosis. Some 10 of 24 tadpoles that survived the operation underwent a second operation removing the hindlimb dorsal root ganglia on the right side at stage XI to eliminate the possibility of any selective innervation of the graft from hindlimb segments.

One-half of the successfully grafted limbs developed into normal-looking hindlimbs though without active movement. Among the remainder, some limbs failed to acquire pigmentation and others did not develop a full complement of limb joints. All, however, developed digits and all acquired sensory innervation. Stimulation of the transplanted limb after metamorphosis resulted in wipe reflexes by an ipsilateral limb in all cases. Responses evoked from the proximal limb segments of the grafted appendage were generally classifiable as thoracic responses. Stroking of the digits, however, resulted in characteristic limb responses by either the ipsilateral forelimb in cases where the dorsal root ganglia were intact or the hindlimb where the ganglia had been excised. Apparently in conformance with the concept of integumental specification, the thoracic nerves under the influence of the distal limb integument had been induced to form limb reflex associations in the spinal centers.

References:

145. TECTAL RETINOTOPIC ORDER OF REGENERATING OPTIC NEURONS IN GOLDFISH

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

Ganglion cells of the goldfish retina connect in topographic fashion with the contralateral optic tectum, the primary visual center of lower vertebrates. Small electrical lesions of the retina produced, as expected, a corresponding denervated tectal zone that was easily visualized by
autoradiographic techniques. Similarly, incisions through optic fibers which transverse the tectum in a near parallel manner result in denervation behind the cut. In both cases the area of denervation was very sharply defined and virtually label-free. By contrast, if the optic nerve was previously severed and allowed to regrow onto the tectum, retinal lesions at first produced no apparent deficit but at longer periods an appropriate denervated zone gradually appeared. However, even at 149 days after nerve interruption fall-off of label occurred over 100
to 200 µ instead of the normal 50 to 100 µ and within this zone light labeling characteristic of fibers of passage could be seen. The denervation produced by tectal incisions was much less marked than normal and showed no improvement with time. Although fairly accurate termination is eventually achieved, there seems to be significant exploratory growth initially, and some fibers appear to grow through abnormal tectal routes to terminate at their correct locus.

146. VISUAL PROJECTION ONTO THE GOLDFISH HALF-TECTUM

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

The retinotopic projection of eye to tectum has been reported to be “compressed” onto a surgically reduced half-tectum following optic nerve section and regeneration by Gaze and Sharma (1970) and confirmed by others since. Until now the somewhat inaccurate eye-in-air electrophysiological method has been used in these studies. A more refined eye-in-water technique recently developed here (Biology 1973, No. 35) shows that initially a relatively uncompressed map is established by the regenerating fibers. This is gradually followed by a more compressed projection, but even at 214 days after surgery evidence for a deficit could be seen. Previously we have shown that in the tree frog the same kind of compression onto a half-tectum does not occur (Meyer and Sperry, 1973). The present results suggest there may not be a basic difference in the mechanism of selective reinnervation between these species but rather some additional factor might be operating in goldfish to eventually permit a partial “compression.”

References:

147. GROWTH OF THE RETINOTECTAL SYSTEM IN JUVENILE GOLDFISH

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

Mitotic growth in the retina and tectum of juvenile goldfish 45 to 90 mm in length was investigated because of the implications for functional plasticity in fiber connectivity patterning. Tritiated thymidine was injected intraperitoneally, and autoradiography of serial sections carried out at periods ranging from 6 hours to several months later. Both retina and tectum were found to be acquiring substantial numbers of new cells, but the pattern of labeled cells in the two was different. The retina was enlarging with radial symmetry by annular growth at the ora serrata. By contrast the concurrent tectal growth was asymmetric occurring primarily at the caudal, medial, and lateral margins but was negligible rostrally with substantial inward migration and was largely, if not entirely, glial. Preservation of the normal topographic retinotectal projection during this growth mismatch requires a gradual inward shift of the projection map like that inferred for amphibian larvae (Gaze et al., 1974). Whereas concomitant function in the amphibian is questionable, in the juvenile goldfish this process must involve plastic changes in neuronal connectivity within a functioning system. It is
suggested that these same growth processes may largely account for previously reported abnormal compression of the retinal map onto the surgically reduced tectum of goldfish.

Reference:

PUBLICATIONS


A protein which relaxes superhelical DNA has been purified extensively from cultured mouse (LA9) and human (HeLa) cells. This activity, which we call superhelix relaxation protein (SRP), was first identified in crude lysates of the nuclei of cultured mouse embryo cells by Champoux and Dulbecco (1972) and is similar to the E. coli $\omega$ protein of Wang (1971).

The purification of SRP was started with the eventual goal of constructing an in vitro DNA replication system using D-loop-containing mitochondrial DNA from mouse cells. This DNA can be isolated as an already primed closed circular duplex. The size of the primer has been postulated by Kasamatsu et al. (1971) to result from a superhelical restriction on further synthesis.

SRP has currently been purified about 500-fold, using ammonium sulfate precipitation, DEAE-cellulose chromatography and Sephadex G-150 filtration. The activity is assayed with closed supercoiled PM2 DNA as a substrate. The conversion to the relaxed closed form of this DNA is assayed either by centrifugation in buoyant CsCl containing ethidium bromide (EtBr) or by a fluorometric method which measures the fluorescence enhancement of EtBr bound to DNA. Both assays are based on the principle that the amount of EtBr bound to circular DNA is a function of the superhelical density. The latter assay makes it possible to define the activity of SRP quantitatively and to study the kinetics of the reaction.

SRP is active both on negatively supercoiled DNA and on positively supercoiled DNA generated by a suitable concentration of EtBr. The protein is active between pH 7.0 and 8.5. It requires either 0.2 M NaCl or 40 mM MgCl$_2$. The molecular weight estimated by gel filtration is about 80,000 to 90,000. The sedimentation coefficient estimated in glycerol gradients is about 4.1. The reaction is inhibited by PCMB. No cofactor requirement is known.

The mechanism of action is under investigation. An endonucleolytic nicking and subsequent rescaling cycle is postulated. The biological function of SRP is not as yet established. It may be involved in DNA replication, relaxing local supertwists induced by unwinding of the replicating strands ahead of a replication fork.

References:
virus population. We have sought to more closely define the parameters of the infection process that are responsible for this effect. It has been found that when a nondefective (passaged at low multiplicity) or a plaque-purified virus stock is used for the infection, the superhelix density of the resulting intracellular DNA is homogeneous with a mean value identical to that of DNA extracted from purified virus. This effect is independent of the multiplicity of infection. However, when a virus stock which has been serially passaged at high multiplicity and is known to contain a high concentration of defective virus particles is used for the infection, the resulting intracellular DNA is heterogeneous in superhelix density and has a mean value lower than that of DNA extracted from purified virus. As above, this effect is independent of the multiplicity of infection. These results suggest that the important parameter is the ratio of nondefective to defective DNA in the infecting virus population, and this, in turn, suggests that a viral function is involved in the supercoiling of SV40 DNA.

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150. DENATURED REPLICATIVE INTERMEDIATES OF SV40

Investigators: David Tang, Jerome Vinograd

Support: National Institutes of Health, Public Health Service

Naturally occurring closed circular DNAs have a limited number of topological turns which are present because of a small deficiency of net turns in the double-stranded molecules. A new set of molecules having greatly increased turn deficiencies has been prepared from SV40 replicative intermediates (RIs) with closed parental strands. These RIs are nicked and closed frequently as replication proceeds with a consequent reduction of net turns. When the progeny strands are removed from the closed RIs by heating, grossly underwound molecules are generated.

In a CsCl gradient containing 5 µg/ml ethidium bromide (EtdBr), the heat-treated RIs were less dense than closed viral DNA and nuclear DNA. The heated RIs with varying degrees of underwinding (derived from molecules with varying degrees of replication) banded differentially in such a low EtdBr gradient. Three fractions spanning the range of 15 to 90% underwinding were isolated.

The underwound circles in the above fractions were resistant to the action of superhelix relaxing protein (SRP) while an internal control of native SV40 DNA was relaxed.

The heated RI fractions were irreversibly denatured at pH 13 and neutralized. In propidium diiodide-CsCl gradients, they formed bands between single-stranded SV40 DNA and denatured viral DNA. The dependence of band position on the extent of turn deficiency is currently under investigation.

151. DNA LIGASE FROM HELE CELL MITOCHONDRIA

Investigators: David Tang, Jerome Vinograd

Support: National Institutes of Health, Public Health Service

An ammonium sulfate extract of sonicated HeLa mitochondria was capable of joining internal nicks placed in T7 DNA by pancreatic DNase. Either ATP or NAD was necessary to demonstrate this enzymatic activity. $^{32}$P-AMP NAD was bound to the extract and was released when NMN was added as indicated by the behavior of acid-insoluble $^{32}$P-radioactivity. These results suggest an initial cofactor binding step similar to the E. coli ligase.

Since the nuclear DNA ligase in rabbit bone marrow was found to be dependent on ATP only (Lindahl and Edelman, 1968), we suspect that the ATP-dependent joining activity in our HeLa mitochondrial extract is a nuclear contaminant,
and that the NAD-dependent ligase is the mitochondrial enzyme. We are at present attempting to separate the two joining activities and distinguish their locations in the cell.

152. SV40 DNA-RNA TRANSCRIPTION COMPLEXES LATE IN INFECTION

Investigators: David Tang, Jerome Vinograd
Support: National Institutes of Health, Public Health Service

Simian virus 40 (SV40) DNA-RNA transcription complexes late in infection were isolated from monkey kidney primary cells lysed with a detergent. In a neutral sucrose gradient, the material sedimenting faster than the replicative intermediates (RIs) was pooled and examined in the electron microscope by the formamide technique. The fields contained circular DNA with two or more attached "bushes." These bushes were removable by prior treatment with RNase or phenol. In order to linearize the bushes, the complexes were treated separately with glyoxal, with high concentrations of formamide, or with T4 gene 32 protein followed by glutaraldehyde fixation. Only the glyoxal technique extended the bushes, which formed linear single-stranded RNAs shorter than genome length. It is planned to map the initiation site(s) of these growing transcripts relative to a unique cut introduced by a DNA restriction endonuclease.

153. DENATURATION OF COVALENTLY CLOSED SUPERHELICAL CIRCULAR DNA

Investigator: Harumi Kasamatsu
Support: National Institutes of Health, Public Health Service

When simple closed circular SV40 DNA molecules are examined by a modified Kleinschmidt technique in the presence of formamide, SV40 DNA molecules with a single-stranded denaturation loop were seen at frequencies of about 5%. They were absent in the open circular DNA population. The denatured region accounted for approximately 4% of the contour length in SV40 DNA. Preliminary studies of covalently closed circular DNA species, PM2, λ, and mitochondrial DNA with high superhelical turns have indicated that these denaturation loops arise as a result of the early melting of the superhelical DNAs having negative superhelical turns. The removal of the superhelical turns results in a relaxed circular conformation and the formation of an unwound duplex region localized in a presumed AT-rich region.

154. MITOCHONDRIAL DNA REPLICATION IN SEA URCHIN OOCYTES

Investigators: Lloyd Matsumoto*, Harumi Kasamatsu, Lajos Pikó*
Support: National Institutes of Health, Public Health Service

During oogenesis the sea urchin oocyte undergoes rapid cytoplasmic growth, with a proportionate increase in the number of mitochondria. We have analyzed the pattern of mitochondrial DNA (mtDNA) replication in growing oocytes of Strongylocentrotus purpuratus.

Mitochondrial DNA replicative intermediates from S. purpuratus oocytes were isolated by ethidium bromide-CsCl density gradient centrifugation and examined by electron microscopy after formamide spreading. In some experiments, the mtDNA was radioactively labeled by exposing isolated oocytes to 3H-thymidine. Oocyte mtDNA replication appears to follow the displacement loop
model outlined in mouse L cells. There are differences in detail. The frequency of D-loop DNA is much lower in oocytes, suggesting that the relative holding time at the D-loop stage is shorter. Duplex synthesis on the displaced strand occurs early and with multiple initiations. The frequency of totally duplex replicative forms, or Cairns forms, is the highest reported for mtDNA. The differences may be related to the fact that oocyte mtDNA replication occurs in the absence of cell division and need not be coordinated with a cell cycle. Molecules with expanded D-loops banded in the intermediate region between the lower and upper bands in an ethidium bromide-CsCl gradient, supporting the notion that displacement replication proceeds on a closed circular template which is subject to nicking-closing cycles. In mature sea urchin eggs, replicative forms are absent and virtually all the mtDNA is stored as clean circular duplexes; the mtDNA remains in this form during early development at least up to the early pluteus stage. In the course of this work some novel structural variants of superhelical circular DNA (molecules with denaturation loops and double-branch-migrated replicative forms) are reported.

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155. RESTRICTION ENDONUCLEASE MAPPING OF ANIMAL MITOCHONDRIAL DNAs USING THE ELECTRON MICROSCOPE

Investigators: Wesley M. Brown, Jerome Vinograd

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

Mitochondrial DNAs (mtDNA) from established cell lines of human (HeLa), African green monkey (BSC-1) and mouse (LA9) origin have been cleaved by the restriction endonucleases Eco RI and Hinf III. Each enzyme recognizes a different specific nucleotide sequence six base pairs long and makes a cut within the sequence in each strand (Smith and Nathans, 1973). The resulting fragments have been sized and their order relative to each other determined by analyzing molecular lengths from partially digested material. The origin and direction of replication has been located on the maps by analysis of D-loop and expanding D-loop-containing fragments. In all cases examined, one end of the D-loop remains fixed relative to the restriction fragment ends, whereas the other end of the D-loop is positioned according to the degree of expansion of the D-loop. This result has allowed us to construct physical maps which orient the restriction sites with respect to the origin and direction of replication. With these maps, it should be possible to orient subsequent positional information, such as the position of transcribed RNAs, promoter regions and fragments obtained with other restriction enzymes, relative to the site of origin of replication for these mtDNAs. Such studies are under way.

Reference:

156. FURTHER MAPPING OF HEla AND LA9 MITOCHONDRIAL DNAs WITH RESTRICTION ENDOUCLEASES

Investigators: Wesley M. Brown, Lawrence I. Grossman, Jerome Vinograd

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

The physical maps of HeLa and LA9 mitochondrial DNA (mtDNA) constructed with the restriction endonucleases Eco RI and Hinf III (see Biology 1974, No. 155) are being extended and refined using Hin dIII, Hpa I, Hae and Eco RII (for terminology, see Smith and Nathans, 1973).
The combined use of these enzymes will provide many additional sites on the map. The refined map will allow more accurate positional assignments of replicative, transcriptional and regulatory loci. In addition, selected fragments, such as those containing the initiation region for DNA replication, can be isolated for nucleotide sequence determination.

The specific restriction fragments are highly labeled in vitro and can be isolated in good yield using an agarose gel preparative electrophoresis method developed by Amy Lee, Caltech Division of Biology (Lee and Sinsheimer, in press).

References:

157. ARE THE RIBONUCLEOTIDES IN MITOCHONDRIAL DNA AT UNIQUE LOCi?

Investigator: Lawrence I. Grossman
Support: National Institutes of Health, Public Health Service

Mature mouse and human mitochondrial DNA (mtDNA) have been shown to contain covalently inserted ribonucleotides, thus explaining their well-known sensitivity to alkali. There is evidence for two populations of molecules: those containing a maximum of about 10 ribonucleotides per DNA duplex, and those containing a maximum of about 30. Whether or not they are located at unique sites on the genome is likely to be important in helping to elucidate their origin and possible functional role.

The location of the ribonucleotides is being investigated during the construction of a restriction endonuclease map. Two primary approaches are being developed. One is the specific labeling of ribonucleotide sites, followed by digestion with a restriction enzyme and analysis using polyacrylamide gel electrophoresis. The other is the scission of the DNA duplex at the site of a ribonucleotide, again followed by gel electrophoresis.

158. IN VITRO LABELING OF RIBONUCLEOTIDES IN HEla MITOCHONDRIAL DNA

Investigators: Paul John Flory Jr., Jerome Vinograd
Support: National Institutes of Health, Public Health Service

A recent study has shown that the rate of alkali nicking of mitochondrial DNA (mtDNA) corresponds to that expected if each molecule contains 10 to 30 ribonucleotides (Grossman et al., 1973). This study also demonstrated the existence of at least one RNase H-sensitive site in the mtDNA. The alkali-sensitive sites appear to be scattered on both strands in about 10 locations, as indicated by the number average molecular weight of a limit alkali digest.

In an effort to find out whether all the alkali-sensitive sites are ribonucleotides, and to determine which ribonucleotides are involved, a method for labeling ribonucleotides in mtDNA has been developed. A limit alkali digest of mtDNA treated with alkaline phosphatase and oxidized with periodate is reduced with high specific activity 3H-sodium borohydride. The mtDNA is then digested with spleen phosphodiesterase to release the 5'-terminal groups as nucleoside derivatives. Two-dimensional chromatography on thin-layer plates followed by fluorography allows the separation and identification of labeled nucleosides (Randerath and Randerath, 1971).

References:
159. AMPLIFICATION OF MITOCHONDRIAL DNA OF ANIMAL CELLS BY E. COLI

Investigators: Wesley M. Brown, Herbert W. Boyer*, Jerome Vinograd

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

The construction of biologically active bacterial plasmids in vitro has been reported (Cohen et al., 1973). E. coli cells, transformed with an in vitro constructed plasmid that contains both of the ribosomal RNA cistrons from the frog, Xenopus laevis, have been shown via DNA-RNA hybridization to transcribe the Xenopus cistrons (Morrow et al., 1974). In a collaborative effort, we have transformed E. coli with reconstructed plasmids which contain all or a specific part of the mitochondrial DNA (mtDNA) of both HeLa (human) and LA9 (mouse) cells. Studies of the structure of the in vitro recombinants and the fidelity of replication, transcription and translation of mtDNA in E. coli are in progress. If replication proves faithful, this method will allow the production of large quantities of animal DNAs and the performance of experiments which are presently impossible due to the lack of sufficient quantity of material. The experiments are viewed as part of the development of procedures for the incorporation of specific genes into eukaryotic cells.

References:

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160. MAMMALIAN CELL CYCLE ANALYSIS BY CULTURE FRACTIONATION

Investigator: John R. Wells

Support: National Institutes of Health, Public Health Service

A technique has been developed for studying the mammalian cell cycle based on culture fractionation. A batch culture in log growth is fractionated by rate-zonal centrifugation in a shallow Ficoll gradient. The cells are separated according to cell size, and therefore a spectrum of fractions of increasing average age is obtained. Relationships between cell size and other parameters of the cell cycles, such as DNA content or synthesis, are then established. Two types of experiments are being carried out. (1) Tests are being run on several different cell types to discover the best gradient and sample conditions to maximize both the quality of the separation and the number of cells which can be separated. (2) Mitochondrial DNA is isolated from age classes of cells following separation, and the frequencies of the replication forms are being determined using the electron microscope.
A technique has been devised which allows buoyant analysis of as little as 0.01 µg DNA per sample on as many as 10 samples per bucket of a Spinco SW-39, 50, or 50.1 preparative rotor in a single centrifuge run. This methodology is currently being extended to allow sedimentation velocity analysis of material in linear sucrose microgradients and the fractionation of the gradients for assay or recovery of the sedimenting material. Thirty µl linear sucrose gradients can be performed by a simple procedure and can be fractionated into as many as 30 fractions.

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**PUBLICATIONS**


THE EARLE M. JORGENSEN LABORATORY
OF INFORMATION SCIENCE

Derek H. Fender

Ken-Ichi Naka
162. MAPPING OF IMAGES BETWEEN THE RETINAL AND CORTICAL STAGES OF THE HUMAN VISUAL SYSTEM

Investigators: Derek H. Fender, Terrance M. Darcey*

Support: National Institutes of Health, Public Health Service
Alfred P. Sloan Foundation

There exists a well-defined point-by-point retinotopic mapping between the retina and visual cortex in which proximal cortical areas arise from proximal areas of the visual field. The existence of this mapping is generally accepted and is supported by many single unit studies in primates and also by recent work involving direct stimulation of the human cortex.

A system which allows the measurement of surface potentials on the head with spatio-temporal resolution has been developed. Data taken by this system in special visual stimulus situations can be analyzed to yield reliable estimates of the spatio-temporal characteristics of equivalent cortical sources (10^5 cells or more) which give rise to the surface activity.

This research involves a careful application of the source localization technique to the study of the point-by-point mapping between retina and cortex in humans. The type of mapping that exists with respect to equivalent cortical sources will be verified by placing stimuli in various areas of the visual field and mapping their respective cortical source locations.

163. INFORMATION PROCESSING IN THE HUMAN VISUAL SYSTEM

Investigators: Derek H. Fender, Stanley Klein*, Thomas P. Santoro**, James G. Moore†

Support: National Institutes of Health, Public Health Service
Alfred P. Sloan Foundation

Results of psychophysical tests analyzed with a Kruskal nonmetric scaling technique have shown promise of unraveling certain aspects of information processing in human visual cortex. These preliminary tests use letters of the alphabet as stimuli. The resultant Kruskal scaling indicates the letters are being handled in at most a 3-dimensional system and gives their configuration in that system. Configurations for different subjects fall into certain classes which are being examined to determine the nature and sources of their differences. Currently various computer graphics and photographic techniques are being implemented to extend this study to textures, which we believe have a representation in the activity of relatively large numbers of cortical cells. If this is indeed the case, this activity may be EEG-significant.

In parallel with the evolving psychophysical facility, development and implementation of EEG hardware is also progressing. A display system in which a PDP 11/45 mini-computer interacts with a two-color CRT is being considered to do the presentation of the processed brain waves. Fluctua-
tions in time and space in the electric field on the surface of the head are visualized as movements of contour lines on the surface of a sphere. A 3-dimensional effect is obtained by using the two-color display to generate an animated anaglyph. An investigator could interact with the display to more closely observe particularly interesting sections of the activity. From these measured electric field patterns, a solution is made for an equivalent source within the cortex which would give rise to such activity. In this way, a sort of crude, but noninvasive, neurophysiology may be performed in which areas of the cortex which respond to a given visual stimulus can be localized. The cortical representation of textures, for example, can be investigated. Such texture localizations, combined with the results of Kruskal analysis of psychophysical tests using textures, may yield a new understanding of the way textures and other visual stimuli are handled.

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164. CHANGES IN HUMAN ELECTRORETINOGRAM CORRELATED WITH RETINAL PATHOLOGIES

Investigators: Derek H. Fender, Arthur J. Koblasz*

Support: National Institutes of Health, Public Health Service

The long-term goal of this project is to apply modern nonlinear systems analysis to the problem of cataloging changes in the human electroretinogram (ERG) correlated with retinal pathologies.

Many retinal diseases manifest known morphological and functional changes. Our improved analysis of the ERG might eventually provide earlier diagnosis of some of these functional changes. For example, monochromatic light directed at a large area of the retina and varying with random-sequence changes in illumination might produce an ERG sensitive to functional changes diffusely distributed over the retina. Examples would be retinal degeneration diseases and occlusive diseases of retinal arteries or veins. On the other hand, many retinal pathologies involve functional changes at discrete focal points; e.g., retinoblastoma, gliomas, focal inflammatory lesions, congenital syphilis, tuberculosis and vascular retinopathies. For these diseases a spot or annulus with random-sequence modulation of position or luminance might be more appropriate. Still other retinal diseases cause functional changes specific to internal retinal layers (e.g., glaucoma, Tay-Sachs disease) or to external retinal layers (e.g., retinitis pigmentosa, choroideremia, Oguchi's disease, Uyemura's syndrome, congenital acro-matopsia). These diseases might be best detected by examining changes in lateral effects derived from the simultaneous application of a spot and annular surround, each with independent random-sequence illumination. The intent of this research is to replace these speculations with fact.

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165. VEC PRODUCED DURING RETINAL IMAGE STABILIZATION

Investigators: Derek H. Fender, Ulker Tulunay-Keesey, Rick A. Williams*, Daniel B. Diner*, C. William Britton*

Support: National Institutes of Health, Public Health Service

It is well known that when a subject fixates on a target, the eyes execute a variety of motions. These cause corresponding motions of the image on the retina, adding a temporal component to retinal stimulation. When the subject is presented with an image which is held stationary on the retina, the subject reports that contrast gradually fades and finally the whole field disappears. This work is aimed at understanding the electrophysiological basis of perceptual disappearance. For this
purpose, movements of the eye and potentials from the cortex will be recorded while the subject views a stabilized image. The eye movement records will be searched for saccades and the cortical signals will be averaged for a 300 msec interval centered around a saccade. The same treatment will be applied to data obtained under unstabilized conditions when the image is free to move on the retina. If image movement is responsible for evoking a retino-cortical response to maintain the visibility of an image a visual evoked cortical potential (VECP) should be recovered by appropriate averaging of the EEG around a saccade, under unstabilized conditions. Potentials evoked by the onset of the stimulus will also be recovered. An experiment where the eye movement and the EEG records obtained in darkness under conditions of no retinal stimulation will supply the control. The evoked potentials then will be subjected to a source analysis to sort out the areas of the cortex which respond to the spatial and temporal (eye movement dependent) aspects of the stimulus.

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166. TRANSIENT REMAPPING OF RETINAL TO PERCEPTUAL LOCALIZATION OF VISUAL INFORMATION

Investigators: Derek H. Fender, Norbert Bischof, Terrance M. Darcey*

Support: National Institutes of Health, Public Health Service
Alfred P. Sloan Foundation
Fairchild Scholars Program

This work is aimed at understanding how the perceptual system differentiates between a target's displacement across the field of view and the retinal image displacement generated by a saccadic eye movement across an immobile target. The method used is to expose a subject to a short light stimulus during or after a saccade. The stimulus is released by the eye movement itself. The subject's task is to localize the stimulus on a fixed luminous scale.

Preliminary experiments have shown that clear translatory illusions occur in these experiments. They depend systematically on the spot on the retina stimulated, and on the time elapsed between the onset of the saccade and the release of the stimulus. These illusions are best interpreted as due to the shift of a "coordinate system" across the retinal field. The shift is initiated by the coordinate of the retinal spot on which the target of the eye movement was projected before the saccade began. This coordinate rapidly swings into the fovea. The processes on the retinal hemisphere opposite to the target occur at a much slower rate. Moreover, temporary changes of the topology of the "coordinate system" may take place during the saccade.

A concurrent investigation is being designed to facilitate EEG recording during the voluntary saccades in which directional perception is studied for a target of sufficient size and intensity to yield a readily detectable evoked potential response in the EEG. The idea is to test whether, during a saccadic eye movement, any spatial remapping of cortical potentials occurs which corresponds to the psychophysical data obtained in the above experiment.

*Division of Engineering and Applied Science, California Institute of Technology.
167. THE STIMULI FOR EYE MOVEMENTS

Investigators: Derek H. Fender, Li-Ho R. Hou*

Support: National Institutes of Health, Public Health Service

The complex eye-brain information processing system includes a decision process. The brain receives input information from the eyes and decides for itself where the eyes will look.

We can postulate at least two kinds of processes that are involved in the problem of how the eye decides where its next point of regard shall be. The visual system should be capable of processing information from different areas in parallel. However, if the subject is given a search task in which all the critical cues are below the threshold for foveal acuity, the subject can be forced into a serial routine, and the order in which the information is handled can be determined from the sequence of eye movements. Experiments are in progress to examine this problem. Certain symbols are first classified by perceptual distance. The stimuli of the experiments include symbols having acuity cues only, symbols that are arranged according to their perceptual classification, and symbols that can be identified in part by context. Also, static and dynamic visual acuity at different areas on the retina are mapped by the display of symbols on a computer graphical terminal.

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168. DISCRIMINABILITY OF RANDOM DOT TEXTURES IN HUMAN VISION

Investigators: Derek H. Fender, Stanley Klein*, James G. Moore**

Support: National Institutes of Health, Public Health Service

Computer-generated random dot patterns with slight correlations are being used to study pattern recognition. Pairs of patterns with slightly different first- and second-order statistics are displayed adjacent to each other, and the distinctiveness of the boundary is rated. From the data on the distances between all pairs it is possible to determine how the objective distance between patterns gets mapped into the subjective distance between patterns. By shortening the amount of time allowed for each perceptual judgment it is possible to determine the temporal properties of the different subjective dimensions. It is expected that certain dimensions can be discriminated by rapid parallel processing while others may require slower serial processing. An attempt is also being made to determine whether the subjective dimensions can be best understood by assuming that the cortex is performing a feature analysis or a Fourier analysis of the patterns.

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169. HUMAN OCULOMOTOR SYSTEM: INTERRELATIONS WITH VISUAL PERCEPTION

Investigators: Derek H. Fender, Syozo Yasui*, Laurence R. Young**

Support: National Aeronautics and Space Administration

Various classes of human horizontal eye movements have been investigated in several areas emphasizing functional relationships.

Perceived Visual Motion as Effective Stimulus to Pursuit Tracking Eye Movements. Perception of visual objects during eye movements has been a classical question for over a century. Vital influence of eye movement information on seen movements is well known as has been explained by the corollary discharge theory. We have examined the converse possibility: might not the perceived target motion (rather than the actual target
motion) in turn influence eye movements? This may be recognized as the perceptual feedback hypothesis or closed-loop extension of the classical corollary discharge theory, where the observer tracks what he perceives as target motion. Experiments involving afterimage tracking under vestibular stimulation were designed to test this motion, and results support the hypothesis.

**Quest for Predictive Behavior in the Oculomotor System.** One of the most intriguing features known to the human eye tracking system is its input-adaptability: the accuracy of visual fixation is improved with increasing stimulus periodicity, a phenomenon attributed to the predictive or learning capacity of man that effectively compensates for the underlying innate neuromuscular delay, as manifested in frequency response by marked reduction of phase lag for periodic target motions as compared with random ones.

This subject was investigated with regard to pursuit and saccadic components individually (as separated from their original composite eye movement by a special data processing) and also the slow phase component of optokinetic nystagmus (OKN) and of vestibular nystagmus. Pursuit frequency response results are characterized by a conspicuous tendency toward large low-frequency phase lead with increasing stimulus bandwidth. A special type of predictive control scheme is suggested in interpreting this finding. Considering the dependence of saccadic response upon the tracking performance by the pursuit subsystem, the notion of effective saccadic input is introduced in order to assess the intrinsic random-input saccadic frequency response, and resultant phase data are compared with the periodic-input counterpart in the literature, confirming the predictive nature of saccadic tracking. OKN slow phase also shows evidence for prediction but no phase lead such as found in the pursuit case. This difference indicates the existence of separate central mechanisms controlling these two classes of visually-evoked oculomotor reactions, despite their similar phenomenological features apparent in the time domain observation. In contrast, vestibular nystagmus slow phase representing a nonvisual oculomotor reflex failed to show a predictive behavior.

**Generation of Fast Phase in Vestibular and Optokinetic Nystagmus.** Behavior of the fast phase in vestibular and optokinetic nystagmus is investigated regarding its characteristic relation to the slow phase movement. To account for the relevant phenomena observed similarly in both types of nystagmus, an efferent feed-forward type model is constructed based on a simple principle envisioned from experimental results, and tested by a series of hybrid computer simulations. The model is intended to predict the fast phase behavior for given slow phase motor information irrespective of its stimulus origin. This view is reinforced by clinical nystagmuographic data from an acoustic neuroma patient. Another pathological case cited is central scotomata, in which reversal of the fast phase direction occurs with OKN. Dependence of OKN mechanism upon central versus peripheral vision as indicated by this example is examined in a normal subject by selective OKN stimulation to the peripheral retina, based on a computer graphic method for stimulus presentation coupled with feedback blanking signals from the eye movement monitor.

**Reference:**

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**Director, Man-Vehicle Laboratory, Department of Aeronautics and Astronautics, Massachusetts Institute of Technology, Cambridge, Massachusetts.*

**PUBLICATIONS**


170. MORPHOLOGICAL CATEGORIZATION OF RETINAL NEURONS

Investigator: Nancy R. G. Carraway*

Support: National Institutes of Health, Public Health Service

Except for the receptors, catfish retinal neurons selectively impregnated by the Golgi method were classified morphologically into categories which correlated roughly with similar cells in other vertebrate retinas. The method of flat-mounting the tissue enabled the stained cells to be evaluated for the usually overlooked aspect of their horizontal expansions. Although the variability of size within each cell type was considerable, there was enough consistency in overall shape and branching patterns to allow the neurons to be organized into generalized classes.

The bipolar and horizontal cells corresponded morphologically to the same types of cells in other vertebrate retinas. The other type of cell found in the inner nuclear layer formed a structural continuum, at the symmetric extreme of which the cell body was centralized with processes radiating laterally and equidistantly as a single layer. Towards the asymmetric extreme, the field of processes becomes increasingly skewed and unidirectional, with the cell body at first removed to one side of a main process and finally isolated altogether by a thin neck. We have described this continuum as extending from a star bursting to an octopus swimming upstream.

The inner synaptic layer consisted of processes from two categories of cells, both of which occasionally appeared to have axons. The spaghetti cell, so called for its long, branchless, processless arms, and the floral cell, so called for its profusion of small, intertwining processes from networks of branches, were more formally arranged into groups on the basis of cell body shape and organization of principal processes emanating from the cell body.

This study has revealed not only the existence of a gradient of size within a particular cell type, but also, unexpectedly, the lack of definite demarcations between cell classifications. Retinal neurons appear to be aligned along a continuous spectrum wherein transitional distinctions between cell types are vague at best. It remains to be seen in further studies if these structural gradients have functional counterparts as well.

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171. COMPUTER-BASED CLASSIFICATION OF THE NEURONS IN THE CENTRAL NERVOUS SYSTEM

Investigators: Lenora Chen*, Ken-Ichi Naka

Support: National Institutes of Health, Public Health Service

In 1952 Ramón-Moliner suggested that the neurons in the central nervous system could be classified by such simple parameters as the shapes of the cell bodies and the position and number of the principal dendrites. In the vertebrate retina a large number of ganglion cells can be easily and selectively stained by methylene-blue dye which especially delineates the cell body and principal dendrites. Prepared in this way, the cells are thus particularly well suited for computer processing and classification according to Ramón-Moliner’s criteria.

The methylene-blue stained ganglion cells are initially scanned by a TV camera; each is aligned...
on a 512 x 512 point array and, according to its density, each point is assigned a value on a 6-bit grey scale. This so-called digitized image is then treated with a threshold program to remove small artifacts such as precipitated dye-crystals; the edges of the neuron are represented by small line-segments and the branching points of the principal dendrites determined. From these computer-abstracted neuron images, the size and shape of the cell bodies and the number and location of the principal dendrites are automatically indicated by a series of algorithms. The whole procedure is fast and requires a minimal amount of human intervention, thus making it possible to analyze a large neuron population—hitherto impossible due to the extent of labor involved.

The resulting groupings can then be processed by the scattergram technique to achieve an objective classification of the neurons based on their morphological traits. Such a classification scheme will serve in the future as a reference for Procion dye-identified neurons or will be correlated to the diversified functions the ganglion cells are known to perform. Further refinement of this automated neuron identification procedure will be done to take advantage of recent progress in artificial intelligence studies.

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

172. COMPUTER-ASSISTED STRUCTURAL ANALYSIS OF GOLGI IMPREGNATED VERTEBRATE RETINAL NEURONS

Investigators: Michael J. Shantz*, Ken-Ichi Naka

Support: National Institutes of Health, Public Health Service

Golgi studies have been carried out on retinal ganglion cells of both adult frogs (Rana catesbiana) and tadpoles. These Golgi-stained cells are scanned using an automated light microscope to produce a three-dimensional digital representation of the neuron. A three-dimensional spatial filtering procedure has been developed to improve z-axis resolution and to give an enhanced data base for the generation of stereo pairs.

Techniques are being developed for the interactive extraction of high level structural parameters from the digitized cell images. Morphological features which are thought to be important and for which quantitative representations are being developed, include: (1) retinal layers containing cell’s dendritic field; (2) size and asymmetry of dendritic fields; (3) degrees of parallel aspects of dendrites; (4) position and orientation of cell in retina; (5) major dendrite cross-sectional area, and volume-to-surface area ratio; (6) cell body size, shape, retinal layer position and number of processes emerging; and (7) axon size and hillock shape.

A unified layer theory based on these data is being developed whereby the input regions, integrating regions and output regions of the cell are characterized structurally. On the basis of these structural characterizations, cells will undergo cluster analysis to outline categories of similar cells. These structural or morphological categories will be compared to functional categories in order to reveal structural-functional correlates.

An analysis of development is in progress which seeks to compare structural categories of tadpole retinal ganglion cells with those of the adult frog. It is thought that such a structural representation with its corresponding functional correlates will form the basis for a unified transformational layer theory model of the functioning vertebrate retina.

*Division of Engineering and Applied Science, California Institute of Technology.
173. FUNCTIONAL AND MORPHOLOGICAL CLASSIFICATIONS OF THE VERTEBRATE RETINAL NEURONS

Investigators: Teruya Ohtsuka*, Ken-Ichi Naka
Support: National Institutes of Health, Public Health Service

The object of this research is to correlate the function with the structure of the retinal neurons in a representative vertebrate system.

The morphological origins of the responses from catfish retinal neurons evoked by step inputs of light were determined by intracellularly injecting a fluorescent dye, Procion yellow M4RAN. A method was devised to view the dye-injected neurons in flat mount to study their dendritic expansions; later the same neurons could be sectioned radially to locate the levels of their somata or dendritic expansions.

Neurons with their cell bodies in the inner nuclear layer gave rise to three types of responses: the bipolar cell type, the sustained type and the transient type. Bipolar cells were identified by their response patterns of center-surround antagonistic polarizations. No morphological differences were found between the two types of bipolar cells.

Sustained type neurons gave rise to maintained depolarizations during illumination. The response of this class of neurons did not vary with the stimulus intensity and/or the spatial distribution of the illumination. These neurons were morphologically similar to what Cajal termed single stratified amacrine cells. Transient types depolarized at both the on- and offset of illumination by spot and/or annulus. In flat-mount view, their common feature was the multiple intertwining of each cell’s dendritic branches. The radial view of these neurons showed their dendritic spread at several levels in the inner plexiform layer. Cajal described similar neurons as two-layered or multipolar cells. The processes of the sustained and transient types proliferated throughout the inner nuclear layer.

In the ganglion cell layer, both a spiking and a slow potential type cell were found. Flat-mount preparations revealed that the spiking neurons had axons. The slow potential type neurons, however, had neither common response patterns nor common morphological features.

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174. SPATIAL AND TEMPORAL PROPERTIES OF RETINAL NEURONS AND THEIR INTERACTION

Investigators: Raymond Y. Chan*, Ken-Ichi Naka
Support: National Institutes of Health, Public Health Service

Retinal neurons respond to intensity (temporal) changes as well as changes in the spatial stimulus pattern. In this study, we first attempted to investigate their spatial response properties by using a sinusoidal input to modulate the size of the stimulus; their temporal response properties can also be studied through sinusoidal intensity modulation. An obvious question would then be, is there any interaction between spatial and temporal response properties of retinal neurons? In a preliminary study neurons were stimulated at the same time by both spatial and temporal stimuli and their responses compared with the responses obtained by using spatial stimulus alone and responses obtained by using temporal stimulus alone. Such a study, however, is quite qualitative since one cannot actually separate the spatial contribution from that of the temporal contribution to the total response.

Fortunately, P. Z. Marmarelis and K.-I. Naka have successfully applied the 2-input white noise technique to retinal neurons, and the contribution due to each input can easily be separated by cross-correlating the stimulus with the total response. Preliminary study has shown that horizontal cells, which behave rather predictably under either spatial or intensity modulation, change their response pattern when stimulated by the combined input, indicating that the spatial and temporal components do not sum linearly to produce the spatial-temporal response. On-center
and off-center bipolar cells are exactly 180° out of phase for both spatial and intensity modulations. Sustained neurons in the inner nuclear layer, which behave linearly with sinusoidal intensity modulation, produce a fast transient component for spatial modulation, offering a first example of the drastic deviation of the spatial response from temporal response. Spatial modulation seems to be a more favorable input for the transient neurons in inner nuclear layer than intensity modulation. As expected, the latency is usually shorter in the case of intensity modulation. Neurons that give rise to spike activity show a similar behavior for both spatial and temporal inputs. However, the responses become transient when they receive both spatial and temporal inputs.

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

**PUBLICATIONS**


WILLIAM G. KERCKHOFF LABORATORIES
OF THE BIOLOGICAL SCIENCES

James Bonner  Ray D. Owen
A. J. Haagen-Smit  Robert L. Sinsheimer
Norman H. Horowitz  Felix Strumwasser
Henry A. Lester  Anthonie Van Harreveld
Edward B. Lewis  C. A. G. Wiersma
Daniel McMahon  William B. Wood
Our efforts are directed towards the understanding of gene expression in the genomes of higher organisms. In a typical mammalian cell, from 1 to 10% of the DNA sequences of the genome are expressed as RNA transcripts. Different DNA sequences are expressed as transcripts in different cell types of the same organism. What determines which DNA sequences, which genes, are expressed and which are not? What determines the way in which an orderly sequential turning on and turning off of the right genes at the right time and the right places results in the development of a normal adult organism—these are the questions to which we address ourselves.

We have already shown, and it is now generally agreed, that DNA complexed with the characteristic higher organism DNA-binding proteins, the histones, are DNA sequences that are not expressed as transcripts. Binding of histones to DNA prevents their transcription but not their replication. Histone chemistry has been extensively developed. The primary structures of histone molecules are amongst the most conserved sequences of any proteins yet studied in higher organisms. We have abandoned the study of histone chemistry since it is a growth industry in other laboratories. Histones lack the ability to bind sequence-specifically to DNA. We have, therefore, concentrated our attention upon trying to discover those elements of the chromosome which tell histone molecules which DNA sequences to combine with and which DNA sequences to leave free for transcription. We show in several different ways, as outlined below, that the transcribable portion (template-active portion) of the mammalian genome consists of DNA sequences complexed not with histones but with nonhistone chromosomal proteins. We have devised a series of methods for the separation of the template-active chromatin from the template-inactive chromatin. We have shown that the template-active chromatin consists of a small subset of the single-copy sequences of whole genomal DNA and of a small subset of the middle-repetitive sequences of whole genomal DNA. What are the sequence-specific elements which tell histones where not to bind? Principal candidates are sequence-specific DNA-binding proteins of the nonhistone chromosomal protein class, and a class of RNA molecules (chromosomal RNA) which we have described in previous reports. We have concentrated during the past year on the study of the nonhistone chromosomal proteins and have prepared in pure form 5 of these components. This is a modest start since the total includes a minimum of about 115 protein species. The isolation in pure form of these 5 protein species has depended upon methods which involve selective dissociation from chromatin of different classes of nonhistone chromosomal proteins, so that the mixture from which an individual protein is to be isolated is less complex than the total mixture. Two of these proteins, found exclusively in the template-active portion of chromatin, have been identified as the RNA-binding proteins, that is, those proteins which bind with RNA as it is transcribed and cause these transcripts to become ribonucleoprotein particles. A third is a highly acidic protein. Its function is yet unknown. A fourth is a highly basic protein. Its function is also not yet known. A fifth is a chromosomal enzyme, also purified to homogeneity. It is a neutral protease with special activity against histones. The properties of the histone protease described above are of interest because this enzyme appears to be involved in the removal of histone from DNA sequences that are activated from the repressed to the derepressed state.

We have also studied the nonhistone chromosomal proteins by isolating from the whole mixture a class which binds sequence-specifically to homologous DNA and not to any other kind of DNA. Thus, rat liver
nonhistone chromosomal proteins contain about 2 to 4% by mass of proteins which bind, in 0.2 M salt, only to rat DNA and not to other kinds of DNA. We have found that these homologous DNA sequence-specific binding proteins bind preferentially to the middle-repetitive sequences of the rat genome. We have found in addition that the middle-repetitive sequences of rat whole genomal DNA may be divided into two classes; namely, a minority which binds to rat liver sequence-specific binding proteins and a majority which does not bind to such proteins. It appears that we have in our hands truly sequence-specific binding proteins. However, the class of proteins thus purified still consists of a mixture of many kinds of proteins, perhaps 200 in all, which bind to perhaps 200 different kinds of DNA sequences. Our next task is to isolate individual species of such sequence-specific binding proteins to determine to which sequence they bind and to determine the properties of both the individual proteins and the individual sequences to which they bind. A further problem is, of course, to determine the extent to which such sequence-specific DNA-binding proteins control gene expression. We have found no simple way to attack this problem. Reconstitution of interphase chromosomes from their components—histones, nonhistone chromosomal proteins, DNA, and chromosomal RNA—has proven to be a difficult task in the sense that the chromatin reconstituted by the methods in hand does not give rise to native chromatin. We have, therefore, embarked upon a new route toward the study of this problem. The strategy, outlined below, is to try to turn on in a particular kind of tissue-cultured cell a gene which is not expressed in that cell. The cell line which we have chosen is a hepatoma, i.e., liver cancer, cell. The gene which is repressed in this kind of cell and which we wish to derepress is one which is turned on in the glial cells of the cortex, namely, the gene which codes for the S-100 protein. We have determined how to detect S-100 proteins in the cytoplasm of cultured hepatoma cells, and we are now supplying such cultured hepatoma cells with the various components of glial nuclei—nucleoplasm, nonhistone chromosomal proteins, brain chromosomal RNA, and so on. This approach should in due course enable us to determine with rigor what class of compounds is required to turn on a particular gene.

We have, of course, already followed some of the parameters associated with the turning on of genes previously turned off. The main finding described in a publication listed below is that in regenerating liver where there is a massive turning on of genes previously repressed, there is a diminution of histone to DNA ratio of the chromatin of the regenerating cells; that is, histones are removed from DNA sequences previously covered with histones and histones are removed by histone protease. What is the signal which tells which histones to be removed by the protease? This we do not know, and that is what we are trying to find out by our tissue-culture system.

Since both chromosomal RNA and the nonhistone chromosomal proteins which bind sequence-specifically to homologous DNA, bind to the middle-repetitive sequences, these sequences are of interest. They are putative regulatory sequences, regulating the expression of the single-copy DNA sequences adjacent to them. We have, therefore, continued on our program of mapping the way in which middle-repetitive sequences are interspersed among the single-copy sequences in mammalian DNA. These studies, summarized below, show that in the rat, middle-repetitive sequences are short, approximately 200 base pairs in length, that they occur in many cases as doublets, that is one middle-repetitive sequence after another, and that such doublets are interspersed by single-copy sequences 500 to 15,000 or more base pairs in length. No hint of longer middle-repetitive DNA sequences has been found in the rat genome. We already know that the bulk of the initial transcripts of rat DNA, the nuclear HnRNA, is on giant transcripts, 15 to 30 kilobases in length. We know also that the messenger RNA molecules which appear in the cytoplasm are much smaller, 1,000 to a few thousand bases in length. Are the messenger RNA molecules derived from the initial giant nuclear transcripts? Are the latter not precursor to messenger but wholly processed and used within the nucleus? We do not know, and nobody knows as yet. We are attacking this problem also with rat liver hepatoma cells.

New methods for the mapping of expressed sequences of DNA and of nonexpressed sequences of DNA are urgently needed. We are approaching this problem in a variety of ways. One is the hybridization of the template-active sequences of DNA, fractionated as we describe below, to whole giant nuclear DNA. A
second is to find a way to label RNA, either giant nuclear or messenger, hybridized to DNA in such a way that it is recognizable as distinct from DNA not hybridized with RNA. In this, too, we have made some progress.

A principal question of the control of gene action is how small molecules, such as hormone and other effector substances, interact with the chromatin to cause particular DNA sequences to become derepressed and therefore expressed. We have studied this manner earlier with hormones such as estradiol, cortisone, etc., and have come up against the same blind alley as everyone else; namely, that the hormone combines to a hormone-binding protein, enters the nucleus, binds to chromatin, probably to a specific nonhistone chromosomal protein bound to DNA, and then causes mysterious events to occur which result in the removal of histone from chromatin and the expression of fresh new DNA sequences. The whole mechanism between hormone binding to chromatin and expression of fresh new DNA sequences is a blackbox mystery. As outlined above, we are trying one particular new way of getting at the matter. A second new way of getting at the matter is by the study which we are now initiating of the way in which liver carcinogens by binding to nuclear proteins cause the expression of previously repressed genes. We don’t know yet, but we hope that the study of the chemical carcinogens which cause liver cancer may provide us with fresh clues about how to find out how genes previously repressed are derepressed.

175. FRACTIONATION OF CHROMATIN

Investigators: Joel M. Gottesfeld, James Bonner

Support: National Institutes of Health, Public Health Service

We are developing methods for the fractionation of chromatin into transcriptionally active and inactive regions. A comparison of the constituents of active and inactive chromatin could shed light on the mechanisms of gene repression and activation in eukaryotes. We present below and in the following section (Biology 1974, No. 176) results obtained with DNase-sheared chromatin fractions; further sections (Biology 1974, Nos. 177 and 178) present two additional methods for chromatin fractionation.

When chromatin from a variety of rat tissues is exposed to the endonuclease DNase II, the transcribed portion of chromatin DNA is attacked more rapidly than the transcriptionally inert DNA sequences. The nuclease-labile fraction of chromatin can be separated from inactive chromatin by virtue of the fact that the latter fraction is insoluble in 2 mM MgCl₂. As reported previously (Biology 1973), the active fraction is depleted in histone protein and enriched in nonhistone chromosomal proteins. Specifically, histone I (lysine-rich) is absent from the active fraction (the other histone species are present in reduced proportions); this fraction is enriched in two nonhistone polypeptides of 30 to 40,000 molecular weight. These latter polypeptides probably correspond to the proteins believed to be involved in the packaging of heterogeneous nuclear RNA (Martin and Swift, 1973). We have shown that the absence of histone I from chromatin does not alter the amount of DNase-labile chromatin or the kinetics of enzyme attack; furthermore, fixation of chromosomal proteins to DNA with formaldehyde does not change the amount of chromatin DNA recovered in the various chromatin fractions. These data suggest that protein movement is not involved in enzyme action on chromatin DNA; moreover, experiments with added radioactive polynucleotides suggest that protein exchange does not take place during nuclease treatment. We conclude, therefore, that the differences in protein composition of the chromatin fraction are real and not produced artifactually by nuclease treatment.

Reference:
176. SEQUENCE COMPOSITION OF THE TEMPLATE-ACTIVE FRACTION OF CHROMATIN

Investigators: Joel M. Gottesfeld, Gyorgy Bagi, James Bonner

Support: National Institutes of Health, Public Health Service

United Nations Educational, Scientific and Cultural Organization

The DNA isolated from the DNase-labile fraction of rat liver chromatin was found to vary in length between 1800 and 50 base pairs (30-second vs. 15-minute exposure to DNase). After 5-minute exposure to DNase, the nuclease-sensitive fraction had a single-stranded length of 400 to 500 nucleotides, as determined by sedimentation under alkaline conditions. The renaturation kinetics of this material were investigated by hydroxyapatite chromatography; three kinetic components were observed: a rapidly reannealing component (10%) renatured by Cot 0.01; an intermediate component (20%) with Cot ½ = 0.27; and a slowly renaturing component (65%) with Cot ½ = 250. The intermediate and slow components renatured 6 to 7 times faster than their respective counterparts in DNA isolated from unfractonated chromatin. This suggests that the DNase-labile fraction contains a specific subset of whole genomic DNA sequences. That the slow component of this fraction corresponds to single-copy DNA was shown by isolating this component, labeling it in vitro with \(^{125}\)I, and reannealing it in the presence of a vast excess of unfractonated rat DNA. The Cot ½ of the labeled DNA was the same as that of single copy of whole DNA (Cot ½ = \(1.5 \times 10^3\)). That the nuclease-labile fraction is enriched in transcribed single-copy sequences was shown by hybridization of the labeled slow component DNA to whole cell RNA under conditions of vast RNA excess; 30% of the single-copy sequences of this fraction were found to be complementary to whole-cell RNA from liver while only 5% of the single-copy sequences of the DNase-resistant fraction were found to be complementary to such RNA. A similar experiment with brain RNA showed that the liver template-active fraction is not enriched in sequences which code for brain RNA. Cross-hybridization experiments with DNA from both the active and inactive fractions indicate that the active fraction is 60 to 70% pure. This value is in agreement with the RNA saturation figure if one assumes asymmetric transcription. Reassociation experiments also indicate that the template-active fraction contains a subset of middle-repetitive DNA families.

177. SUCROSE FRACTIONATION OF CHROMATIN

Investigators: Joel M. Gottesfeld, William R. Pearson, James Bonner

Support: National Institutes of Health, Public Health Service

When Virtis-sheared (30 V, 90 seconds) rat liver chromatin is centrifuged through a 15 to 30% sucrose gradient two fractions are obtained, a pellet which contains the bulk of the input material and a 25 to 30S fraction which comprises about 5% of the input chromatin DNA. This latter fraction has a slightly reduced histone to DNA ratio (0.44, as compared to 1.06 for bulk chromatin) and is enriched in nonhistone chromosomal proteins (NHC protein/DNA = 1.25, as compared to 0.65 for bulk chromatin). As compared to the DNase fractionation method, all five major species of histones were found in the light chromatin fraction. When chromatin was treated with the intercalative dye ethidium bromide prior to centrifugation, essentially all the dye was found associated with the light chromatin fraction. A correlation between template activity and ability of chromatin to bind ethidium has been reported (Seligy and Lurquin, 1973). When the light chromatin fraction was assayed for its susceptibility to DNase II, it was found that the kinetics of attack were similar to the action of the enzyme on protein-free DNA. The pellet fraction and unfractonated chromatin were attacked by the enzyme much more slowly. These data suggest that the DNA of the sucrose light
fraction is in a conformation similar to protein-free DNA, and that this fraction might also correspond to template-active chromatin.

Reference:

178. UREA-MgCl₂ FRACTIONATION OF CHROMATIN

Investigators: Gyorgy Bagi, Joel M. Gottesfeld, James Bonner

Support: United Nations Educational, Scientific and Cultural Organization
National Institutes of Health, Public Health Service

It has been shown (Biology 1974, No. 177) that sedimentation of chromatin in a sucrose gradient yields a bimodal distribution of nucleoprotein complexes: the majority of the material sediments rapidly with a sedimentation coefficient of greater than 100S; however, a minor fraction (5% of the total DNA) sediments more slowly with an S value of 25 to 30. When chromatin is treated with 5 M urea prior to centrifugation, the nucleoprotein is converted almost entirely to the 25S form (Chalkley and Jensen, 1969). This finding and the fact that inactive chromatin is precipitated by Mg ions lead us to the following fractionation scheme: chromatin is suspended in 5 M urea and insoluble material is removed by low speed centrifugation. MgCl₂ is added to 4 mM and the chromatin is stirred for 15 minutes at 4°C. Next, the chromatin is centrifuged at 27,000 x g for 15 minutes yielding a pellet and a supernatant fraction. The nucleoprotein of the supernatant is concentrated by centrifugation overnight at 40,000 rpm in the Spinco 50.1 rotor. The yield of DNA and protein compositions of the chromatin fractions are presented in Table I. The Mg-soluble fraction corresponds in amount to the sucrose-light (25S) fraction; furthermore, this fraction is depleted in histone and slightly enriched in nonhistone protein.

When chromatin from ³H-uridine pulse-labeled hepatoma cells was fractionated in the manner described above, and the distribution of labeled RNA in the fractions was determined, it was found that the specific activity of the Mg-soluble material was 10-fold greater than that of the Mg-insoluble fraction. The Mg-insoluble pellet had a specific activity of 365 cpm/µg DNA while the Mg-soluble chromatin had a specific activity of 3120 cpm/µg DNA. Thus it appears that the Mg-soluble fraction may also correspond to the minor portion of chromatin which is transcriptionally active in vivo.

Reference:

Table I. Urea-MgCl₂ Fractionation of Chromatin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% of total DNA</th>
<th>Histone DNA</th>
<th>Nonhistone DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellet</td>
<td>94.5</td>
<td>0.97</td>
<td>0.70</td>
</tr>
<tr>
<td>Mg-soluble</td>
<td>5.5</td>
<td>0.22</td>
<td>0.79</td>
</tr>
</tbody>
</table>
SEQUENCE ORGANIZATION OF THE RAT GENOME

Investigators: Mahlon M. Wilkes*, Tom St. John**

Support: National Institutes of Health, Public Health Service

We have continued our electron microscopic studies of the size and distribution of the various sequence elements of the rat genome. By mass this genome consists of 70% single-copy DNA, 20% middle-repetitive (MR) and 10% highly-repetitive (HR) sequences as defined by kinetic criteria. The various sequence classes may be isolated in pure form by appropriate hybridization and hydroxyapatite chromatography procedures of sheared DNA preparations. We have self-reassociated 1.2 kilobase (kb) MR DNA fragments to Cot 50, mounted for electron microscopy by the formamide technique, and scored “forked” structures representing duplex regions of MR sequence with four radiating single-copy single strands. The statistical length distribution of the duplex regions corresponding to the MR sequence lengths falls within the range of the smallest observable lengths (ca. 50 nucleotides) to about 500 nucleotides, with a modal average in the range of 100 to 200 bases. Following a similar experimental procedure, we found a length distribution of HR fragments (reassociated to Cot 0.005) also in the range of the smallest observable lengths to 500 nucleotides, with a modal range of 100 to 200 bases. To appraise the distribution of these sequence elements in the nuclear DNA, we reassociated sheared DNA fragments of defined length and sequence composition in vast excess to 7.5 kb unsheared, unfractionated single-strand DNA and visualized hybrid structures consisting of duplex regions and radiating single-copy branches. A frequency histogram of more than 500 of such structures reveals a broad maximum in the range 50 to 500 nucleotides in the case of MR DNA, representing the lengths of the sequences themselves. Greater lengths represent the interspersion distances of the sequences and fall into two broad classes: (1) MR sequences interspersed with each other over fairly short distances of 750 to 2000 bases and (2) sequences of long range interspersions 2 to 30 kb. Recent studies employing long single-strand DNA of greater than 100 kb length prepared by special mild isolation procedures (see Biology 1974, No. 180) reveals qualitatively similar results. These experiments also revealed that a large fraction (60% by number) of MR elements is found in close contiguity to each other as doublets. To address the question whether longer MR sequences exist in the rat, we reassociated several MR or MR+HR DNA preparations of succeedingly greater fragment length (2.5, 5 and >10 kb) and scanned for long duplex regions. Again we found only double-strand sequences 500 nucleotides or shorter. Similar interspersion and self-reassociation experiments with HR material revealed that these are not extensively interspersed, but are found on long DNA as occasional networks indicating sequence clustering. No HR duplexes longer than 500 bases were found. Self-reassociated (Cot 0.005), dilute preparations of giant (>100 kb) single-strand DNA display numerous palindrome sequences, which are highly interspersed over distances widely ranging from a few to more than 100 kb. These elements are generally 200 to 1000 base pairs long. We conclude that all the repetitive and palindrome sequence elements in rat are short. The MR and palindrome elements are highly interspersed with single-copy material, while the existing evidence favors the view that HR sequences are serially repeated in clusters.

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Figure 1. Electron micrographs of rat DNA visualized by the Kleinschmidt procedure and prepared under conditions such that both double- and single-stranded DNA are visible. *Left:* Denatured DNA fragments about 1.2 kilobases in length and containing middle-repetitive sequences were reassociated to a middle-repetitive Cot. A double-stranded section in the center is attended by 4 single-stranded (single-copy) branches. The double-stranded region of this H form structure is approximately 200 base pairs long. *Right:* Denatured DNA fragments about 1.2 kilobases in length and each containing a middle-repetitive sequence were hybridized (in vast excess) to a middle-repetitive Cot to denatured unfractionated DNA of very great length (approximately 100 kilobases). The trunk DNA runs from upper left to lower center of the picture. In the center may be seen a U structure on the trunk DNA, consisting of a double-stranded region approximately 200 base pairs in length and attended by two radiating branches.
180. PREPARATION OF GIANT DNA FOR HETERODUPLEX MAPPING EXPERIMENTS

Investigators: Mahlon M. Wilkes*, David Holmes*

Support: National Institutes of Health, Public Health Service

For an analysis of long-term organization patterns of various sequences by heteroduplex mapping techniques, it is useful to have preparations of very long single-stranded DNA available. We have developed a technique for facile isolation of DNA, which when denatured, reassociated and visualized in the electron microscope has a length in excess of 100 kb. Some strands as long as 1000 kb have been seen. The technique involves suspension of packed rat ascites cells in 2.67 M CsCl, 6 M GuHCl, 0.005 M carbonate buffer (pH 9) at 60°C. The suspension is mixed by gentle swirling and centrifuged 48 hours at 60 K rpm in a titanium rotor. The DNA, which forms a sharp band in the center of the tube, may be recovered by meticulous use of a wide-bore pipette. We currently judge the location of the DNA band by viscosity, though we are now investigating the possibility of a more precise estimation by addition of ethidium bromide to the preparation. After dialysis into a buffer appropriate for hybridization experiments, this preparation has an ultraviolet absorption spectrum indicative of high purity.

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181. KINETIC STUDIES ON THE LENGTH AND DISTRIBUTION OF REPETITIVE SEQUENCES IN RAT DNA

Investigator: William R. Pearson

Support: National Institutes of Health, Public Health Service

Two techniques can be used to study the distribution of repetitive DNA sequences, visualization with the electron microscope using the Kleinschmidt technique (see Biology 1974, No. 179) and reassociation of different lengths of DNA to a repetitive Cot (Davidson et al., 1973; Graham et al., 1974). It is useful to compare the data on repetitive sequence structure using both techniques.

Previous studies have used the reassociation of long, labeled fragments driven by short, unlabeled DNA to determine the repetitive interspersion distance. However, when the DNA sequences are more than 1000-fold repetitive and also short (500 base pairs) a simple self-reaction of different length DNA strands can be used to quantitate both the interspersion distance and the length of the repetitive sequence.

Rat DNAs sheared to 350, 1600 and 3200 nucleotides were reassociated to Cot 50, a point at which all repetitive DNA has hybridized. These behave as 33%, 63% and 73% double-stranded on hydroxyapatite. This indicates that about 75% of rat DNA has a repetitive sequence within 3200 bases, in agreement with electron microscopy data. The decrease in hyperchromicity of the hybrids has been shown to be an accurate measure of the amount of the hybrid double-stranded, or in this case the length of the repetitive sequence (Graham et al., 1974). About 150 of the 350-nucleotide DNA are in hybrid while 250 nucleotides of the 1600-long reassociated fragments are in duplex. Thus the repetitive sequence length is probably around 200 nucleotides average, again in good agreement with electron microscope data.

References:
182. ISOLATION OF RAT ASCITES MESSENGER RNA

Investigator: William R. Pearson

Support: National Institutes of Health, Public Health Service

A procedure has been developed for isolation of the poly A-containing fraction of rat ascites messenger RNA free from heterogeneous cytoplasmic RNA or nuclear RNA. Studies on the DNA coding for this RNA and its relationship to high molecular weight RNA have begun.

Messenger RNA has been isolated by lysis of ascites cells without detergent, fractionation of the cytoplasm into 80S and >80S material on a sucrose gradient, conversion of the >80S material to ribosomal subunits with puromycin and fractionation of this material into 80S and <80S. This material is then passed through a poly U Sepharose column and the bound material eluted and again bound to poly U Sepharose. When ascites RNA is labeled with 3H-uridine for 4 hours in the absence of any drugs, about 10% of the polysome-associated label is bound to poly U Sepharose. The poly A-containing message has a specific activity of 20,000 cpm/µg and comprises about 2% of the mass of the polysomes.

183. NONHISTONE CHROMOSOMAL PROTEIN-DNA INTERACTION: CONDITIONS FOR SELECTIVE INTERACTION

Investigator: J. Sanders Sevall

Support: Damon Runyon Memorial Fund for Cancer Research

National Institutes of Health, Public Health Service

We have isolated a group of rat liver nonhistone chromosomal (NHC) proteins which shows preferential interaction with rat DNA (van den Broek et al., 1973). By use of an assay which depends on the retention of protein-DNA complexes on nitrocellulose filters (Riggs et al., 1970), the specificity of the DNA-protein interaction has been investigated. A constant amount of labeled, sheared (350 base pairs) rat 3H-DNA was saturated with increasing amounts of NHC proteins. At 50 mM ionic strength, 35% of the DNA was retained on the filter at saturation of the binding sites. When the unbound DNA was again mixed with the NHC protein mixture, none of the DNA was bound. Therefore, the NHC proteins of rat liver bind specific subsets of DNA sequences.

The effect of ionic strength on specificity of the DNA-protein interaction was investigated by competition experiments. Limiting concentrations of nonhistone protein and 3H-rat DNA (350 base pairs) were held constant while the concentration of competing unlabeled DNA was varied over a 25-fold range. Six competitor DNAs were used; those of rat, M. lysodeikticus, D. melanogaster, D. discoideum, calf thymus and E. coli. At low ionic strength, the eukaryotic DNAs all showed competition equal to that of rat. At high ionic strength (200 mM), the competition became specific for rat DNA and the other eukaryotic DNAs became inefficient competitors. The bacterial DNAs were poor competitors under all conditions tested. No correlation can be made between efficiency of competition and DNA base composition.

Our data show that an ionic strength of the reaction medium near that of the cell increases specificity of NHC protein-DNA interactions. Thus, the investigation of the DNA sequences which bind the NHC protein under high criterion may add to the knowledge of the molecular structure of eukaryotic chromatin.

References:


184. NONHISTONE CHROMOSOMAL PROTEIN-DNA INTERACTION: SEQUENCE RECOGNITION

Investigators: J. Sanders Sevall, Andrew Cockburn*, M. Savage**

Support: Damon Runyon Memorial Fund for Cancer Research
National Institutes of Health, Public Health Service

Analysis of the sequence structure of rat DNA indicates that it is organized into interspersed segments of middle-repetitive and single-copy sequences (Biology 1973, No. 105). The protein-binding characteristics of our homologous DNA-binding proteins to the purified components of the rat genome were therefore investigated.

Rat DNA, sheared to 350 base pairs, was fractionated into its middle-repetitive and unique DNA sequences by virtue of their reassociation kinetics and hydroxyapatite chromatography.

The second-order rate constant for the unique fraction is $K = 0.00105$ and for the middle-repetitive $K = 1.27$ as determined by their reassociation kinetics. The degree of duplex formation of the renatured fractions was determined by their hyperchromicity. Native rat DNA sheared to 350 base pairs had a $T_m$ of 84.5°C and a hyperchromicity of 0.337. The unique fraction had a $T_m$ of 81.0°C and 0.265 hyperchromicity while the repetitive fraction had a $T_m$ of 74.5°C and 0.256 hyperchromicity indicating that 75% of the renatured complex is in duplex form. The single-stranded nucleotide regions were removed by Aspergillus oryzae S-1 nuclease digestion. After nuclease treatment, both fractions showed 0.312 to 0.330 hyperchromicity, or over 91% duplex content. The nucleotide base pair length by electron microscopy was now 218.

Saturation of the two fractions of DNA by the DNA-binding NHC proteins at low ionic strength (0.05 M) retained 20% of the middle-repetitive DNA sequences and 30% of the unique sequences. Increased ionic strength (0.2 M) had no effect on the retention of the middle-repetitive DNA but decreased the retention of unique DNA to less than 10%. Under both ionic strength conditions, competition between the various fractions showed that middle-repetitive DNA is a highly efficient competitor for middle-repetitive labeled-DNA protein complexes while the unique DNA is a very inefficient competitor. With labeled unique DNA, the opposite is true. NHC protein binding to unique sequence DNA may be wholly due to the contamination of the latter by middle-repetitive sequences. Our studies indicate that the repetitive sequences of the rat genome include the selective binding sites for a class of DNA-binding nonhistone proteins.

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185. PREPARATIVE AMOUNTS OF NATIVE NONHISTONE CHROMOSOMAL PROTEINS

Investigators: J. Sanders Sevall, M. Savage*

Support: Damon Runyon Memorial Fund for Cancer Research
National Institutes of Health, Public Health Service

We have earlier reported that a small fraction (4%) of native rat liver nonhistone chromosomal (NHC) proteins interacts preferentially with rat DNA (Biology 1972, No. 165). To facilitate investigation of these proteins, a procedure to isolate them in large quantities has been developed. Chromatin is purified from rat liver by several washes of the crude nuclear pellet in 0.1% Triton X-100, 10 mM tris pH 8.0. Chromatin of rat liver prepared by standard procedures has a chemical composition of DNA:NHCP:histone ratio of 1:0.653 ± 0.022:1.12 ± 0.019. Our chromatin has the chemical composition 1:0.61:1.10. Comparison of the NHC polypeptide composition of detergent-purified chromatin with that of sucrose gradient-purified chromatin indicates that the former shows a decrease in the high molecular weight polypeptides but that all components are represented.

Chromosomal proteins were next dissociated
from DNA in 4 M NaCl at pH 8.0. DNA was separated from the chromosomal proteins by centrifugation at 255,800 x g for 14 hours. Histones were removed from the chromosomal protein pool by dialysis to 0.4 M NaCl, tris pH 7.0 in the presence of Bio-Rex 70 and elution of the NHC proteins with 0.4 M NaCl. Some basic nonhistone protein is retained on Bio-Rex 70 as are the histones.

Fractionation of the total NHC proteins by tandem series of E. coli-DNA cellulose columns followed by rat DNA-cellulose columns resulted in 4% of the total NHC protein remaining bound only to rat DNA. The total yield of such homologous DNA-binding protein is 3.5% of total NHC protein. The procedure outlined above makes it possible to isolate large quantities of rat DNA-binding NHC proteins.

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186. PURIFICATION AND STUDY OF INDIVIDUAL NONHISTONE CHROMOSOMAL PROTEINS

Investigators: Angeline Douvas, Christina Harrington*

Support: National Institutes of Health, Public Health Service

Efforts to isolate nonhistone chromosomal (NHC) proteins from rat liver chromatin have yielded four proteins in pure form. (1) A basic protein of molecular weight 37,000 daltons as estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. This protein is found exclusively in the nonhistone complement of template-active chromatin (Bonner et al., 1973) and may be an “informomer” or RNA packaging protein (Martin et al., 1973). (2) A basic nonhistone of approximate molecular weight 20,000 daltons. (3) A major nonhistone (molecular weight = 45,000 daltons) of which there may be as many as 10^6 copies per genome (Garrard et al., 1974). This protein is a major component of template-active chromatin. (4) A highly acidic nonhistone of molecular weight 31,000. We are now beginning to study the distribution of these proteins in chromatin by electron microscopy. Hopefully the nonhistones specific to template-active chromatin will be useful as markers for mapping chromosomal regions active in RNA synthesis.

Apart from the work with individual proteins we are studying a class of basic nonhistones ranging in molecular weight from 18,000 to 30,000 daltons. The DNA-binding capabilities of these proteins are indistinguishable from those of histones with respect to ionic strength dependence and DNA-binding capacity. By conventional techniques used for separating histones from nonhistones (acid extraction, Bio-Rex 70 chromatography) we find that the basic nonhistones segregate partly or entirely into the histone fraction. The effect of these proteins on the template activity of DNA is now being investigated.

References:

*Undergraduate, California Institute of Technology.
Rat liver chromatin contains a neutral protease with a marked preference for chromosomal proteins as substrate. The enzyme has been purified to homogeneity. This involved about a 700-fold purification from chromatin proteins. The enzyme has a molecular weight of 200,000 and contains 2 subunits of equal size; it is inhibited by classical serine protease inhibitors such as diisopropylfluorophosphate; it requires divalent ions as activators. The isolated enzyme appears to be similar to that responsible for the endogenous degradation of chromosomal proteins. The susceptibility of the 5 histone species to proteolysis by this enzyme is dependent on whether the histones are complexed to DNA. In intact nucleohistone the 4 major histones are rather resistant to proteolytic attack while histone I is rapidly attacked. If histones are freed from DNA, all the histone molecules are attacked at about the same rate with the exception of histone I, which is degraded more slowly than are the others. The enzyme is present to the extent of about 10,000 copies per genome.

Slime mold chromatin is isolated by modifications of procedures published by Sussman and Rayner (1971) and Bonner et al. (1968). This strain of *D. discoideum* (AX-3) yields about 6.5 g of cells at late log stage for every liter of axenic medium. The cells are harvested and washed twice in 7% sucrose. A nonionic detergent, Triton X-100, is used to lyse the cells in buffered sucrose. The lysis is much more effective if the solution is slightly warm (10 to 15°C). The crude nuclear pellet is then washed with saline-EDTA, followed by buffered sucrose with Triton X-100. Finally, centrifugation through a 1.7 M sucrose cushion yields purified chromatin. Alternatively, chromatin is prepared from cells harvested and stored frozen at -80°C. The frozen cells are disrupted by homogenization in a Waring blender in saline-EDTA.

Two primary difficulties have arisen in this purification. First, slime mold contains a high level of DNase activity. Approximately 75% of this activity can be attributed to the highly basic lysosomal enzyme DNase II; the rest is due to the nuclear DNase I. To eliminate degradation from either DNase the nuclei are quickly removed from the cytoplasm, while EDTA and a pH of 8 are used to inhibit the enzymes. Since some degradation still occurs, a high ionic strength is maintained to aggregate the chromatin for sedimentation. Secondly, slime mold has a very high lipid to DNA ratio and the nuclear membrane seems intimately associated with the chromatin. Triton X-100 solubilizes some of the lipid and the remainder is extracted with a low concentration of 2-octanol.

Chromatin purified in this manner has an ultraviolet spectrum similar to that from other eukaryotic chromatin. However, it contains more RNA and protein than do other chromatin. This may be simply due to the greater expression of single-copy DNA in *Dictyostelium* than in other creatures (Firtel, 1972). Less than 1% of the total lactate dehydrogenase activity co-purifies with the chromatin. Cytoplasmic contamination is therefore minimal. Additional controls are needed to deter-
mine if there is any mitochondrial or ribosomal contamination.

Once the purity of this chromatin is established, its proteins will be extracted and characterized. *Dictyostelium* has several basic, nuclear proteins, one of which is similar to histone I on urea gels. The nonhistones are a complex group of proteins.

The chemistry and functions of these proteins are yet to be determined.

References:


189. A PROGRAM TO STUDY DEREPRESSION OF A SPECIFIC REpressed GENE

Investigators: Keith E. Peters, Andrew Cockburn*

Support: National Institutes of Health, Public Health Service

Smith, Kline and French

We have started on a new way to try to find what chromosomal, nuclear, or other components are responsible for gene activation. We are trying to switch on a specific (previously repressed) gene in a Novikoff hepatoma cell line (#NTS7-67 from Dr. P. G. Plagerman). This gene codes for a nervous tissue-specific protein, S-100, initially characterized by Moore (1965). This protein is exclusively of nervous tissue origin, is present in relatively high concentrations, is well characterized and is immunochemically highly cross-reactive between species. The attempted activation of the suspension cell culture genes will be done by supplying them with materials from isolated rat brain nuclei. Such fractions include brain chromatin, its nonhistone chromosomal proteins, its chromosomal RNA, and its nucleoplasm. These are being added separately and in combinations to early log phase suspension culture hepatoma cells. One to two generations later (12 to 24 hours) the cells are labeled with 3H-leucine or a tritiated protein hydrolysate of high specific activity and 12 to 24 hours later the cells harvested. Extraction of soluble proteins with phosphate buffer will be followed by centrifugation at 105,000 x g. To this supernatant is added a small amount of antibody to S-100 (which we have prepared in rabbits using electrophoretically pure S-100 obtained from bovine brain) together with a known amount of cold S-100 carrier protein. After incubation at 37°C, followed by 4°C, the washed precipitate is dissolved in SDS buffer, electrophoresed on polyacrylamide gels and the gels sliced and counted to separate nonspecifically precipitated counts from those in S-100. The sensitivity of this method is such that nanogram quantities of protein should be detectable. Assuming the S-100 gene is activated, the scale of operation will be increased sufficiently to provide enough "inducer" material such that further separation and characterization by standard biochemical procedures may be carried out.

Reference:


*Undergraduate, California Institute of Technology.

PUBLICATIONS


Professor Emeritus: A. J. Haagen-Smit

Dr. Haagen-Smit continued his efforts to bring clean air to California as Chairman of the State Air Resources Board. In January 1974 he retired from this Board but continued his activities for the State as Chairman of its Research Committee. At the Federal level, he served as Chairman of the National Air Quality Committee of the Environmental Pollution Administration and as a member of its Science Advisory Board.

PUBLICATIONS


The germination factor of *Neurospora crassa* (GF) is 20 to 25 times more concentrated in conidia than in the mycelium. Treatments which increase membrane permeability—e.g., lowering the water activity of the medium—cause loss of GF from the conidia and thereby prevent their germination. GF is synthesized by growing mycelia and may be essential for hyphal growth as well as conidial germination, but there is no direct evidence on this point. GF is a dialyzable, acid-labile substance of unknown structure with an estimated molecular weight on the order of 1000. It has been found in various species of *Penicillium* and *Aspergillus*, as well as *Neurospora*.

The Carbon Assimilation Instrument is one of three biological experiments which will be carried to Mars aboard the Viking spacecraft to be launched in 1975. It is designed to detect the incorporation of carbon from CO$_2$ or CO into soil organic matter. Since it does not depend on aqueous media, it can operate under Martian conditions of temperature, pressure, and atmospheric composition. The other two Viking biological experiments utilize aqueous solutions. In addition to these instruments, Viking will carry an automated pyrolysis-gas chromatograph-mass spectrometer which will perform atmospheric and soil organic analyses. Together with a pair of facsimile cameras, these make up the complement of life-seeking experiments on the Viking lander.

The photocatalytic synthesis of organic compounds on silicate surfaces under Martian conditions was discovered in the course of developing the Carbon Assimilation experiment. We believe that this reaction is taking place on Mars at the present time.

### 190. NEUROSPORA GERMINATION FACTOR

**Investigators:** Gisela Charlang, George Horn, Norman H. Horowitz

**Support:** National Aeronautics and Space Administration

As reported last year, conidia actively concentrate the germination factor (GF). Further experiments on the uptake mechanism show that it is manifested about 2 hours after conidia are inoculated into growth medium, or about 1 hour before germination. Development of the uptake mechanism is inhibited by cycloheximide or by omission of either the carbon source or the salt component of minimal medium. Addition of GF to the medium does not hasten the appearance of the uptake mechanism. If supplied with exogenous GF, conidia will take up about 5 times their normal complement of the factor.

Attempts to induce a GF-destroying enzyme in *Neurospora* have not succeeded. A variety of polyols have been used as inducing agents, since GF appears to be a polyol. No GFase activity is detectable in either young or old cultures. *Neurospora* excretes GF into the medium. Thus, 48-hour cultures contain as much GF in the medium as in the cells.

An assay procedure for GF which uses EDTA in place of NaCl to induce a GF requirement has been developed. It has the advantage of greater sensitivity and a shorter incubation time (48 hours as against 96 hours for the NaCl assay).

A germination-defective mutant (cgr) discovered and kindly supplied by J. Schmit of the University of California, San Diego, has shown a germination and/or growth response to GF in preliminary tests. Since cgr conidia appear to contain normal amounts of GF, the significance of the positive response is not clear. Studies are continuing.

Although GF is apparently a polyol, it does not form a charged complex with borate. This suggests that it does not possess cis-hydroxyl groups, or alternatively that it contains cis-hydroxyl groups in a pyranose ring. In either case, a weak or negative reaction with borate would be expected.

Chromatography of GF concentrates on silica gel, with ethanol and methanol as solvents, yields a product with some increase in specific activity.

The best preparations are active at concentrations of less than 0.01 µg/ml in the NaCl assay.
191. THE CARBON ASSIMILATION EXPERIMENT FOR MARS

Investigators: George L. Hobby, Jerry S. Hubbard, Paul K. Cartier III, Norman H. Horowitz

Support: National Aeronautics and Space Administration

The experiment was described in last year's report. The instrument that will perform this experiment on Mars is being built by TRW. During the year a variety of technical problems were encountered involving the thermal design, heater bonding, high temperature seals, electromagnetic noise and liquid transport systems. The packaging design of the xenon arc lamp was one major problem. Lamp and anode lead breakages occurred in several assemblies due to thermal stresses that developed in the elastomeric potting compounds used to provide electrical insulation. Viton seals used on the pyrolysis and incubation chambers failed during testing on flight prototype instruments, and the carbon-14 detection system proved to be excessively susceptible to electromagnetic interference. A critical heater in the pyrolysis system failed to provide required temperature profiles.

Currently, six instruments are being built: four for flight and two for engineering tests. These instruments incorporate the revised designs that are solutions to the earlier problems.

A follow-up study was made during the year of a problem arising at the beginning of 1973. This involved excessive retention of $^{14}\text{CO}_2$ on the gas chromatographic column that is supposed to pass $^{14}\text{CO}_2$ and $^{14}\text{CO}$ and retain volatile organic molecules. It was found that the effect is associated with the CuO that is incorporated into the column packing material (Chromasorb). The retention can be prevented by heating the column at 950°C in O$_2$, with subsequent cooling in CO$_2$.

192. PHOTOCATALYTIC SYNTHESIS OF ORGANIC COMPOUNDS ON SILICEOUS SURFACES

Investigators: Jerry S. Hubbard, Gerald E. Voecks*, George L. Hobby, Norman H. Horowitz

Support: National Aeronautics and Space Administration

The President's Fund

The photocatalytic conversion of CO to simple organic compounds can be effected on a variety of siliceous particles (Hubbard et al., 1973). A unique aspect of these syntheses is that they are catalyzed by UV of wavelengths longer than those absorbed by the gaseous reactants. Thus, these syntheses may have relevance to the questions of chemical evolution on contemporary Mars and on the primitive Earth because of the abundance of solar energy available at the active wavelengths. The following results were obtained in the past year.

Irradiation of $^{14}\text{CO}$ in the presence of substratum yields $^{14}\text{C}$-formate ester of the silica substratum as the major product. Protic solvents hydrolyze the ester yielding $^{14}\text{C}$-formic acid.

When substratum containing surface-adsorbed NH$_3$ is irradiated in the presence of $^{14}\text{CO}$, the major products are $^{14}\text{C}$-urea and $^{14}\text{C}$-formamide with smaller amounts of $^{14}\text{C}$-formaldehyde and unidentified compounds. UV-catalyzed reactions involving NH$_3$ and surface formate ester also yield the organic nitrogen products. The rate of the CO-NH$_3$ reaction increases with decreasing temperatures, being almost proportional to the absorption isotherm for NH$_3$. Apparently the photolysis of surface-adsorbed NH$_3$ to NH$_2$· and H· is the initiating step in the reaction. Substrata with greater availability of surface hydroxyl groups give higher yields of formamide. Hydrogen bonding between the formate ester and adjacent hydroxyl groups apparently determines the preferred site of attack of NH$_2$·. In addition to its possible importance in chemical evolution, the CO-NH$_3$ reaction could provide a mechanism for the synthesis of organic molecules on grain surfaces in interstellar space.
A combined gas chromatograph-radiation detector has been constructed for use in identification of other products of these syntheses.

Reference:

*Jet Propulsion Laboratory.

PUBLICATIONS


Assistant Professor: Henry A. Lester

Graduate Student: Robert E. Sheridan Jr.

Research Staff: W. Larry Crowder, Ruth E. Estey, Donna R. Williams

Our nascent laboratory is interested in synaptic transmission: how synapses function and how they develop.

This year we have concentrated on the physiology of cholinergic transmission. The electroplaque of the electric eel, *Electrophorus electricus*, contains about $10^{11}$ nicotinic acetylcholine receptors on its innervated membrane. These receptors, similar to those at the endplate region of skeletal muscle, are studied by bath application of agonists (acetylcholine and such) to the entire face. We are busily investigating physiological influences on the way the receptor functions.

The program on synaptic development is supported by a fellowship from the Alfred P. Sloan Foundation. We have two presumptive projects, neither sufficiently differentiated to warrant an abstract of its own. The first involves our stock in trade, the nicotinic synapse. We intend to follow the establishment of neuromuscular synapses in cultures of chick myoblasts and in the tails of South African clawed toad (*Xenopus*) tadpoles. The second project involves possible synaptic reorganization during amphibian metamorphosis. We have been studying the Mauthner neurons of bullfrog tadpoles with light microscopy and shall probably bring electrophysiology, electron microscopy, and histochemistry to bear on them as well.

**193. CONDUCTANCE INCREASES PRODUCED BY BATH APPLICATION OF CHOLINERGIC AGONISTS TO ELECTROPHORUS ELECTROPLAQUES**

**Investigators:** Henry A. Lester, J.-P. Changeux*

**Support:** Sloan Fund for Basic Research
Robert E. and May R. Wright Foundation Fund
National Institutes of Health, Public Health Service

Experiments performed previously in Paris have been analyzed in terms of the steady-state chord conductance induced by two esterase-resistant agonists, carbachol and decamethonium. For a linear instantaneous current-voltage curve (*Biology 1974*, No. 194), these data measure the number of ion channels in the membrane produced by agonists.

In the presence of saturating agonist concentrations and at normal resting potential, we find roughly 3 milliamperes of voltage-clamp current in an entire electroplaque's face. This is equivalent to an average of 180 monovalent ions per millisecond for each receptor in the membrane. An elementary event, lasting a millisecond but involving a net flux of about 12,000 ions, underlies agonist-induced conductance (*Katz and Miledi, 1972*). To reconcile these two findings, we therefore hypothesize that even when a molecule of agonist is bound to the receptor, the ion “channel” opens infrequently.

The chord conductance induced by agonists varies with membrane potential: a shift of 62 mV in potential in the negative direction causes an e-fold increase in conductance. The following data strongly suggest that membrane potential affects receptor function at some site which is distinct from the interaction between the agonist and its binding site: (1) The e-fold change for 62 mV occurs for both the monoquaternary, carbachol, and for the bisquaternary, decamethonium. (2) The dose-response curve for both these agonists has a sigmoid beginning, with a Hill coefficient of 1.9 for carbachol and 1.45 for decamethonium. The Hill coefficient does not vary with potential. (3) The dissociation constant for the competitive inhibitor, d-tubocurarine, is also not dependent upon potential.

Thus, we hypothesize that membrane potential influences the probability of opening for the channel. Variations in membrane potential may
provide a selective probe for the ion channel (see Biology 1974, No. 194).

Reference:

*Institut Pasteur, Paris, France.

194. RELAXATION SPECTROSCOPY OF THE ACETYLCHOLINE RECEPTOR IN EEL ELECTROPLAQUE

 Investigators: Robert E. Sheridan Jr., Henry A. Lester

Support: National Institutes of Health, Public Health Service
          National Science Foundation
          Sloan Fund for Basic Research
          Robert E. and May R. Wright Foundation Fund

Instantaneous current-voltage curves have been measured for agonist-induced conductances. These are linear at negative potentials and indicate a reversal potential of +10 to +12 mV at 15°C for the concentrations of carbachol and decamethonium so far studied. Therefore, the synaptic conductance appears to involve both sodium and potassium components. Over the range of 14°C to 26°C, the steady-state agonist-induced conductance at -100 mV has a Q10 of approximately 1. This value is characteristic of a simple Boltzmann equilibrium between “open” and “closed” populations of receptor channels.

We have begun studies of the kinetics of transitions in the acetylcholine receptor. The agonist concentration is held constant and the membrane potential is jumped (under feedback control, see Biology 1974, No. 195) from one value to a second. As a result, the agonist-induced conductance “relaxes” at measurable rates which probably reflect the time course of opening and closing for the individual ion channels (see Biology 1974, No. 193). Typically, the conductance relaxes exponentially with time to the new level. The rate constant, τ, of this change does not depend on the past history of the membrane potential and is characterized only by the value of the second potential, the temperature, and the type of agonist. The variation with the second potential (V) is given by: \( \tau = A \cdot e^{(B \cdot V)} \). At 15°C, A equals 0.59 msec for carbachol and 0.35 msec for decamethonium; B equals 0.0054 mV\(^{-1}\) in carbachol and 0.0059 mV\(^{-1}\) in decamethonium. At -100 mV in carbachol, the Q10 of \( \tau \) lies between 2 and 4. Comparable values have been reported in experiments on jumps in acetylcholine concentration at constant transmembrane potential (Magleby and Stevens, 1972a,b).

We conclude that while the equilibrium between “open” and “closed” receptor channels does not depend strongly on temperature, the kinetics of the transition between “open” and “closed” channels does vary a great deal with temperature.

References:
195. HIGH SPEED, COMPUTER-CONTROLLED VOLTAGE CLAMP OF EEL ELECTROPLAQUE

Investigators: Fred Sigworth*, Robert E. Sheridan Jr., Henry A. Lester

Support: National Institutes of Health, Public Health Service
National Science Foundation
Sloan Fund for Basic Research
Robert E. and May R. Wright Foundation Fund

One of the most useful techniques in cellular neurophysiology has been the "voltage clamp": current is injected by feedback to maintain a constant potential across a cell membrane. Thus, one can monitor the time- and potential-dependent conductances of the membrane, without the difficulties of simultaneous changes in time and membrane potential which occur during normal, "unclamped," cell activity. The temporal resolution of a voltage clamp is the time necessary for the feedback network to stabilize. This cannot occur faster than the time constant of the potential sensing system, which in turn is limited by the high capacitance and resistance of glass microelectrodes necessary for intracellular recording. The best clamp times which have been obtained with such electrodes are approximately 200 microseconds—no faster than the rate constants in a number of neuronal processes. Thus, a faster clamp is desirable.

One way to speed up the clamp is to reduce the resistance of the potential recording electrodes. This can be accomplished with the eel electroplaque as an experimental preparation. The cell is shaped roughly like a thin pancake with all active elements on one planar symmetric face. The passive face is highly convoluted, with a very low total resistance. Thus, when recordings are made across the entire cell, with suitable corrections for the resting potential and series resistance between the electrodes, the system accurately measures events at the active face. In this way, low resistance electrodes may be used to record the potential across the active face, without damaging the cell membrane by penetrating it with these large-tipped electrodes. A voltage clamp has been constructed in accordance with these principles and tested on an electrical analog of the eel electroplaque. We note a reduction in the "settling time" of the clamp to less than 60 microseconds. We look forward to considerably better time resolution of processes associated with synaptic transmission.

Since acetylcholine receptors desensitize rapidly in the presence of high agonist concentrations, the voltage-clamp trials must be performed within a few seconds. We therefore are preparing our Nova 2 computer to generate the desired sequence of membrane potential waveforms. The computer controls the entire experiment, collects the data on voltage-clamp currents, and also performs a small amount of on-line analysis.

*Undergraduate, California Institute of Technology.
A STUDY OF THE ACETYLCHOLINE RECEPTOR IN ARTIFICIAL PHOSPHOLIPID BILAYER MEMBRANES

Investigators: Donald Miller*, Fred Sigworth*, James Hall**, Carver Mead**, Daniel Michaelson†, Michael Raftery†

Support: California Foundation for Biochemical Research
National Institutes of Health, Public Health Service

Most studies on isolated acetylcholine receptors have explored the binding ability of various drugs, without regard to the receptor's action in ion transport across cell membranes. A technique is necessary to incorporate receptors into artificial phospholipid bilayer membranes and to measure ion permeation through such structures.

In forming these artificial phospholipid bilayers, the method of Montal and Mueller (1972) is employed. There is a small hole in a partition which separates two pools of liquid; there are electrodes in each pool. First, a monolayer of phospholipid is formed on the fluid surfaces of the two pools. The phospholipids arrange themselves so that their hydrocarbon tails stick up into the air. The fluid levels are then raised until the monolayers rise past the hole in the partition; the two monolayers fuse to form a bilayer membrane.

Using this technique, attempts are being made to fuse receptor-rich membrane fragments or liposomes into the monolayer; the resulting bilayer appears to contain active receptors.

This technique will allow the study of the channel properties of individual receptors.

Reference:

*Undergraduate, California Institute of Technology.
**Division of Engineering and Applied Science, Department of Electrical Engineering, California Institute of Technology.
†Division of Chemistry and Chemical Engineering, California Institute of Technology.

PUBLICATIONS


We are analyzing a cluster of bithorax genes in *Drosophila* whose function is to control the level of development achieved by certain body segments. Flies which carry mutant genes belonging to this cluster have striking transformations of the normal body segmentation, such as four wings instead of two, or eight legs instead of six. These mutant effects arise in general as the result of inactivation or loss of the normal or wild-type genes. We are trying to learn more about how the bithorax genes are regulated by increasing the number of doses of the wild-type cluster of genes. Thus far, there are only slight effects associated with adding three doses to the two doses that the fly normally carries. We are using X-rays to induce chromosomal rearrangements which should provide the tools for greatly increasing the number of doses of bithorax gene clusters. Another approach that we are using to study the bithorax cluster is outlined below.

**197. SCANNING ELECTRON MICROSCOPE STUDIES OF BODY SEGMENT TRANSFORMATIONS IN DROSOPHILA**

*Investigators:* Edward B. Lewis, M. Terri Marton

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

We are using the scanning electron microscope (SEM) to investigate the fine structure of the cuticle of the *Drosophila* larva and adult. Our purpose is to determine how the pattern of differentiation is affected by certain mutant genes which profoundly alter the segmentation pattern of the organisms. It has already been determined that one such mutant, bithoraxoid, bxd, a recessive gene in the middle of the right arm of the third chromosome, is able to modify the first abdominal segment of the adult into a thoracic-like structure (notably producing flies with seven or eight legs instead of the usual six). Although no trace of an effect of bxd on other abdominal segments can be seen in the adult, we have identified, with the aid of the SEM, a transformation of additional abdominal segments of the larva towards a thoracic-like state. We have discovered that the *Drosophila* larva normally has a row of four very minute hairs arising dorsally on the cuticle of each of the three thoracic segments of this organism, whereas each of the eight abdominal segments normally has only two such hairs. We have found that the bxd mutant causes three to six of the larval abdominal segments to develop three to four, instead of two, hairs. Thus the bxd mutant effects a generalized transformation from an abdominal to a thoracic state.

Another use of the SEM is to analyze the segmentation pattern of the adult fly. Dorsally, the fly has an enormous overdevelopment of its second or mesothoracic segment. The third or metathoracic segment bears only the tiny halteres in place of wings and the first or prothoracic segment has been thought to be represented only by a humerus, a structure resembling an epaulet arising at the anterior edge, and on each side of, the mesothorax. We have found that a specific bithorax mutant which transforms the metathoracic segment into a mesothoracic one is able to produce a humerus-like structure in the metathoracic region. The presumption is strong that the humerus is really an offshoot of the mesothorax rather than the prothorax. Our results suggest that...
Figure 1. An SEM photograph of the head of a *Drosophila* larva (photograph by M. Terri Marton).
there are no prominent features in the dorsal thoracic region of the fly that are clearly attributable to the prothoracic segment. Instead it may be that the prothorax has become fused with the head segments. If so, a possible explanation is then seen for the mutant ophthalmoptera which causes wings to begin to grow out of the head region. These wings could represent a modification of the prothoracic tissue in the head segment in much the same way that the halteres can be converted in the metathorax to wing-like structures in the presence of specific mutants belonging to the bithorax gene complex.

**PUBLICATIONS**


Assistant Professor: Daniel McMahon

Graduate Students: John W. Cross Jr., Stanley R. Hoffman, Mark J. Miller

Research Staff: William G. Fry

We are continuing our empirical and theoretical studies of cellular interactions in development. Our experimental studies of the plasma membrane of D. discoideum have shown that it is extensively remodeled during development. Some of these changes are described below. We also include evidence which indicates the changes are coupled to development. One of the most important of these investigations has been the discovery of a developmental mutant whose plasma membrane contains identified molecular defects.

Our studies of the action of two drugs, chloral hydrate and actinomycin D, on C. reinhardi have progressed substantially. We discovered some time ago that chloral hydrate (knockout drops) was an inhibitor of protein synthesis in C. reinhardi and in cultured mouse neuroblastoma. Our recent studies have begun to uncover the sequence of biochemical events that are involved in this effect of chloral hydrate.

Although actinomycin D is an important agent for chemotherapy of cancer, the mechanism by which it kills cells is unknown. We have been able to isolate mutants which are resistant to actinomycin D. The interesting properties of these mutants are proving useful for the elucidation of the effects of actinomycin D and for studying the control of RNA synthesis in C. reinhardi.

198. CHARACTERIZATION OF D. DISCOIDEUM MEMBRANE PROTEINS AND GLYCOPROTEINS

Investigators: Stanley R. Hoffman, Daniel McMahon

Support: National Institutes of Health, Public Health Service

D. discoideum cells alter their adhesiveness during development suggesting that changes in the composition of the plasma membrane occur. In order to verify this, we have characterized the plasma membranes of D. discoideum purified from cells at 3 different stages of development. We have analyzed the protein and glycoprotein composition of these membranes by SDS gel electrophoresis.

SDS gels of log phase membranes, stained for protein with Coomassie blue, show 32 peptides with molecular weights ranging from 10,000 to 258,000 daltons. During development from log phase amoebae to preculmination, 5 polypeptides increase in amount and 6 disappear or decrease. SDS gels of log phase membranes stained by the PAS technique for glycoproteins, show about 15 bands of apparent molecular weights 15,000 to 170,000 daltons and an intensely stained region that runs with the tracking dye, and seems to be glycolipid. Almost every glycopeptide changes in relative amount during development. In addition 3 major new glycopeptides appear.

If intact log phase cells are treated with pronase, 5 polypeptides are destroyed. We believe that these are on the external surface, exposed to the medium. In preculmination, 4 new polypeptides are sensitive. Glycopeptides are not sensitive to pronase in intact cells until the preculmination stage when 2 of the new glycopeptides are degraded. Pronase treatment of purified membranes destroys almost all high molecular weight peptides, suggesting that they are on the membrane's cytoplasmic face. One high molecular weight peptide, 121,000, is resistant to this treatment. For a number of reasons we believe that it is the intramembrane particle of log phase cells which Gregg has described.

Reference:
199. EFFECTS OF CYCLOHEXIMIDE ON DEVELOPMENTAL CHANGES IN THE D. DISCOIDEUM PLASMA MEMBRANE

Investigators: Stanley R. Hoffman, Daniel McMahon

Support: National Institutes of Health, Public Health Service

Cycloheximide inhibits macromolecular synthesis and the development of *D. discoideum*. We wished to see if changes occur in the plasma membrane composition when normal morphogenesis is blocked by cycloheximide.

Cells were prevented from aggregating by treating with cycloheximide. Analysis of the plasma membranes by SDS gel electrophoresis showed that all glycopeptide changes were blocked. One polypeptide change was blocked but 2 were not. In the presence of cycloheximide, cells that would have developed to the pseudoplasmodium stage formed patches of structures typical of late aggregation interspersed with regions of no visible development. Membranes prepared from these plates showed inhibition of all polypeptide changes characteristic of the pseudoplasmodium stage except one. All the characteristic decreases in glycopeptides seem to have occurred except one. The synthesis of 3 new glycopeptides is inhibited. In addition a glycopeptide which is a minor component in untreated aggregation stage membranes and which is absent in log or pseudoplasmodium stage is a major component of this kind of preparation.

200. A DEVELOPMENTAL MUTANT OF D. DISCOIDEUM WITH AN ALTERED PLASMA MEMBRANE

Investigators: Daniel McMahon, Stanley R. Hoffman

Support: National Institutes of Health, Public Health Service

We have isolated a spontaneous mutant of *D. discoideum* which does not aggregate normally and whose terminal state of development is small, loose aggregates of 10 to 100 cells. When this mutant is mixed with wild-type cells it interferes with their development although mutant cells are incorporated into the sorocarps and differentiate into spores.

Plasma membranes purified from log phase cells of this mutant are deficient in a “spectrin-like” internal, extrinsic protein, and in addition, are deficient in a high molecular weight intrinsic glycoprotein while a new low molecular weight glycoprotein has appeared.

The simultaneous appearance of the developmental and biochemical defects and the general pattern of the properties of this mutant suggest that the membrane defect may be the cause of the developmental defect. We are currently mapping the location of this mutant in collaboration with Dr. R. Kessin, Harvard University, to obtain some additional insight into this relationship.

201. CHEMICAL MESSENGERS IN DEVELOPMENT: A HYPOTHESIS

Investigator: Daniel McMahon

Support: National Institutes of Health, Public Health Service

The chemical basis for the formation of pattern in developing systems has not been resolved although it is a problem of some interest. There appear to be two classes of phenomena involved in pattern formation: morphogenetic field organiza-
induction, i.e.,

1) a) External inducers interact with receptors on the plasma membrane of developing cells.
   b) Inducers are peptide hormones and neurotransmitters.

2) These generate internal changes in ion and cyclic nucleotides in the affected cells.
3) The temporal pattern of ion and cyclic nucleotide changes determines the developmental path of the cell.

Application of these postulates to some developing systems has generated some interesting predictions that are discussed in a paper presenting this model.

References:

202. THE MECHANISM OF ACTION OF CHLORAL HYDRATE

Investigator: John W. Cross Jr.

Support: National Institutes of Health, Public Health Service

Chloral hydrate has long been known as an anesthetic and an inhibitor of mitosis. We have recently shown that it also inhibits protein synthesis in Chlamydomonas at concentrations similar to those causing anesthesia and mitotic arrest (McMahon and Blaschko, 1971). Our efforts are now directed to finding the molecular basis of this effect.

We find that 50% of the polysomes in chloral hydrate-treated cells break down to monoribosomes within minutes after the drug is added. However, the polysomes gradually reform, and after several hours are at a higher percentage of the total ribosomes than initially. This behavior occurs at a concentration which inhibits 85 to 90% of the incorporation of radioactive amino acids into protein. The transient breakdown is prevented by cycloheximide or by chilling the cells, so the cause is unlikely to be simply reduced ribosomal stability or RNase action. The reformation phase occurs slowly or not at all in ts13, a mutant defective in initiation of protein synthesis (see Biology 1974, No. 205). In addition if ts13 cells treated with chloral hydrate for 8 or 9 hours are raised to the restrictive temperature, the polysomes break down just as they do in the undrugged control. Thus, the polysomes in drug-treated cells are unlikely to be some special aggregation not formed by ordinary initiation. We also find that the monoribosomes formed during the transient breakdown will dissociate into subunits if treated with high salt concentrations. This result is conventionally interpreted as meaning that these monoribosomes are not bound to mRNA and are hence inactive for protein synthesis (Martin, 1973).

Chloral hydrate itself is responsible for these effects since we do not find any significant amounts of its metabolic products, trichloroethanol or trichloroacetic acid, in cells over a 24-hour period. Nevertheless, trichloroethanol, which is also an anesthetic, inhibits protein synthesis and causes polysome breakdown at similar concentrations and with similar kinetics as chloral hydrate.

Polysome breakdown is not accompanied by normal peptide release, as equal numbers of peptidyl puromycin molecules are formed after incubation of chloral hydrate-treated and untreated ribosomes with radioactive puromycin.

References:
203. SYNTHESIS AND PROCESSING OF CHLOROPLAST RIBOSOMAL RNA IN CHLAMYDOMONAS REINHARDI

Investigator: Mark J. Miller
Support: National Institutes of Health, Public Health Service

The chloroplast possesses many properties which are similar to those found in prokaryotes. Among these are the chloroplast's sensitivity to certain drugs and the size of its ribosomal RNA. The plant alkaloid camptothecin will inhibit RNA synthesis in eukaryotes, but prokaryotes are resistant. I have found that camptothecin will inhibit the synthesis of cytoplasmic ribosomal RNA (rRNA) in the protozoan Chlamydomonas reinhardi while still allowing chloroplast rRNA synthesis to continue, though at a lower rate than in uninhibited cells. This has made it possible to determine the processing scheme for chloroplast rRNA. Pulse incorporation of $^{32}$P0$_4$ shows that label first appears simultaneously in peaks of molecular weight 1.14 M and 0.64 M ($M = 10^6$ daltons), which appear to be precursors of the mature 1.07 M (23S) and 0.54 M (16S) rRNAs respectively. Label is also found in a 0.56 M species which is presumed to be an intermediate in the synthesis of the mature 16S rRNA. By chasing short pulses of $^{32}$PO$_4$ with rifamycin SV, an inhibitor of chloroplast rRNA transcription, label found in the 1.14 M and 0.64 M peak migrates into the mature chloroplast rRNA, thus demonstrating the precursor nature of these RNAs. There is no evidence of a single precursor for both species of rRNA as is found in the synthesis of cytoplasmic rRNA (Biology 1972, No. 23).

The results demonstrate two new ways in which the chloroplast resembles prokaryotes: (1) in the way in which it processes its rRNA; and (2) in its resistance to inhibition by camptothecin.

204. STUDIES ON TEMPERATURE-SENSITIVE MUTANTS OF CHLAMYDOMONAS REINHARDI WHICH ARE RESISTANT TO ACTINOMYCIN D KILLING

Investigator: Mark J. Miller
Support: National Institutes of Health, Public Health Service

I have previously reported (Biology 1973, No. 253) the isolation of temperature-sensitive mutants in the protozoan Chlamydomonas reinhardi which are resistant to killing with actinomycin D (AMD). These cells are resistant to AMD at both the permissive (22°C) and nonpermissive (33°C) temperatures. At 22°C the mutant incorporates $^3$H-AMD at the same rate as do wild-type cells. Hence they are not defective in their permeability to AMD. At 33°C, however, while wild type increase their rate of $^3$H-AMD uptake by 2- to 4-fold, the rate of uptake in the mutant cells becomes only half that of the wild type. The mutant's sensitivity to AMD killing also increases only marginally while wild-type cells die at nearly a 10-fold greater rate. Hence, the lethal effects of AMD appear to involve a temperature-sensitive cellular function which is defective in the resistant cells.

The mutants appear to be defective in their synthesis of RNA when placed at 33°C. My previous report (Biology 1972, No. 24) that, in one mutant, there was a preferential inhibition of incorporation into the large ribosomal RNA (rRNA) subunit of both the cytoplasmic and chloroplast ribosomes, appears now to have been an artifact due to differential extraction of the rRNAs. However, analysis of the incorporation rates of $^{32}$PO$_4$ into the rRNAs of these mutants indicates that they are defective in their rRNA transcription and/or its processing at 33°C. Experiments are currently in progress to determine the locus of these defects.
205. TEMPERATURE-SENSITIVE MUTANTS OF CHLAMYDOMONAS

Investigator: John W. Cross Jr.

Support: National Institutes of Health, Public Health Service

We have continued to characterize two temperature-sensitive mutants, tsl and tsl3, which are defective in protein synthesis. As reported in Biology 1973, tsl is defective in initiation of protein synthesis. This conclusion was based on the rapid breakdown of polysomes into 80S ribosomes which occurs after shift of the cells to the restrictive temperature (33°C). The breakdown was completely prevented by cycloheximide, an inhibitor of peptide elongation. To confirm this result, tsl3 protein synthesis was tested in vitro. The test system was based on that of Schreier and Staehelin (1973) and used endogenous messenger. In this system incorporation of labeled amino acid by tsl3 ribosomes is quite low compared with the parent strain, arg2, or with tsl ribosomes, and is not further reduced at 33°C. Preliminary data indicate that addition of an arg2 ribosomal extract enriched in initiation factors stimulated incorporation by tsl3 ribosomes. The tsl3 ribosomes were also stimulated but to a lesser extent by postribosomal supernatant proteins from arg2. In addition, supernatant proteins from tsl3 fail to stimulate arg2 ribosomes as do arg2 supernatant proteins. We also know that none of the aminoacyl tRNA synthetases are defective (Biology 1973). Together, these results indicate involvement of a supernatant factor in the molecular defect of tsl3, but the results do not yet distinguish between initiation and transfer factors. However, in view of the nearly complete breakdown of tsl3 polysomes at 33°C, we believe that an initiation factor is most likely defective.

Tsl ribosomes have been tested in the same in vitro system, but they are consistently more active than even arg2. Tsl supernatant factors are equally as active as those of arg2 tested with arg2 or tsl ribosomes. At 33°C the tsl ribosomes and supernatant factors are not inactivated. Since tsl polysomes showed only partial breakdown at 33°C, we believe that peptide elongation is most likely the site of the lesion, but we have not been able to find an in vitro assay which is sensitive to the defect.

Reference:


Professor: Ray D. Owen
Gosney Research Fellow: Joan Klotz
Graduate Students: Tommy C. Douglas, Susan L. Melvin, Suzanne Ostrand-Rosenberg
Research Staff: Elizabeth P. Blankenhorn
Laboratory Staff: Kathleen Gallaher, Nancy E. Johantgen, Donna R. Williams

The Immunogenetics group works at one of the busiest intersections in current biomedical sciences—the confluence of genetics and immunology. Our main interests are in cell membrane specificities detected by the sensitive methods of serology and manipulated wherever possible with the tools of genetics. We deal with “differentiation antigens,” specificities that appear on cells, or others that disappear, in association with the tuning of particular cell populations for the performance of defined functions in the organism. Cells in the immune system itself are objects of our curiosity; we are interested in brain and blood cells, too. Antigens, cellular or soluble, that mark stages of development, and which may reappear during the course of tumor formation, are especially provocative to us. With help from Jean-Paul Revel and some of his people, we have enjoyed visualizing specific cell surface sites with electron microscopy; we have been the recipients of extraordinary generosity from Professor Clyde Stormont and his group at the University of California, Davis; and we profit through active collaboration with Dr. Ray Teplitz at the City of Hope Medical Center and Dr. Murray Gardner at the University of Southern California School of Medicine. Some of the fun we have had during the past year is soberly summarized in the following abstracts.

206. FUNCTIONAL EFFECTS OF ANTIBODIES ON RAT PERIPHERAL LYMPHOCYTES

*Investigator:* Tommy C. Douglas

*Support:* U.S. Atomic Energy Commission, National Institutes of Health, Public Health Service

The theta antigen of the mouse is carried by one or more molecules, possibly glycolipid in nature, which can be detected on the surface membranes of thymus-derived lymphocytes (T-cells) but not on antibody-producing cells or their precursors (B-cells). The two genetic variants of theta, Thy-1.1 and Thy-1.2, are the products of two autosomal codominant alleles, Thy-1<sup>a</sup> and Thy-1<sup>b</sup> respectively. Antibodies specific for one form of the antigen can be obtained by immunizing animals homozygous for one Thy-1 allele with T-cells from those homozygous for the other. In the presence of a series of serum proteins collectively referred to as the complement system, antibodies directed against the theta antigen present on a given cell can cause membrane damage and eventually cytolysis.

Peritoneal cells from inbred AKR/J mice (homozygous for Thy-1<sup>a</sup>) previously immunized with EL4 mouse leukemia cells are capable of killing these tumor cells in vitro in the absence of complement. That this is dependent upon the action of T-cells is indicated by the observation that pretreatment of the peritoneal cells with anti-Thy-1.1 and complement completely abolishes their anti-tumor activity.

Despite the fact that rat thymocytes possess an antigen indistinguishable from Thy-1.1, rat peripheral T-cells were not killed by anti-Thy-1.1 antibodies and complement. But the possibility remained that such treatment might inhibit the anti-tumor activity of the peritoneal cells even though it did not cause cell death. Therefore peritoneal cells from rats immunized against mouse EL4 leukemia cells were pretreated with complement and either mouse normal serum, mouse anti-Thy-1.1 serum, or mouse antiserum against both Thy-1.1 and rat species antigens. Only the latter gave strong inhibition of peritoneal cell activity, indicating that antibodies against Thy-1.1 cannot be used for the selective inactivation of rat peripheral T-cells. The alternative explanation that tumor cell killing in this system might be mediated by cells other than T-cells is currently under investigation.
207. THE EXPRESSION OF THETA-LIKE ANTIGEN BY PERIPHERAL LYMPHOID CELLS OF THE RAT

Investigators: Andrew Dowsett*, Tommy C. Douglas

Support: U.S. Atomic Energy Commission
National Institutes of Health, Public Health Service

In the mouse, less theta antigen is expressed by peripheral T-cells than by thymocytes. Although theta-like antigen can be detected on rat thymocytes by either antibody absorption or complement-dependent cytotoxic tests, neither of these methods gives positive results for rat peripheral T-cells. Others (Acton, 1974) have reached the same conclusion using radioactive antibodies, although theta antigen has apparently been detected using an indirect immunofluorescence assay (Micheel et al., 1973).

Another way to approach the question of whether the theta-like antigen is present on rat peripheral T-cells is to raise antibodies against these cells. If part of the resulting antibodies is specific for theta, then this antigen must have been present on the cells in question.

Groups of AKR/Cum mice were injected weekly with 2.5 x 10^6 nucleated cells from rat thymus, spleen, lymph node, Peyer’s patches, bone marrow, peritoneal exudate, or blood. During the six-week interval studied, all groups produced antibodies which were cytotoxic for thymocytes of the closely related strain AKR/J (Thy-1^a) but not for those of AKR/Cum (Thy-1^b). When tested against thymocytes from backcross mice, these sera were found to be cytotoxic for cells from Thy-1^a/Thy-1^b heterozygotes but not Thy-1^b homozygotes. It therefore appears that the theta-like antigen is present on rat peripheral T-cells. The possibility that transplanted rat cells might have developed the theta-like antigen after injection into mice is being examined.

Unexpectedly, animals in three of the groups described above were found to be producing low titers of antibody against AKR/J thymocytes before immunization. These groups made significantly stronger responses during the first week as would be expected in a secondary antibody response. Further experiments have indicated that approximately 18% of adult male AKR/Cum mice spontaneously make low titers of antibodies against Thy-1.1. The presence of “natural antibodies” against the theta antigen may be part of the reason that theta generally evokes stronger antibody responses than do most other mouse lymphocyte alloantigens.

References:

*Undergraduate, California Institute of Technology.

208. IN SEARCH OF NEW GENETIC POLYMORPHISMS IN THE RAT

Investigator: Tommy C. Douglas

Support: U.S. Atomic Energy Commission
National Institutes of Health, Public Health Service

Studies of the genetic polymorphisms for theta antigen, cytoplasmic malic enzyme, and transferrin (an iron-binding protein) of the mouse have been made previously in this laboratory (Biology 1973, No. 133). To date no corresponding polymorphisms have been reported for the common laboratory rat, Rattus norvegicus, although transferrin polymorphism has been observed in the black rat, Rattus rattus. The discovery of “mutant” forms of these genes, all three of which are linked in the mouse, would be very useful for genetic studies in the rat.

In complement-mediated cytotoxic tests thymocytes from rats of 18 inbred and two random-bred populations were all killed by antibodies specific for Thy-1.1 (θ-AKR) but not by
those specific for Thy-1.2 (θ-C3H). Thus, no qualitative differences in the theta-like antigen of the rat were found. In order to look for possible quantitative differences in antigen expression, the number of thymocytes from a given strain required to absorb a standard amount of anti-Thy-1.1 antibody was determined. The amount of antigen present on the thymocytes of five different strains of rats (A/2, AUG, BN.B2, COP, WF/fz) was found to differ by less than 2-fold, indicating no large differences in antigen expression.

209. WILD MOUSE THETA (Thy-1) ANTIGENS

Investigators: Elizabeth P. Blankenhorn, Murray Gardner*, John Estes*

Support: U.S. Atomic Energy Commission

We have recently completed the first experiments (Biology 1973, No. 136) designed to investigate the incidence of Thy-1 alleles in two feral populations of mice, and the possible representation on lymphoma cells of Thy-1 antigens. The study of the functional and morphological cellular origins of leukemias and lymphomas is currently of great interest to medical researchers. In the mouse, there are a number of antigens characteristic of certain cell types, and it was felt that the above approach was one good way of determining whether or not the lymphomas found in one wild mouse colony arose from a normal clone of Thy-1 positive, thymus-derived cells.

Animals for this project were obtained from wild mouse colonies maintained at the University of Southern California Special Cancer Virus Program facilities. Two colonies were studied: the Bouquet Canyon colony, which has a very low incidence of spontaneous lymphomas, and the Lake Casitas colony, which has a lymphoma incidence of 17% over a 12-month observation period. Serological techniques revealed that the Thy-1 antigen can be easily detected on brain and lymphoid tissue of normal young mice. Genotypes for 57 normal and lymphoma-bearing animals from the Lake Casitas colony were obtained. The observed frequency of the Thy-1\textsuperscript{a} allele is 0.43, and the frequency of the Thy-1\textsuperscript{b} allele is 0.57. The observed frequency of heterozygotes is 0.40, which is not significantly different from that expected in a population in Hardy-Weinberg equilibrium. The frequency of the Thy-1\textsuperscript{b} allele in the lymphomatous population (0.70, n = 25) was significantly different from that of the normal mouse group (0.47, n = 32); there were also fewer heterozygotes (29\% vs. 46\%). However, the presence of the Thy-1\textsuperscript{a} allele is restricted to the Lake Casitas colony: the Thy-1\textsuperscript{b} allele is apparently fixed in Bouquet Canyon mice. The implication of this result will be investigated in a proposed study of wild mice from other trapping areas, to see if the presence of the Thy-1\textsuperscript{a} allele is related to the presence of lymphomas. Our observation that the Mod-I\textsuperscript{a} allele and the Trf\textsuperscript{b} allele (two loci linked to Thy-1 on chromosome 9) are fixed in both Lake Casitas and Bouquet Canyon mice makes the segregation of Thy-1 in the Lake Casitas colony even more interesting.

Thy-1 antigen expression on brain tissue showed some variation with gene dosage (as detected by the α-Thy-1\textsuperscript{b} reagent) and with the day on which the experiment was conducted, but it did not vary with sex, age, or disease state. Thy-1 expression on normal lymphoid tissue did decrease somewhat with age, but not below detectable levels. 13 of 20 histologically-confirmed lymphomatous spleens (65\%) were completely negative for Thy-1 antigen, Starch block electrophoresis of serum samples from the 15 rat populations described above revealed no variation in the electrophoretic mobility of transferrin. Electrophoretic analyses of kidney cytoplasmic malic enzyme samples from the same rats detected no variant forms. Enzymes from eight selected strains also ran similarly when a second buffer system was used.

Thus, no genetic variation in these traits has been found to date.
4 spleens were positive, and 3 gave ambiguous results. Thus, it does not appear that lymphomas from this colony are typically Thy-1 positive or thymus-derived. Other antigenic markers and functional characteristics of these lymphoma cells will have to be tested to determine their cellular origins.

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210. THYMUS AND BURSA-SPECIFIC ANTIGENS OF THE PIGEON

Investigator: Susan L. Melvin
Support: U.S. Atomic Energy Commission
National Institutes of Health, Public Health Service

Lymphocytes of thymus-dependent and thymus-independent origins have separate roles in the immune response, but may cooperate in the antibody response to certain antigens. The lymphocytes look alike, but they can sometimes be distinguished from each other by serological means. The immune systems of birds are unique in that the functionally distinct populations of lymphocytes are anatomically separated in the central lymphoid organs—the thymus and the bursa of Fabricius. The avian immune system is used as a model for understanding mammalian immune systems. Cooperation between lymphocyte populations has been demonstrated in the avian antibody response although not with the two anatomically separate lymphocyte populations. Few serological markers are known for avian lymphocytes. Additional markers could more clearly identify distinct interacting cells and determine their roles in synergistic immune responses.

Antisera were prepared against pigeon thymus and bursa lymphocytes and against saline extracts of thymus and bursa. All antisera were prepared in rabbits—some as standard anti-lymphocyte reagents and some in rabbits injected neonatally with large doses of pigeon cells. The neonatal injections were made to reduce the rabbits’ antibody response to species-specific antigens upon challenge with pigeon lymphoid tissue.

All unabsorbed sera were screened for differential reactivity against thymus and bursa-derived tissue by passive hemagglutination, immunodiffusion, leukoagglutination and complement-dependent lymphocytotoxicity. Sera identified in this manner were absorbed with thymus and bursa tissue to demonstrate specificity. One serum with specificity against thymus extracts was identified by immunodiffusion. The thymus specificity was absent from extracts of pigeon bursa, brain, liver and breast muscle, but present in spleen extracts. The thymus specificity was shared by extracts of thymus from two different breeds of pigeon but not by extracts of chicken lymphoid tissue. This thymus specificity does not appear analogous to lymphocyte specificities identified in other species.

Shared tissue specificities and a possible quantitative antigenic difference among the tissue extracts were also demonstrated by immunodiffusion and absorption analysis. In lymphocytotoxicity tests, fresh rabbit normal serum is highly toxic for pigeon thymus and bursa cells. This toxicity resembles the natural antibody present in rabbit and guinea pig serum against heterologous thymus cells. The activity against pigeon lymphocytes can be absorbed by pigeon thymus and bursa cells and is heat labile. Unlike the natural antibody against thymus cells of other species, the toxicity against pigeon lymphocytes could not be absorbed by agarose.

A rabbit anti-pigeon gamma globulin serum was rendered specific for bursa cells by absorption with thymus cells. Some standard anti-lymphocyte sera were shown to contain an antibody fraction specific for thymus cells.

Some or all of these reagents may be useful for distinguishing cooperating cell populations in a variety of immune responses. Avian species, in addition to the chicken, may provide additional data on the phylogeny of functionally distinct lymphocyte populations.
211. DEVELOPMENTAL RED CELL ANTIGEN IN THE PIGEON

Investigator: Susan L. Melvin
Support: U.S. Atomic Energy Commission
National Institutes of Health, Public Health Service

Several fetal red cell antigens are described in the literature for species other than the pigeon. In this study, a fetal red cell antigen was sought as a possible serological marker for white cells as well. There are several reasons for thinking that such an antigen might be present on white cells. First, these two cell types are derived from a common stem cell. Second, a number of other antigens are known to be shared by white cells and red cells. In addition, the thymus and the bursa of Fabricius, which are the primary lymphoid organs in birds, are transient sites of erythropoiesis. Both lymphoid organs could be classified as developmental structures. At least one fetal red cell antigen is known to exist on cells other than mature red cells and to persist in these sites even after the antigen is no longer detectable on red cells.

The studies reported here did in fact reveal an antigen on red cells from all pigeon squabs less than four days old. The antigen appears not to be secondarily adsorbed to the red cells from the fluids of the egg or the embryo. In vitro, masking of the antigen by components of adult serum does not occur under the conditions tested, nor can the antigen be made to reappear in the adult after frequent bleedings. Although the squab antigen behaves similarly to a known fetal red cell antigen in doves, it is probably qualitatively different from that antigen and from the known chick red cell antigen.

The squab antigen is not detectable on lymphocytes from the bursa or the thymus by either leukoagglutination or complement-dependent lymphocytotoxicity. The antigen does not appear to be a lymphocyte marker. It is, however, an additional developmental antigen. If it is like other developmental antigens, it could be an interesting marker in both normal development and some disease states.

212. THE RELATIONSHIP BETWEEN THE CHICKEN FETAL ANTIGEN AND AVIAN MYELOBLASTOSIS VIRUS

Investigators: Suzanne Ostrand-Rosenberg, Raymond L. Teplitz
Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

Chickens possess a fetal red blood cell antigen which is present at the time of hatching but which disappears during the early development of the animal. The fetal antigen is present on certain adult tissues and particularly on cells in the bone marrow, presumably erythrocyte precursors. The fetal red blood cell antigen reappears on the red cells of adult chickens which have leukemia induced by avian myeloblastosis virus (AMV). It has been shown that leukemic chickens had the chicken fetal antigen at one day post-hatching, subsequently lost the antigen during development, and reacquired it upon contracting leukemia (Teplitz et al., 1974). Because of the association of leukemia onset and reappearance of the chicken fetal antigen we are investigating the possibility that the chicken fetal antigen is the receptor for the AMV virus.

Hemocyanin has been glutaraldehyde-coupled to goat-anti-rabbit-IgG (Hcy-GAR IgG) to serve as an electron microscopic visual label for the chicken fetal antigen. The AMV virus serves as the marker for the viral receptor. Examination of cell surface replicas by transmission electron microscopy with the Hcy-GAR IgG reagent shows large quantities of the fetal antigen on young chick red cells and only
background levels on the adult chicken red cells. We are currently carrying out competition and blocking experiments with the hemocyanin reagent and AMV virus to study the cell surface topological relationship of the AMV receptor and the chicken fetal antigen.

Reference:

*City of Hope National Medical Center, Duarte, California.

213. A SEARCH FOR MOUSE ALPHA-FETOPROTEIN VARIANTS

Investigators: Elizabeth P. Blankenhorn, Vanessa P. Skedzeleski*

Support: U.S. Atomic Energy Commission

A number of proteins found in mammalian and avian fetal development appear again (in "retrogenic" expression) just prior to or during malignant tumor growth. The term carcino-embryonic antigen (CEA) is applied to such cell-bound or soluble antigens, and their detection in adults, especially human adults, may be correlated with or even diagnostic of tumor formation. One such molecule is alpha-fetoprotein (AFP), found in embryos of many mammalian species and in primary carcinomas of the liver. It is a protein of "alpha" mobility on agar or starch electrophoretic gels, and has a molecular weight of about 70,000 daltons. It closely resembles albumin, and it has been suggested that AFP is a fetal analog of adult albumin. AFPs from two different species may be cross-reactive in immunoprecipitation reactions with antiserum prepared in a third species.

Our purpose in this study is to investigate possible antigenic differences between AFPs from different inbred strains of mice. It would be useful to find molecular variants of AFP in order to prepare specific alloantiserum to AFP and eventually to locate the gene(s) responsible for its production.

Mouse AFP is prepared from homogenates of mouse 19-day embryos and partially purified by Sephadex gel chromatography. AFP-containing fractions from four different inbred mouse strains (A/J, CBA/J, C57Bl/6J and AKR/Cum) have been prepared. There is no difference in mobility between them when the samples are subjected to electrophoretic separation on starch gels. Rabbit antiserum to AKR/Cum mouse fetal homogenate (containing AFP, albumin, transferrin, hemoglobin, and other minor proteins) was prepared and absorbed with adult mouse serum proteins which had been glutaraldehyde-cross-linked by the method of Avrameas (1969). The twice-absorbed reagent reacts with AFP, but not albumin, from column-purified fetal homogenates of all four strains of mice. Experiments are in progress to test for strain-specific antigens detected by this reagent, by absorbing it with AFP preparations from each of the four strains of mice.

Reference:

*Undergraduate, California Institute of Technology.

214. EFFECT OF IMMUNOSUPPRESSION ON PRIMARY TUMORS

Investigator: Susan L. Melvin

Support: U.S. Atomic Energy Commission
National Institutes of Health, Public Health Service

If immune surveillance suppresses spontaneous tumors which result from somatic mutation, environmental carcinogens, and oncogenic viruses, antithymocyte serum treatment should increase the tumor incidence and shorten the latent period. It might also allow the expression of tumors not normally seen in unsuppressed populations.

Virgin female mice of a single inbred strain,
CBA/J, obtained after a variety of treatments, were observed for primary tumors until either tumor onset or death. Included were mice which were: (1) immunosuppressed as adults by injection of rabbit anti-mouse thymocyte serum (RAMTS), (2) injected with normal rabbit serum, (3) immunized with an irrelevant antigen (mouse serum β-lipoprotein) or (4) untreated. Data were collected on tumor histology, incidence and time of onset for all groups.

No tumors appeared during the period of ATS-immunosuppression or for several months following treatment. There was no evidence from this study or from the literature that anti-thymocyte serum has any carcinogenic effect in the absence of oncogenic viruses or carcinogens.

This strain carries mammary tumor virus as a result of neonatal infection via milk from infected mothers. The most frequently observed tumor, the mammary tumor, appeared at an average age of slightly less than two years. Although the first tumors appeared in ATS-treated mice, the mean age at tumor onset was not significantly affected by ATS-immunosuppression. No unusual tumors and no lymphomas were observed.

Tumor incidences among groups of mice purchased at different times were different, but unrelated to ATS-immunosuppression. Tumor incidence was not depressed by failure of mice to reach tumor age; the mean life span of non-tumor-bearing mice was as long as or longer than that of tumor-bearing mice. Cause of the fluctuation in tumor incidence is not known.

Although tumor immunity reportedly exists at a time when palpable mammary tumors are present, immune surveillance does not appear to be the determining factor in the long tumor latent period. This conclusion is supported by published data from other laboratories which indicate that cellular immunity to mammary tumors is often compromised by immunological tolerance and/or the presence of blocking factors in the serum. The failure of ATS-immunosuppression to affect mammary tumor growth is consistent with these observations.

215. A POSSIBLE LINKAGE ASSIGNMENT FOR 6-PHOSPHOGLUCONATE DEHYDROGENASE IN MUS MUSCULUS

Investigators: Joan Klotz, Debra Dison*

Support: Gosney Fund
U.S. Atomic Energy Commission

The availability of numerous inbred strains of mice, each differing from others at one or many genetic loci, has facilitated gene mapping and linkage studies in this organism. However, no variants for 6-phosphogluconate dehydrogenase (Pgd-1) have been reported in inbred mice, although electrophoretic variants have been detected in wild populations (Selander and Yang, 1969). It is probable that this lack of variation in inbred mice has delayed the assignment of the structural gene for this enzyme to a linkage group. While studying progeny of a cross between wild mice and the inbred strain C57Bl/10Sn we noticed a marked tendency for two alleles from the wild population, Pgd-1a and Pgm-2b (phosphoglucomutase-2), to segregate together. This linkage was detectable in only one family, the only family segregating for electrophoretic variants of Pgm-2. We have initiated crosses between F2 progeny, heterozygous for both of these markers, and the C57Bl/10Sn strain. The linkage between Pgd-1, Pgm-2 and other genes known to be on the same linkage group as Pgm-2 will be analyzed in more detail, using the progeny of this cross.

Reference:

*Undergraduate, California Institute of Technology.
GENETIC STUDIES ON SUSCEPTIBILITY TO LYMPHOMA IN MUS MUSCULUS

Investigators: Joan Klotz, Debra Dison*

Support: Gosney Fund
U.S. Atomic Energy Commission

Investigations of susceptibility to leukemia and lymphoma using inbred strains of mice have implicated a number of genetic loci (Lilly and Pincus, 1973). We have been collaborating with Dr. Murray Gardner, University of Southern California School of Medicine, on such a study using a colony of wild mice which has been shown to have a high frequency of spontaneous lymphoma (Gardner et al., 1973). The wild mice have been mated to members of an inbred strain of mice, C57Bl/10Sn, which is known to be highly resistant to leukemia and lymphoma. At six weeks of age, F1, F2 and backcross progeny are splenectomized and the tissue examined for the presence of murine C-type virus group specific (gs) antigen and for virus particles. The mice are then maintained in the laboratory to determine whether or not they develop lymphoma in later life. We have been testing blood samples obtained from these mice for a number of genetically determined protein polymorphisms, looking for linkage between any of these and gs antigen expression. Our preliminary data suggest a possible linkage between the structural gene for 6-phosphogluconate dehydrogenase (Pgd) and the locus controlling expression of gs antigen. We have data indicating that Pgd is located on chromosome 4, a chromosome which is also known to contain the locus Fv-1, which controls susceptibility to Friend leukemia virus (Rowe et al., 1973). If these preliminary data are confirmed by additional work now in progress, it would seem possible that the same genetic controls which regulate susceptibility to leukemia in inbred mice are operating in this feral population.

References:

*Undergraduate, California Institute of Technology.

DO THE AUTOANTIBODIES OF MYASTHENIA GRAVIS PATIENTS INHIBIT SARCOPLASMIC RETICULUM FUNCTION?

Investigator: Tommy C. Douglas

Support: U.S. Atomic Energy Commission
National Institutes of Health, Public Health Service

Autoantibodies reactive with muscle as well as a variety of other tissues of human or animal origin are frequently found in the blood of patients suffering from myasthenia gravis (MG). Recently it was reported (Mendell et al., 1973) that these autoantibodies could be localized to the sarcoplasmic reticulum membranes of striated muscle. The ATP-dependent active transport of Ca++ ions by these membranes is necessary for muscular relaxation. Any interference with this process might contribute to the muscle dysfunction seen in this disease.

Partially purified sarcoplasmic reticulum membranes were prepared from rat striated muscle by differential centrifugation. These membranes were intact as judged by their ATPase activity and ability to accumulate Ca++ in vitro in the presence of ATP. By electron microscopy this preparation could be seen to be composed of a mixture of thin- and thick-walled vesicles.

So far, the exposure of these membranes to sera from MG patients has not been found to affect their capacity to accumulate Ca++ in vitro. Further experiments along these same lines are
currently in progress. The development of other methods for detecting the binding of autoantibodies to sarcoplasmic reticulum membranes is also planned.

Reference:

218. SEROLOGICAL INVESTIGATION OF ELECTROPHORETICALLY DETERMINED ISOZYMES

Investigators: Phyllis Frostenson*, Andrew E. Senyei**

Support: National Science Foundation
U.S. Atomic Energy Commission

Electrophoretic variants of enzymes have proven to be valuable markers in many types of genetic and developmental studies. Current methods used to detect the presence of enzymes employ indirect measurements of products and cofactors involved in the enzymatic process. Though colorimetric or spectrophotometric analysis coupled with electrophoresis is adequate for some investigations, a direct immunoassay would be more desirable, especially in histochemical studies requiring visualization or microquantitation of enzymes present in small amounts of tissue. We attempted to prepare specific antibodies against the electrophoretically defined genetic variants of two enzymes, glucose phosphate isomerase (GPI) (E.C.5.1.1.9) and malic enzyme (Mod-1) (E.C.1.1.1.40).

In order to detect possible antigenic differences between the electrophoretic variants both hetero- and alloantiserums were prepared. The enzymes from AKR/Cum (GpiA and Mod-1A) and CBA/J (GpiB and Mod-1B) mice were partially purified by ammonium sulfate precipitation and Sephadex column chromatography. Preparation of alloantiserum involved cross-immunization of AKR/Cum and CBA/J mice with appropriate isozyme extracts. Rabbits were injected with the extracts in order to prepare heteroantiserum.

Analysis of the alloantiserum by immunodiffusion and immunoelectrophoresis failed to demonstrate the presence of allotypic antibodies. The heteroantiserums were found to contain antibodies to all the variants as shown by immunodiffusion. However, they were highly cross-reactive and specificity for individual isozymes could not be shown even after extensive adsorptions. The lack of demonstrable allotypic antibodies may in part be due to an insufficient quantity of enzymes used in the immunizations. It is conceivable that given an adequate antigen dose allotypic antibodies could be elicited.

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219. CYTOTOXIC AND IMMUNOLOGICAL PROPERTIES OF IONENE POLYMERS

Investigators: Andrew E. Senyei*, Alan Rembaum**

Support: JPL-Caltech President's Fund
U.S. Atomic Energy Commission

The immune system affords a good model with which to investigate the relative contributions of specific as well as nonspecific cell surface characteristics involved in cell-to-cell interactions. The necessity for specific cell surface markers in immune recognition has been well documented. The role of nonspecific surface parameters, such as cell surface charge, has also been examined but to a lesser extent. Most investigators have employed enzymes (e.g., neuraminidase) to change the surface charge of cells. Another approach to modifying cell surfaces is the addition of polyanions or polycations which strongly adhere to cells and also change their net charge. In our investigations we
have been using ionenes, a class of quaternary ammonium polymers, to alter the surface properties of lymphocytes and EL4 leukemia cells. We are attempting to assess the behavior of these modified cells in a variety of in vivo and in vitro immune mechanisms.

Previous work (Biology 1973, No. 145) examining the immunological properties of these polymers showed that they were not immunogenic by themselves. However, they did show interesting cytotoxic and cell agglutinating behavior. We found that by varying the charge density and molecular weight of the polymer different biological activities could be conferred. Ionenene 3,3 (I3,3), the most densely charged, agglutinated murine erythrocytes and peripheral white blood cells to a greater extent than I6,10, which is less charged. However, the toxicity of the polymers with respect to the cells is inversely correlated. The differential surface activity of the polymers suggested examining the ability of various ionenes to modify either afferent or efferent immune cell interactions.

Examinations of in vitro cell mediated immunity against ionene-coated EL4 cells showed no significant difference from controls (i.e., not coated EL4 cells). We are currently looking at the ability of ionenes to alter afferent immune mechanisms by means of in vitro mixed lymphocyte reactions and also by means of in vivo response to ionene-coated EL4 cells.

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**Jet Propulsion Laboratory.

### 220. QUANTITATIVE CELL SURFACE EXPRESSION OF GENETICALLY DEFINED BOVINE RED CELL ANTIGENS

**Investigator:** Suzanne Ostrand-Rosenberg  
**Support:** National Institutes of Health, Public Health Service  
U.S. Atomic Energy Commission

We have used the electron microscope to investigate the quantity and distribution of blood group antigens on the surface of bovine red cells. The antigens were visualized for electron microscopy (EM) by an indirect labeling method using hemocyanin from the snail *Busycon canaliculatum*, glutaraldehyde-coupled to rabbit-anti-bovine immunoglobulin. Gene dosage effects, reflected serologically in time of complement-mediated lysis for cells negative, heterozygous, and homozygous for the Z allele, were examined for the quantity of Z antigen per unit area of cell surface, on genetically defined cell populations. Homozygous cells were found to have approximately twice as much Z antigen as heterozygous cells. Cells negative for the Z antigen showed only background levels of label.

The J antigen is a soluble serum glycoprotein which secondarily adsorbs to the red cell surface. Some bovine red cells display a very low J titer and require large amounts of anti-J antibody to lyse them, while other cells have high J titers and require only minute amounts of antibody for cellular lysis. We have studied the cell surface quantitative expression of the J antigen using our EM labeling reagent, and have found that the J titer of a cell corresponds to the quantity of J antigen present on that cell's membrane. Anti-J antiserum was fractionated into the IgG and IgM components and these fractions were assayed for binding activity by EM. During the course of this study, we have identified an additional antigen on a population of J negative cells which does not appear to elicit lytic or direct hemagglutinating antibody, but which can be serologically detected by an indirect Coombs reaction with anti-immunoglobulin.

The expression of antigens on the cell surface in the presence of bound antibody directed against another antigen was studied by a sequential double labeling technique. Two specificities in the same blood group complex (C complex) as well as antigens in different complexes (C and Z complexes) were examined. In two experiments it was found that bound antibody directed against one
specificity did not inhibit binding of antibody against another specificity. In another experiment with different red cell antigens it was found that bound antibody directed against one specificity enhanced the adsorption of antibody directed against a second specificity.

221. THE EFFECTS OF ENZYME TREATMENT ON THE EXPRESSION OF CELL SURFACE ANTIGENS AND CONCANAVALIN A BINDING SITES ON BOVINE RED CELLS: IMPLICATIONS FOR MEMBRANE STRUCTURE

Investigator: Suzanne Ostrand-Rosenberg
Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

Using an indirect labeling technique and hemocyanin as the visual marker for transmission electron microscopy, bovine red cells have been examined for their Concanavalin A (Con A) binding. A report by Hines et al. (1972) suggested that for bovine red cells the Con A receptor and the A antigen may be molecularly the same structure. Electron microscopic, hemolytic and agglutination experiments in this laboratory using Con A and anti-A antiserum demonstrate that the two binding activities are involved with separate red cell surface structures. Red cells were either sensitized with Con A or anti-A antiserum and then tested for additional uptake of the other reagent in the above-mentioned assay systems. Untrypsinized bovine red cells were not agglutinated by Con A, but trypsinized A positive cells were agglutinated, as reported by Hines et al. (1972). Both A positive and A negative untrypsinized cells bound Con A, although the untrypsinized A positive cells bound more lectin than the untrypsinized A negative cells. Following trypsinization the Con A uptake of both cell types was increased, but the trypsinized A positive cells still bound more lectin than the trypsinized A negative cells. The uptake of immunoglobulin by both types of trypsinized cells was found to be largely nonspecific and due to an increased "stickiness" of the trypsinized cells.

Additional studies using the freeze-fracture technique for examining the internal structure of membranes have shown that for the bovine red cell A antigen the A antigenic determinants associate physically with the intramembranous particles seen on freeze-fracture replicas. This observation supports the theory of membrane structure proposed by others.

Reference:

222. BOVINE LEUKOCYTE ANTIGENS

Investigators: Suzanne Ostrand-Rosenberg, Clyde Stormont*
Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

Bovine red blood cell surfaces display over 60 serological specificities grouped into 11 genetic systems. These specificities and the phenogroups that they form have been well characterized serologically. Believing that a study of bovine leukocytes for the presence of bovine red cell antigens is in order, we have surveyed 28 bovine red cell typing reagents for their leukocytotoxicity in a microcytotoxicity system. Previously we have used the Amos one-step microcytotoxicity for our tests, but we found that additional positive leukocyte reactions were present using the two-step assay (Biology 1973, No. 140). We have now tested leukocytes from a panel of 13 animals from herds at University of California, Davis, and California Polytechnic Institute at Pomona. Using the Amos two-step assay we have tested representative reagents from each of the bovine red cell blood
groups including 8 B complex antisera, 3 C complex antisera, F antiserum, Z antiserum, 7 S complex antisera, 2 A complex antisera, Lx, J antiserum, M, and R' and S' antisera. In all cases where the test panel consisted of cells from donors whose red cells displayed plus and minus reactivity patterns with the test reagents (28 of the 29 reagents), the pattern of leukocytotoxicity did not match the pattern of red cell hemolysis. Also, reagents that were not cytotoxic for leukocytes were nonreactive with leukocytes from donors whose red cells possessed the antigen, as well as with leukocytes whose donors lacked the antigen. These data indicate that bovine leukocyte antigens, as detected by the two-step microcytotoxicity assay, are distinct from red cell antigens. Because there is no apparent association of leukocyte reactivity with bovine red cell antigens, we are probably detecting different antigenic systems on leukocytes and red cells.

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223. H-2 AND Ia ANTIGEN TOPOLOGY ON THE CELL SURFACE OF MURINE LYMPHOCYTES

Investigators: Suzanne Ostrand-Rosenberg, Carol H. Sibley, Jack Silver

Support: National Institutes of Health, Public Health Service
U.S. Atomic Energy Commission

Within the past year a new system of murine lymphocyte cell surface alloantigens has been described by David et al. (1973) and Sachs and Cone (1973). The genetic region which controls the production of the Ia gene products is closely linked to the Ir region of the H-2 major histocompatibility locus in the mouse (see Biology 1974, No. 120) for additional information on the Ia antigen). The Ia antigens have been detected both serologically (David et al., 1973; Sachs and Cone, 1973) and chemically (Cullen et al., 1974) and shown to be distinct from the H-2 gene products.

Because of the close genetic linkage of the Ir region genes and the H-2 genetic region we are studying a possible cell surface association of the H-2 and Ir gene products. An electron microscopic labeling reagent has been prepared in which rabbit-anti-mouse immunoglobulin is coupled to hemocyanin (RAMIgG-Hcy). This reagent is being used to tag indirectly the cell surface H-2 and Ia antigens. Specific H-2K and H-2D end antisera from the National Institutes of Health and an anti-Ia antiserum from Dr. D. Shreffler are available. Cells are treated with the appropriate antiserum, reacted with the RAMIgG-Hcy, and prepared for electron microscopy by making carbon replicas of the cell surfaces. In some experiments the cells are treated twice with antisera, each time with a different antiserum followed by the hemocyanin reagent, to assess the blocking capacity of a particular antibody. Capping experiments using the hemocyanin reagent are also being undertaken to see if the Ia and H-2 products cap (e.g., are mobile on the cell surface) and if they co-cap. If the Ia and H-2 antigens are located on the same molecule they may cap together. If they are close to each other on the cell surface, but not on the same molecule they may block binding of antibody to one another but not co-cap. If they are sterically independent on the cell surface they may show neither blocking nor co-capping.

References:
224. ENZYME STUDIES IN CAENORHABDITIS ELEGANS

Investigators: James Loddengaard*, Carl Johnson, David Dusenbery**

Support: U.S. Atomic Energy Commission
National Science Foundation

We have examined seven strains of Caenorhabditis elegans for genetic polymorphisms in seven enzymes using starch gel electrophoresis. No substantiated polymorphisms were found. Crosses for several apparent polymorphisms produced no evidence of Mendelian segregation.

In the process of screening for genetic polymorphisms, we observed two apparently exclusive forms of the enzyme acid phosphatase in populations of animals separated into large vs. small by filtration through a nylon mesh. This developmental polymorphism is being examined further.

In order to examine the development of the separable forms, we have devised a technique to grow large populations of nematodes synchronously in liquid culture. This is accomplished by taking heterogeneous cultures, separating gravid adults by settling through a 2 to 4% sucrose gradient and resuspending them in fresh culture media. After a suitable time for egg laying (2 to 6 hours), the adults are removed by settling. Synchronous progeny are maintained in liquid cultures and can be harvested at any desired developmental stage. In one such experiment six stages in development were collected and analyzed for enzyme.

Initial experiments have used α-naphthyl acid phosphate to stain for acid phosphatase. As an alternative, we are experimenting with 5-bromo-4-chloro-3-indolyl-phosphate as a possible higher resolution substrate.

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**Georgia Institute of Technology, Atlanta, Georgia.

PUBLICATIONS


The application of “restriction” nucleases to ΦX RF DNA has enabled us to obtain specific sectors of this DNA corresponding to any desired portion of the genome. These in turn are enabling us to study the structure and function of this DNA in considerably greater detail than has heretofore been possible.

The specific DNA fragments produced by these different restriction nucleases have been ordered into a physical map that has been correlated with the well established genetic map of ΦX174. With this basis the fragments are employed to advance our knowledge of such subjects as location of the one methylcytosine in ΦX DNA, electron microscopic study of this DNA, sites of origin of RNA transcription from this DNA, the DNA sequence in the region thought to be involved in the origin of DNA replication, etc.

Studies of in vivo and in vitro transcription and of the process of progeny single-strand synthesis have been continued. Preliminary evidence has been obtained that suggests post-transcriptional processing of ΦX messenger RNA by RNase III.

225. A CLEAVAGE MAP OF BACTERIOPHAGE ΦX174 GENOME

Investigator: Amy Shiu Lee
Support: McCallum Fund
National Institutes of Health, Public Health Service

The genome of bacteriophage ΦX174 is a covalently closed circular molecule consisting of a single strand of DNA of about 5500 nucleotides. Several bacterial restriction deoxyribonucleases have been described which cleave the double-stranded, circular ΦX174 replicative form DNA into specific fragments. These enzymes are particularly useful since they hydrolyze DNA at specific sites. In order to use these specific fragments to study the structure and function of ΦX, it is necessary to order these fragments with respect to the ΦX genetic map.

Restriction endonucleases isolated from Hemophilus influenzae (Hin), Hemophilus parainfluenzae (Hpa) and Hemophilus aegyptius (Hae) were used to cleave ΦX174 replicative form (RF) DNA into 3 sets of specific DNA fragments, ranging in size from 1700 to 100 base pairs, as determined by polyacrylamide gel electrophoresis. Fragments were isolated individually by continuous electrophoresis through 2.5% agarose gels. The order of these fragments and their location with respect to the ΦX genome were determined by: (1) analysis of partial digest products; (2) analysis of overlapping sets of fragments produced by two different restriction enzymes; and (3) the ability of individual fragments to complement various ΦX mutants.

On the basis of these results, a detailed physical map of the ΦX174 genome has been constructed with respect to the cleavage sites of all 3 enzymes (Figure 1).

Reference:
Methylation of DNA occurs in viruses, bacteria and higher organisms, and has been the topic of intensive study. Experiments carried out in bacteria have demonstrated that methylation of DNA is related to DNA replication, and that this process normally occurs in the nascent DNA strand close to or at the replication point (Billen, 1968).

The discovery of a unique methylcytosine group in the progeny phage ΦX DNA by Razin et al. (1970) prompted us to localize it with respect to restriction enzyme fragments and thereby on the ΦX genetic map.

Bacteriophage ΦX174 DNA was labeled in vivo with 3H-methyl methionine. The methyl-labeled progeny DNA was extracted from purified bacteriophage ΦX174 particles and used as template for in vitro synthesis of the complementary strand in the presence of the nucleoside triphosphates and *E. coli* polymerase I. The resultant replicative form DNA was then cleaved, in separate experiments, with restriction endonucleases from *Hemophilus influenzae* and *H. aegyptius*. The DNA fragments were analyzed by polyacrylamide gel electrophoresis. It is concluded that the single methylcytosine in the viral DNA is located at a specific region of the ΦX174 genome, very likely in gene H.

References:

**227. A CONTINUOUS ELECTROELUTION METHOD FOR THE RECOVERY OF DNA RESTRICTION ENZYME FRAGMENTS**

*Investigator:* Amy Shiu Lee  
*Support:* McCallum Fund  
National Institutes of Health, Public Health Service

Bacterial restriction enzymes are deoxyribonucleases which cleave double-stranded DNA at specific sites and produce unique, high molecular weight fragments. The principal method for separation of the DNA fragments is electrophoresis through polyacrylamide or agarose...
gels. However, when fragments are to be isolated for further analysis, continuous elution from long cylindrical gels allows maximum separation and recovery. We have adapted a continuous electroelution system modified from that designed by Popescu et al. (1971) to separate and recover \( \Phi \times 174 \) DNA fragments produced by *Hemophilus* restriction endonuclease. The high conductivity of this continuous electrophoresis system in comparison with others provides good separation of fragments at relatively low voltage, therefore minimizing heating effects. The pumping speed used for elution can be as low as 4 ml/hour without loss of sample into the anode buffer chamber. A slow pumping speed and collection of small volume fraction minimizes the dilution of the eluting fragments.

References:


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**228. THE INSERTION OF SPECIFIC SINGLE-STRANDED DNA FRAGMENTS INTO \( \Phi \times 174 \) REPLICATIVE FORM II MADE IN VITRO**

*Investigator:* J. Lee Compton

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

\( \Phi \times 174 \) negative (complementary) strand DNA was purified from \( \Phi \times 174 \) replicative form II (RF II). When linear negative strands were mixed with circular viral strands under renaturing conditions circular double-stranded molecules were formed. The presence of this *in vitro* RF II was confirmed by electron microscopy.

A specific double-stranded DNA fragment, purified from a digestion of \( \Phi \times 174 \) replicative form DNA by *Hind* of *Hemophilus influenzae*, was mixed with \( \Phi \times 174 \) viral and negative strand DNA. The mixture was denatured, renatured, and mounted for electron microscopy. In addition to the molecules seen in the control, RF II molecules containing a loop of the size of the added DNA fragment were seen. The loop size distributions measured for two different inserted fragments were distinct, indicating the specific origin of the insert loop.

When two distinct *Hind* fragments were added simultaneously, molecules containing both one and two loops were seen, with the frequency of two-loop molecules approximately the product of the frequencies of the two distinguishable one-loop molecules. The loop sizes of the one-loop molecules had a bimodal distribution as expected for the two fragments. Both the loop sizes and the distance between the two loops in the two-loop molecules corresponded to the values expected from the *Hind* cleavage map (see Figure 2).

This technique will be useful for mapping specific DNA fragments relative to each other, and for providing internal markers for mapping various events in the reproductive cycle of \( \Phi \times 174 \).

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**229. USE OF DNA FRAGMENTS PRODUCED BY CLEAVAGE OF \( \Phi \times 174 \) DNA WITH RESTRICTION ENDONUCLEASES FOR DETERMINATION OF NUCLEOTIDE SEQUENCES IN ADJACENT REGIONS**

*Investigator:* Karel Grohmann

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

Fragment R8 was produced by cleavage of \( \Phi \times 174 \) RF I by *Hin* restriction endonuclease and purified by continuous electroelution from agarose and polyacrylamide gels. This fragment was then annealed to \( \Phi \times 174 \) viral DNA at 65°C. The annealed product was then used as the template for a pulsed synthesis reaction catalyzed by *E. coli* polymerase I. This reaction introduced label specifically into the R3 fragment adjacent to R8 (Lee and Sinsheimer, 1974), as was shown by cleavage of newly synthesized RF by *Hin* restriction endonuclease. Determination of the nucleotide sequence of this fragment will be useful for mapping specific DNA fragments relative to each other, and for providing internal markers for mapping various events in the reproductive cycle of \( \Phi \times 174 \).
sequences adjacent to the 3'-end of the complementary strand of fragment R8 by restricted repair reaction and ribo-substitution technique is in progress.

Reference:

Figure 2. A ΦX174 RF II molecule showing insert loops produced by Hind fragments R1 and R4.

230. PYRIMIDINE TRACTS IN ΦX174 DNA FRAGMENTS PRODUCED BY Hin ENDONUCLEASE

Investigator: Karel Grohmann
Support: National Institutes of Health, Public Health Service

ΦX174 RF II selectively labeled in the complementary strand was prepared by an in vitro polymerization reaction, using E. coli polymerase I, ΦX174 viral DNA as a template, d(pApG)₄ as a primer and α⁻³²P·dTTP or α⁻³²P·dCTP as a source of label. This DNA was cleaved by Hin restriction
endonuclease into fragments which were separated by continuous electroelution from agarose and polyacrylamide gels. Pure fragments R1, R2, R3, R4, R5, R8, R9, and R10, and fragment mixtures R6(3) and R7(2) were then depurinated by Burton's procedure. The pyrimidine tracts thus obtained were separated by a 2-dimensional fingerprinting procedure, using electrophoresis on cellulose thin-layers at pH 3.5 for separation in the first dimension and TLC on DEAE-cellulose for separation in the second dimension. Fingerprints of the individual fragments demonstrated the unique distribution of pyrimidine tracts among the several fragments. Determination of nucleotide sequences in some of the larger pyrimidine tracts (n = 7-11) is in progress.

231. RNA POLYMERASE BINDING SITES ON ФX174 RF II

Investigator: Karel Grohmann
Support: National Institutes of Health, Public Health Service

Under conditions where only initiation of messenger RNA synthesis was allowed, DNA-dependent RNA polymerase from E. coli protected approximately 1 to 2% of the ФX174 genome against exhaustive digestion by pancreatic DNase. The protected pieces were purified by column chromatography on Sephadex G-200, deproteinized by extraction with phenol-SDS, and precipitated with ethanol. Analysis on calibrated SDS-acrylamide gels indicated the presence of two classes of protected pieces, a major class 39 base pairs long and a minor one 25 base pairs long. The relative proportions of these two classes seem to be strongly dependent upon the age of the RNA polymerase preparation, the smaller size class becoming predominant as the enzyme is stored over a period of several months. Analysis of the protected pieces on calibrated denaturing urea-acrylamide gels showed a sharp major component (single-strand) approximately 35 nucleotides in length with a broad shoulder of polynucleotide material extending toward smaller size, peaking at approximately decamer length. The protected pieces hybridized primarily to Hin fragments R4 and R3 in nitrocellulose disc assay.

232. PURIFICATION OF OLIGONUCLEOTIDES FROM 5'-END OF mRNA BY HYDROXYAPATITE COLUMN CHROMATOGRAPHY

Investigator: Karel Grohmann
Support: National Institutes of Health, Public Health Service

Bernardi (1964) has shown that ribonucleoside mono-, di-, tri-, and tetraphosphates can be efficiently separated by column chromatography on hydroxyapatite. Soave's results (Soave et al., 1973) on separation of SS RNA species indicated that this separation technique is sensitive to the length of the polyphosphate chain in the molecule, but relatively insensitive to the molecular weight. Application of this technique to the isolation of oligonucleotides derived from 5'-ends of messenger RNA (mRNA) and containing triphosphate end groups, was investigated. Separations were tested with T1 RNase digests of an artificial mixture of 3H-uniformly-labeled mRNA and mRNA selectively labeled with γ-32P-ATP or GTP at the 5'-end. All mRNAs were prepared in vitro on ФX174 RF I template, using DNA-dependent RNA polymerase from E. coli. The digests were fractionated on hydroxyapatite columns with KPO4 buffer gradient. Oligonucleotides from the 5'-ends of these RNAs elute at much higher molarity than oligonucleotides lacking the triphosphate end groups. In cooperation with L. H. Smith, work is in progress on optimization of separation conditions and the extension of this technique to sequence studies of 32P-labeled ФX174 mRNAs.

References:
233. 5'-END SEQUENCES OF \(\Phi X174\) mRNAs MADE IN VITRO

**Investigators:** Lloyd H. Smith, Karel Grohmann  
**Support:** National Institutes of Health, Public Health Service

Previous studies (Biology 1971; Biology 1972) have shown that \(\Phi X174\) RF DNA directs the synthesis of several distinct mRNA species in vitro with \(E.\) coli RNA polymerase. The 5'-terminal sequences of these have been determined in the following manner. \(\Phi X174\) in vitro mRNA is synthesized using highly labeled \(\alpha-^3P\)-ribonucleotide triphosphates. The RNA is cleaved with pancreatic or TI ribonuclease (Barrell, 1971).

Oligonucleotides not containing 5'-phosphates are then degraded to mononucleotides using bovine spleen exonuclease (Bernardi and Bernardi, 1968).

The remaining oligonucleotides, which contain terminal 5'-triphosphate, are fractionated and sequenced by the standard techniques (Barrell, 1971). The sequences thus determined are:

- \(\text{pppA}\text{pUp}(\text{Cp})\)
- \(\text{pppA}\text{p(Ap)}_2\text{Up}(\text{Cp})\)
- \(\text{pppA}\text{p(Ap)}_3\text{Up}(\text{Cp})\)
- \(\text{pppGpApUp(Gp)}\)

**References:**  

234. LOCALIZATION OF THE 5'-ENDS OF THE IN VITRO mRNAs ON THE PHYSICAL MAP OF \(\Phi X174\)

**Investigator:** Lloyd H. Smith  
**Support:** National Institutes of Health, Public Health Service

In vitro mRNA labeled either with \(\gamma-^3P\)-ATP or \(\gamma-^3P\)-GTP has been hybridized to purified \(Hind\) fragments of \(\Phi X174\) RF I. All three ATP initiations (sequences given in accompanying report, Biology 1974, No. 233) arise from transcription within the "R4" region, near the 5'-end of gene A. The major GTP initiation hybridizes within one of the three "R6" regions. The site of the GTP initiation may be identical to the RNA polymerase binding site detected by Chen et al. (1973) near the 5'-end of gene D.

**Reference:**  

235. \(\Phi X174\)-SPECIFIC mRNA: SYNTHESIS IN TOLUENE-TREATED CELLS

**Investigator:** Aladar A. Szalay  
**Support:** Gosney Fund, National Institutes of Health, Public Health Service

A more exact study of \(\Phi X174\)-specific mRNA initiation, synthesis and termination would be possible if (1) the 5'-ends of the newly synthesized RNA could be specifically labeled in an in vivo system, and (2) host RNA synthesis could be eliminated.

We have found that cells made permeable to XTPs by toluene treatment (Moses and Richardson, 1970) provide the best system for specific in vivo labeling. Cells, both infected and uninfected, were treated with toluene and purified by repeated centrifugation in basic medium (Vosberg and Hoffmann-Berling, 1971). The toluene-treated cells required the XTPs, dXTPs, amino acids, tRNAs and PEP. The system is sensitive to 10 \(\mu\)g/ml rifampicin.

Both \(\alpha-^3P\)-GTP incorporation are linear for 30 minutes at 37°C in infected and uninfected cells. The incorporation of \(\alpha-^3P\)-GTP is 55 to 60 pmoles/10^8 cells in 20 minutes. 10 to 12% of the labeled RNA extracted from \(\Phi X174\)-infected,
toluene-treated cells hybridized to ΦX174 RF immobilized on filters. The molecular weight of the
ΦX174 RNA labeled for 5 minutes was determined by sedimenting the total RNA through sucrose
gradients with marker RNAs and hybridizing the fractions to filters containing ΦX174 RF. 13 to 14%
of the RNA sedimenting at 23S or faster hybridized to the filters whereas only 2 to 3% of the RNA
sedimenting slower than 23S hybridized. The synthesis of the full length ΦX174 mRNA in
toluene-treated cells is similar to the synthesis of full length ribosomal RNA in osmotically shocked cells
as reported by Lazzarini and Johnson (1973).

Combining 5'-end labeling of ΦX174 mRNA in vivo as described, with current experiments to
eliminate host RNA synthesis by UV irradiation (Studier, 1973), provides the possibility of mapping
of the in vivo ΦX174 mRNAs.

References:

236. POST-TRANSCRIPTIONAL CLEAVAGE OF IN VITRO SYNTHESIZED ΦX174 mRNA BY RNase III

Investigator: Aladar A. Szalay
Support: Gosney Fund
National Institutes of Health, Public Health Service

It is known that RNA polymerase from E. coli recognizes and transcribes in vitro the early region
of the relaxed T7 DNA. It also transcribes supercoiled ΦX174 DNA (RF I), most likely in its
entirety.

The T7 early mRNA is polycistronic (2.5 x 10^6 daltons) and is cleaved by RNase III
post-transcriptionally in vitro and in vivo (Dunn and Studier, 1973). Experiments investigating the in
vitro initiation, termination and post-transcriptional RNase III cleavage of the ΦX174 mRNA were
carried out in collaboration with Dr. John J. Dunn at Brookhaven National Laboratory (using the T7
system as a control). We found that the RNA made in vitro in 20 minutes at 37°C from ΦX174 RF I
was heterogeneous in size whereas the T7 mRNA was homogeneous. The ΦX174 mRNA electrophoresed as three major and five minor bands on 2% acrylamide, ranging in size from 0.245 to
2.05 x 10^6 daltons. The gel pattern was constant in the presence or absence of the ρ factor.

Adding 10 units of RNase III to 100 µl mRNA synthesized in vitro for 20 minutes at 37°C and
incubating an additional 20 minutes at 37°C causes the three highest molecular weight RNAs to
disappear. In their place four new bands with molecular weights ranging from 0.205 to 0.97 x 10^6
daltons appear. These bands can now be used to locate promoter and RNase III cleavage sites on the
ΦX174 genome by hybridizing them (after labeling the 5'-ends with γ-^32P-GTP or ATP) to specific
restriction endonuclease fragments made from ΦX174 RF I.

The combination of post-transcriptional cleavage by RNase III with multiple initiation and
termination may be responsible for the 18 different species of in vivo ΦX mRNA found by Clements and
Sinsheimer (1974).

References:
237. INTERMEDIATES IN ΦX174 SINGLE-STRANDED DNA SYNTHESIS

Investigator: Romilio T. Espejo
Support: National Institutes of Health, Public Health Service

When infected cells are pulse-labeled during single-stranded ΦX DNA synthesis, part of the label sediments as a broad peak with rates between 25S and 18S. This labeled DNA consists at least in part of double-stranded ΦX DNA rings accompanied by a single-stranded branch of different length, an expected intermediary according to the rolling circle model for ΦX DNA synthesis.

We have now been able to resolve further this pulse-labeled material by equilibrium centrifugation in CsCl-PDI which allows separation of circular closed double-stranded ΦX DNA (RF I), ΦX DNA and nicked RF I (RF II) by their different densities due to their distinctive PDI-binding capacities. The heaviest DNA species isolated in these gradients has not been previously described; because of its density, higher than RF I, it probably consists at least partially of circular closed double-stranded DNA or other form with restriction for dye intercalation. It sediments as a homogeneous component at a rate of 23S; upon treatment with S1 single-stranded-specific nuclease it is converted to RF II plus low molecular weight DNA. The presence of a single-strand portion in this DNA species is also indicated by its density in neutral CsCl, slightly higher than RF II or RF I. Sedimentation in alkaline sucrose gradient suggests that the label is almost exclusively present in linear single strands longer than ΦX DNA. The simplest explanation for the properties of this DNA is that it consists of a circular double-stranded DNA with a single-strand branch; the dye binding restriction resulting in a higher density in CsCl-PDI is not understood. Electron microscopy has confirmed our present interpretation of the structure of this component and a detailed study of the molecules observed will help to understand their unusual properties.

That this DNA species is indeed an intermediary during ΦX DNA synthesis has been suggested by pulse and chase experiments.

PUBLICATIONS


Past work from this laboratory has shown that the direct measurement of three neuronal circadian rhythms (CR) is possible in isolated parts of the nervous system of *Aplysia*. In the last year Mr. Gerald Audesirk has discovered that the eyes (which contain one of the known circadian oscillators) are necessary for the entrainment, by photoperiod in the intact organism, of another CR in the neuron R15. He has also discovered that the pathway for entrainment in the intact organism need not be synaptic but that a humoral route is sufficient. He has now demonstrated appropriate humoral effects of intact eyes (from phase-advanced organisms) on the timing of the CR in the R15 neuron (from other animals) kept together in the same recording chamber. Mr. Duncan Stuart in collaboration with Audesirk is examining the nature of the material released from isolated eyes. Mrs. Alvarez has continued an electron microscopic study of the *Aplysia* eye. We describe gap junctions between the photoreceptor cells there. The nature and possible mechanisms of long-term effects of synaptic stimulation of R15 (*in vitro*) are described in two papers published this year in the *Journal of Neurophysiology*. Mr. David Armstrong has been further examining the mechanism of the long lasting synaptic inhibition of R15. During the last year, I performed experiments on the regulation of the surface topography of acetylcholine receptors by action potentials and contraction in isolated mammalian muscle while in the Department of Biophysics, University College London.

### 238. PHOTENTRAINMENT OF THE NEURON R15 IN VIVO

**Investigator:** Gerald J. Audesirk  
**Support:**  
Earle C. Anthony Fellowship  
National Institutes of Health, Public Health Service  
Sloan Fund for Basic Research

The identified neuron R15 in the parieto-visceral ganglion (PVG) of *Aplysia* shows a circadian rhythm of spike output recorded in the isolated ganglion (Strumwasser, 1965). The timing of this rhythm can be altered by changes in the lighting regime under which the animal was kept prior to dissection. It has been reported that the eyes of the animal are not necessary for the entrainment of the R15 rhythm by light (Lickey and Zack, 1973). However, in this experiment, both blinded and normal animals were housed together in the same aquaria during entrainment, giving rise to the possibility of "social entrainment."

In the present experiments, *Aplysia* were kept for 1 to 2 weeks on a light schedule of LD 12:12, with lights on at 0800 and off at 2000. Some animals were then moved to individual 15 gallon aquaria and exposed to a 6 hour advanced light cycle, that is, one with lights on at 0200 and off at 1400, for 1 to 2 weeks. Light intensity in the tank varied between 250 and 3000 lux, depending on position and orientation. Prior to placement in the phase-advanced light cycle, animals were: (1) blinded by removal of the eye; (2) sham-operated by removal of a similar-sized piece of skin just anterior to the eye; or (3) left intact. PVGs from all groups, including some intact animals from the original light schedule, were dissected at 0900 ± 100 hours and R15 activity recorded for the subsequent 24 hours or more. Five animals were used in each group. The results (see Table I) clearly show that only the blinded animals were unable to entrain their R15s to the new light schedule.

References:  
239. HUMORAL INFLUENCES OF THE EYE ON R15 IN VITRO

**Investigator:** Gerald J. Audesirk

**Support:**
- Earle C. Anthony Fellowship
- National Institutes of Health, Public Health Service
- Sloan Fund for Basic Research

The circadian rhythm of the neuron R15 can be entrained by light, and in *vivo* the photoreceptor required for this entrainment appears to be the eye (see Biology 1974, No. 238). Further, the nervous pathways between the eye and R15 do not seem to be necessary for entrainment to occur. An inference which can be drawn from these data is that the eye, directly or indirectly, can humorally entrain R15. Experiments were therefore designed to investigate this possibility *in vitro*.

Eyes and R15s were incubated together and recorded from *in vitro* in constant darkness. Isolated eyes show a strong circadian rhythm for several days in constant darkness (Jacklet, 1969). In one series of experiments, eyes and PVG from the same animal were kept together. In this situation it was found that the peak of R15 activity occurred earlier (very near projected dusk) than in R15s recorded without eyes present (difference of 4.7 hours; t = 3.02; p <0.025). In some experiments this circadian peak repeated itself up to three days *in vitro*. Another series of experiments involved eyes and PVGs from different animals. Eyes were dissected from animals whose light cycle was six hours advanced relative to the light regime of those animals from which the PVGs were taken. In this instance, it was expected that, by analogy with the previous experiments, the R15 peaks would occur near “dusk” of the eye, not PVG, projected light cycle. This in fact does occur in most experiments for up to three days. The presence and size of the R15 peaks seem to depend upon the level of activity shown by the eyes. The eyes therefore appear to release spontaneously a humoral factor capable of influencing the activity of R15 *in vitro*.

Preliminary short-term experiments indicate that R15s are responsive to aqueous extracts of *Aplysia* eyes (R15 activity is suppressed in the presence of the extract) and that the active fraction of the eyes is excluded from Sephadex G-10, indicating that it is not the transmitter content of the eye.

**Reference:**
It has been shown (see Biology 1974, No. 239) that the eyes are the most probable photoreceptors for the entrainment of R15 by light. Since R15 is only known to receive a few synaptic inputs from the circumesophageal ganglia (into one of which the optic nerves enter), it was decided to see if these inputs were necessary for the photoentrainment of the cell in vivo.

The same protocol was used as in the previous study. A new set of controls were run, to correct expected peak times for possible seasonal changes (Lickey, 1969). In the experimental animals, both pleuro-visceral connectives were cut prior to placement in the phase-advanced light cycle. A small slit was made in the skin between the anterior attachments of the parapodia, the parieto-visceral ganglion was located, and the connectives cut. The animals were not anesthetized for the operation. The wound was sutured, and the animal injected with ca. 20,000 units each of streptomycin and penicillin. After initial failures with slightly different protocols, only one animal died as a result of the last eight operations.

Results (see Table II) indicated that, of the eight successful operations, five animals entrained to the new light cycle, two did not, and one case was ambiguous. (R15s are reported to show peak activity in the day or night; considering this last R15 to be a day peak, 12 hours later than its corresponding night peak, would put this cell also into the entrained group.) Therefore, it appears that the nervous connections between the eyes and R15 are not necessary for the photoentrainment of the neuron. Humoral influences are apparently sufficient for entrainment.

Reference:

<table>
<thead>
<tr>
<th>Table II</th>
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<tr>
<td><strong>Control</strong></td>
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<tr>
<td>Time of peak</td>
</tr>
<tr>
<td>(PDT)</td>
</tr>
<tr>
<td>-1830</td>
</tr>
<tr>
<td>0100</td>
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<tr>
<td>0030</td>
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<tr>
<td>Mean ± S.D.</td>
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<td>±0254</td>
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<tr>
<td>Predicted peak</td>
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*Control light schedule: LD 12:12; light on at 0800, off at 2000.*
*Shifted light schedule: LD 12:12; light on at 0200, off at 1400.*
*Predicted peak time based on a 6 hour advance over the control peak average.*

The eye of *Aplysia* is interesting because it contains a circadian neuronal oscillator which is expressed in vitro for one to two weeks and which is entrainable by either light or depolarizing agents such as high external [K⁺]. The mode of communication from the eye to the cerebral ganglion is unusual in that compound action potentials (CAPs) are used to transmit light intensity information as well as the circadian rhythm of spontaneous discharges. The mechanisms of synchronous discharge of neural elements in the eye are not known, but a total substitution of...
proprionate for chloride in the bathing medium is known to block CAPs, but not the slow receptor potential as evidenced by an intact ERG (Audesirk, 1973). Hence a detailed light and electron microscopic investigation of the eye has been undertaken to extend our earlier studies (Jacklet et al., 1972).

Gap junctions have been frequently demonstrated between nerve cells in which electrical transmission is known to occur (see Bennett, 1973). We have found gap junctions between photoreceptors in the Aplysia retina. These gap junctions have been studied with ultra-thin sections and the freeze-etching technique (the latter in collaboration with Prof. J.-P. Revel). The morphological characteristics of a photoreceptor cell and its processes are different, but identifiable, so it was possible to identify gap junctions at three different sites between the photoreceptors: (1) between two receptor cell bodies, (2) between the cell body and a process, and (3) between processes. Sometimes the contacts between the processes occur in series (three to four). The gap junction displays the same ultrastructural features as described in other species. The two unit membranes are closely apposed with a narrow gap between them (20 to 25 Å wide). The inner and outer leaflets of the unit membrane are both electron dense, but the inner leaflet appears twice as thick as the outer leaflet.

Thus it appears likely that photoreceptors are electrically coupled in the eye of Aplysia, which may insure synchrony at the earliest level of integration but with a likely loss of acuity and form vision. The complexity of cell types within this eye (not reported here) therefore seems likely to be unrelated to processing of object recognition.

References:

242. PROLONGED INHIBITION OF SPONTANEOUS NEURONAL ACTIVITY

Investigators: David Armstrong, John Lehmann*
Support: National Institutes of Health, Public Health Service
Sloan Fund for Basic Research

Parnas et al. (Biology 1973, No. 27) described a complex synaptic potential which can inhibit the spontaneous firing of R15 in the parieto-visceral ganglion in Aplysia for extended periods, and eliminated the possibility that this hyperpolarization was due to reverberatory synaptic activation. Therefore the mechanism by which the release of inhibitory transmitter for less than a minute affects the ionic permeability of the postsynaptic membrane for hours remains to be elucidated. There are reports that cyclic nucleotide concentrations of nervous tissue can be altered by synaptic stimulation and by the application of putative transmitters, and that such changes mediate directly the synaptic activation of transient changes in neuronal permeability. However, these physiological and chemical changes have never been measured simultaneously in a single neuron. Therefore, we have developed methods to evaluate the hypothesis that the long lasting hyperpolarization in R15 results from an increase in cyclic AMP concentration produced by synaptic stimulation. Less rigorous support for this hypothesis is provided by the observation that bath application of 5 mM dibutyryl cyclic AMP inhibits the spontaneous firing of R15. Furthermore, 10 mM Li⁺⁺, a potent inhibitor of adenyl cyclase in vertebrate brain, blocks the synaptic activation of the long lasting hyperpolarization.

Reference:

*Undergraduate, California Institute of Technology.
243. REGULATION OF THE ACETYLCHOLINE RECEPTOR ON THE SURFACES OF DENERVATED MAMMALIAN MUSCLE

Investigators: Felix Strumwasser, Jean-Jacque Dreifuss

Support: Muscular Dystrophy Associations of America, Inc.
NATO Senior Fellowship

The rat diaphragm, a relatively thin muscle, can be maintained in organ culture for a few days (Miledi and Trowell, 1962) to about two weeks (Purves and Sakmann, 1974). This preparation has allowed an in vitro study of the effects of muscle action potentials and contraction on the distribution of extrasynaptic acetylcholine (ACh) receptors caused to develop by prior denervation for 6 or more days in the intact rat. Purves and Sakmann (1974) had reported that electrical stimulation (in vitro, 37°C) for 6 to 7 days caused a 95% reduction in ACh sensitivity of extrasynaptic sites as assayed by muscle membrane potential response to iontophoretically applied ACh. Stent (1973) has suggested that muscle action potentials (APs) directly displace extrasynaptic ACh receptors from the membrane by effects of the electrical field during the AP. One test of Stent's hypothesis is to prolong the AP whereby it would be expected that a more favorable elimination of ACh receptors might occur for the same number of impulses. A 10°C drop in temperature prolongs the action potential by a factor of 2 to 3 in a variety of excitable cells. We found that at 25°C, the stimulation paradigm used by Purves and Sakmann (1974) produced an increase (20 to 25%) in $^{125}$I-α-Bungarotoxin (BuTX) binding of stimulated diaphragm strips compared with unstimulated (stretched) control strips. In addition, we found that at 35°C there was a 35% reduction in $^{125}$I-α-BuTX binding of stimulated versus control strips. It therefore seems likely that action potentials do not directly cause an elimination of ACh receptors from the surface membrane because the sign of the response to stimulation is a function of the temperature, whereas it is of course well known that the sign of the AP remains the same under these conditions. It is more likely, from other data, that the relationship between APs and receptor topography in these experiments is a complex one involving contraction and the metabolism of the system at the two temperatures.

This work was done at the Department of Biophysics, University College London.

References:
*Department of Biophysics, University College London.

244. EVIDENCE FOR A POSSIBLE SHORT-LIVED 18,000 DALTON INTERMEDIATE IN THE SYNTHESIS OF EGG-LAYING HORMONE IN THE BAG CELLS OF APLYSIA

Investigator: Jeffrey L. Ram

Support: National Institutes of Health, Public Health Service

Arch (1972) showed that the 6000 dalton egg-laying hormone (ELH) synthesized in the bag cells of Aplysia is derived from a precursor of approximately 25,000 daltons. The 25K (K = 1000 daltons) precursor breaks down 30 to 60 minutes after synthesis into 12K and 6K subunits, the latter of which is presumed to be ELH. This could take place in two possible sequences: $25K \rightarrow 12K + 12K + 12K + (6K + 6K)$ or $25K \rightarrow 18K + 6K \rightarrow (12K + 6K) + 6K$. A pulse-chase experiment was performed which supports the existence of an 18K intermediate, as required by the latter sequence.

After rinsing for 3 hours in unlabeled media, parieto-visceral ganglia were labeled for 4 hours in media containing $^{14}$C-leucine, rinsed 4 hours in
unlabeled media, and then labeled 4 hours in media containing \(^3\text{H}\)-leucine. 18 to 33 minutes after the second incubation bag cells were dissected from their connective tissue sheath, and prepared for running on SDS polyacrylamide gels as described elsewhere (see Ram, 1974). Although incorporation at 18K never accounts for a very large proportion of the total counts in the gel (always 9% or less), the second label always showed a higher percent of total counts at 18K than the earlier label (mean difference was 3\%, significant at \(p < 0.01\) on a t test of paired variates, \(N = 6\)). A plot of the ratio of \(^3\text{H}\) counts to \(^{14}\text{C}\) counts produces a distinct and large peak at 18K. Experiments in which both \(^3\text{H}\) and \(^{14}\text{C}\) were incorporated simultaneously produced level plots of the ratio of \(^3\text{H}\) to \(^{14}\text{C}\) (\(N = 2\)), indicating that the effect was not due merely to the difference in label.

The 18K peptide has the following properties expected of a short-lived intermediate in the processing of 25K material into 12K and 6K products: (1) the expected molecular weight, (2) a change in percent of total counts between recent and not so recent labels, and (3) this change being only a small percentage of the total counts at 25K, 12K, and 6K. A fourth property, namely disappearance of the increase at 18K with increasing time after the second labeling, has not yet been tested.

References:

PUBLICATIONS


Work on the effects of visual deprivation and eye enucleation on the peripheral and central components of the visual system was continued. Lid suture and eye enucleation had different effects on the synaptic morphology of the visual cortex. Experiments on the effect on memory formation of proline and glutamine, which counteract the response of dendritic structures to glutamate, were concluded. These experiments support the postulate that glutamate is involved in memory formation. A consequence of this postulate is that neuronal activation causes an electrolyte and water uptake of dendritic spines. An attempt is being made to demonstrate such a change. As a model the monosynaptic activation of the dendrites of granular cells in the fascia dentata by perforant fibers was chosen. In a preliminary investigation the exact location of the endings of these fibers on the granular cell dendrites was determined. In another preliminary investigation the use of freeze-substitution was extended to deep brain structures such as the fascia dentata. Experiments are now in progress to determine the morphological effects of stimulation of the perforant fibers.

245. PLASTIC AND DEGENERATIVE CHANGES IN THE VISUAL CORTEX

Investigator: Eva Fifkova

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

In monocularly lid-sutured albino rats under moderate illumination (500 lux for 8 hours per day) the retina of the occluded eye becomes affected by deprivation of form vision, and the retina of the functional eye by light. These changes also involve the visual cortex. Changes in the synaptic organization of the visual cortex connected with the deprived eye were a mirror image of changes in the cortex connected with the functional eye as compared with the values in unoperated rats. There was a decrease in the density together with an increase in the size of axodendritic synapses and a decrease in size of axosomatic contacts in the cortex connected with the lid-sutured eye with respect to the unoperated controls. Contrary to this in the undeprived cortex there were more and smaller axodendritic synapses and larger axosomatic contacts. In the functional eye a change occurred in the visual receptors and its magnitude was related to the length of daily light exposure, suggesting that enhanced stimulation of the functional eye had occurred resulting in damage to the receptors. To prove this, the daily light exposure was doubled (500 lux for 16 hours per day). Under these conditions severe shrinkage of the receptor layers occurred with a consequent enhanced reduction of the synaptic density in the inner plexiform layer of the retina. In the occluded eye protected by the sutured eyelids the receptor damage was less severe. In addition to the transsynaptic changes caused by the receptor damage, the inner plexiform layer of the sutured eye revealed in the retinas subjected to prolonged light exposure a synaptic rearrangement similar to that observed in the moderate light regimen (i.e., an increased density of amacrine contacts). Two questions arose from these results: (1) How are the changes caused in the retina by light transferred to the visual cortex? (2) Do or do these changes not interfere with the effect of deprivation on the visual cortex?

Thirteen albino rats (8 monocularly sutured at the 14th postnatal day and 5 unoperated) were illuminated daily for 16 hours at 500 lux for 2 months. In semiserial sections cut tangentially through the visual cortex as described previously (Fifkova, 1970) the density of axodendritic contacts was counted in a total surface of 200,000 µ². The size of 34,400 axodendritic and 7300 axosomatic
contacts was measured. In the cortex connected with the sutured eye the total density of the axodendritic synapses was 17% lower than that in the 8-hour illuminated group. A similar decrease in the synaptic density (by 23%) was observed in the inner plexiform layer of the retina in 16 hours as compared to the 8 hours light regimen. Even though the 16 hours illumination affects the receptors it does not change the magnitude of the difference between the deprived and undeprived cortex as compared to the 8 hours daily light exposure. There was a decrease in density (by 9%) together with an increase in size (by 9%) of axodendritic synapses and a decrease in size (by 10%) of axosomatic synapses in the deprived cortex as compared with the unoperated controls. In the cortex connected with the functional eye there were more (by 8%) and smaller (by 5%) axodendritic synapses and larger (by 11%) axosomatic synapses.

Assuming for the axodendritic cortical synapses an excitatory, and for the axosomatic contacts an inhibitory function, then the inverse reaction of these two types of cortical synapses suggests a disturbed balance of the processes of excitation and inhibition in the visual cortex by the changed visual input. Form vision deprivation diminishes the excitability of the visual cortex as has been demonstrated in electrophysiological experiments (Yinon and Auerbach, 1972), which may be caused by the loss of part of axodendritic (excitatory) synapses. The remaining axodendritic synapses may grow bigger by spreading over the dendritic surfaces vacated by the lost contacts (Raisman, 1969). The inhibitory axosomatic synapses may suffer from disuse and grow smaller as the process of inhibition may be of little importance in the cortex with an abnormally low input from the deprived eye. A change opposite to that found in the deprived cortex was found in the cortex connected with the functional eye. Since the difference between the deprived and undeprived visual cortex is not affected by the length of daily light exposure the changes in the undeprived cortex could be attributed to enhanced stimulation of the functional eye before the light causes damage to the receptors.

Summing up: the effect of increased light exposure (1) is translated from the retina to the visual cortex and reduces the density of axodendritic synapses, and (2) does not interfere with the effect of form vision deprivation on the visual cortex.

References:

246. PLASTIC CHANGES INDUCED IN THE VISUAL CORTEX BY EYE REMOVAL

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

The aim of these experiments was to investigate the mechanism underlying the post-deprivation changes in the visual cortex described previously. It has been assumed that these changes are the result of disuse in the deprived visual pathway rather than the result of degeneration. If this assumption were correct then the visual cortex, after eye removal, should show transsynaptic changes which differ from those caused by lid suture. To prove this the visual cortex has been studied after the eye connected with it was enucleated.

In three albino rats the right eye was enucleated under ether anesthesia at the 14th postnatal day. Animals were kept together with those from the group described in the preceding abstract (illumination 500 lux for 16 hours per day) for 2 months. The material was processed in the same way as described in the preceding abstract. The density of axodendritic synapses was counted in a total surface of 75,000 µ². The size was measured in 12,900 axodendritic and in 2440 axosomatic contacts.

In the visual cortex related to the enucleated eye the density and size of axodendritic synapses did not differ from unoperated controls raised under moderate illumination regimen (500 lux for 8 hours per day); it differed therefore considerably from the deprived cortex. The volume reduction of the visual cortex is similar after deprivation and enucleation. The unchanged density of synapses after the latter procedure therefore suggests a moderate loss of synapses, while the pronounced decrease in density
after deprivation is indicative of a considerable synaptic loss.

The moderate change in density together with the unchanged size of axodendritic synapses after enucleation compared to the pronounced changes after deprivation leads to the conclusion that an abnormal visual input is more damaging for the fine structure of the visual cortex than a complete absence of this input. That the enucleation at the 14th postnatal day does not markedly affect the size and density of the synapses in the visual cortex may indicate that in this structure the synaptic development is already completed by this time. No final conclusion can, however, be drawn as long as there are no data available on the synaptic pattern of the visual cortex at the day of enucleation. If the synaptic pattern were not complete at the 14th postnatal day, then it would seem that its further development goes on irrespective of the presence of the visual input. Whether synapses developed under such conditions are silent or can respond to stimulation remains to be established in electrophysiological experiments.

247. THE TERMINATION OF PERFORANT FIBERS IN THE FASCIA DENTATA

Investigators: Eva Fifkova, A. Van Harreveld

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

It has been shown that the fine structure of synapses can be changed by modifying the input presented over longer periods of time. It is generally believed that the process of memory formation is in some way coded in the neuronal connectivities of the brain. Since synapses are capable of plastic reactions which can be recognized in the ultrastructure, it can be postulated that the morphological basis of memory formation also leads to microstructural changes in the synapses. In the brain there are systems displaying considerable functional modifiability which could serve as a model for the changes postulated to occur during memory formation. High frequency stimulation may facilitate synaptic transmission (potentiation) for long periods of time (minutes to hours). Such an effect was demonstrated in a monosynaptic pathway—the pathway of perforant fibers—which originates in the entorhinal area and terminates in the dentate gyrus of the hippocampus (Lömo, 1971a,b). In view of this finding, the objective of the experiments reported below was to study possible morphological correlates of potentiation. For such an investigation the precise knowledge of termination of the perforant pathway within the dentate gyrus is indispensable. The origin, course and termination of this pathway has been studied with the light microscope in normal material and in material after lesions of the entorhinal area. The degenerating pathway was traced by silver impregnation techniques along the hippocampal fissure in the stratum moleculare-lacunosum of the CA1 field of the hippocampus and in the upper half of the stratum moleculare of the dentate gyrus (Raisman et al., 1965; Hjort-Simonsen and Jeune, 1972). However, the termination of this pathway can hardly be identified by the light microscope where it is difficult to make a definite distinction between degenerating axons and degenerating terminals. In the only electron microscopic study dealing with this subject the result of destruction of one part of the entorhinal area was examined (Nafstad, 1967). An electron microscopic study after lesion of the entire entorhinal area which would correspond to the extent of stimulation in the planned experiments was therefore indicated.

Thirteen albino rats were used. Electrolytic lesions were placed in the entorhinal area by a triple electrode introduced from the temporal side in such a way that it followed the curvature of the pyriform lobe. Animals survived this procedure for 16, 24, 36, 48 and 72 hours. Three unoperated animals were used as controls. After appropriate times the anesthetized rats were fixed by perfusion, the brain was dissected and a 1 mm thick coronal section was isolated. From this section a block from the superior hippocampus including the dentate gyrus was prepared for electron microscopy. During the trimming of the block 1 μ sections were stained with azure and toluidine blue for the purpose of general orientation. The final trim, from which the thin sections were cut, included the molecular layer of the upper blade of the dentate gyrus with the hippocampal fissure at one side and the granular...
cells at the opposite side, thus enabling the orientation in the thin section. To get a survey of the lesion, the caudal part of the operated hemisphere including the entorhinal area was examined in paraffin sections stained after Nissl.

(1) **Time of survival.** If there are any indications of degeneration 16 hours postoperatively, they could not be distinguished from the normal variations of the structure. The signs of degeneration became identifiable after 24 hours and increased in severity after 36, 48 and 72 hours.

(2) **Location of degeneration.** The degenerated terminals were confined to the outer third of the molecular layer of the dentate gyrus. The degenerated terminals contacted exclusively dendritic spines of the granular cells and represented about one-third of the total synaptic population of this area.

(3) **Appearance of degeneration.** The terminals showed two types of degeneration: (a) The common type occurring in the central nervous system and generally referred to as dark, electron dense degeneration. The background matrix of the terminal became increasingly dark and the synaptic vesicles, sometimes enlarged, sometimes shrunken, tended to clump together. Also the mitochondria were either swollen or shrunken and the outline of the terminal was collapsed. Subsequently the vesicles and mitochondria became more difficult to identify. The region of the synaptic thickening was the last one to be absorbed, so that the postsynaptic side could still be identified at this stage of degeneration. (b) The second type of degeneration, until now not commonly observed in the brain, consisted of a changed appearance of synaptic vesicles (irregular shape) which subsequently disintegrated. The mitochondria disappeared as well, and the terminal became greatly swollen. The ground matrix of the terminal was pale and contained vacuoles of different shape and size. In spite of these changes the region of the synaptic thickening remained preserved so that the postsynaptic element could be identified. Neurofibrils were never observed in these terminals. The density of dark polygonal granules (probably glycogen) increased in the microglia and astrocytes 24 hours postoperatively. The cytoplasm of both these elements became swollen. The processes of reactive astrocytes, often containing neurofibrils, surrounded the degenerating terminals.

It appears that the axon terminals of fibers from the entorhinal area have two distinct patterns of degeneration, which occur simultaneously. They are likely to be two parallel processes rather than two stages of one process.

**References:**

**248. EXTENSION OF FREEZE-SUBSTITUTION AND ELECTRON MICROSCOPY TO DEEP STRUCTURES IN THE BRAIN**

**Investigators:** A. Van Harreveld, Eva Fiškova

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

Freeze-substitution for electron microscopy had to be applied exclusively to superficial layers of tissues due to the fact that only the superficial 10 to 15 µm can be frozen with sufficient rapidity to prevent ice crystal formation which destroys the fine structure of the tissue. The possibility has been considered that deeper brain structures might be made accessible to freeze-substitution by exposing them before freezing. It was felt, however, that in view of the lability of the brain water distribution the unavoidable mechanical disturbance would introduce serious changes in the brain tissue. The effect of the mechanical disturbance may have been overestimated, however, since Krigman and Bernard (1974) observed a rather normal water distribution in small tissue samples punched out of deeper brain structures and subjected to freeze-substitution. We decided therefore to reinvestigate this problem. The method finally adopted consisted in cutting the head off a small animal (mouse) in a guillotine-like apparatus so that the desired brain structure is exposed in a clean and flat surface of cut tissue. This
The surface is then frozen in the usual way by contacting it with a cooled silver surface protected from the condensation of water, CO₂ and O₂ by a stream of cold helium (Van Harreveld et al., 1965). The electron micrographs prepared from this material showed a surface layer, 1 to several µm thick, in which the tissue elements were abnormally elongated and closely adjoined each other. Under this layer was tissue resembling that observed previously in surface frozen samples of cerebral and cerebellar cortex in which the tissue elements in the fields of thin nonmyelinated axons were rounded and exhibited a rather abundant extracellular space. It is believed that the abnormal surface layer is caused by a shearing action due to the friction of the blade as it cuts through the brain. As usual the tissue in layers more than 10 to 15 µm from the surface is deformed by ice crystals.

It has been possible to investigate in this way a number of previously inaccessible structures such as the deeper layers of the cerebral cortex, the hippocampus and the brain stem. By judiciously locating the cut with the guillotine it should be possible to investigate in this way the entire central nervous system. In previous experiments with surface structures, asphyxiation of the brain tissue caused an uptake of the extracellular material by cellular elements. This has been shown to occur also in the deeper structures. In the hippocampus of a brain asphyxiated for 6 minutes, for instance, there was very little extracellular space and certain tissue elements (dendrites, dendritic spines and glia) appeared electron-transparent and swollen.

References:

249. ELECTRON MICROSCOPIC EXAMINATION OF THE FASCIA DENTATA AFTER STIMULATION OF THE PERFORANT FIBERS

Investigators: A. Van Harreveld, Eva Fifkova
Support: National Science Foundation
National Institutes of Health, Public Health Service

A transport of extracellular material into the intracellular compartment occurs during spreading depression (SD) resulting in a change in size and shape of the structures involved. There is evidence that this transport is caused by an increase in Na permeability of the plasma membrane of dendritic elements and that this permeability increase is caused by a release of glutamate from the intracellular compartment where it is present in high concentration (10 mM). The physiological significance of SD is not well understood. We have considered the possibility that SD is an abnormal mass activity of a process which has its functional meaning on a microscale. Spreading depression can be elicited by transcallosal stimulation and by convulsive activity, suggesting that neuronal activation results in a release of glutamate. This release may occur not only at synapses, but anywhere in the course of the neurons involved since a glutamate release has been demonstrated even from excited peripheral nerve. A physiological activity could release glutamate in a pattern determined by the pattern of incoming impulses, causing a localized uptake of extracellular material by dendritic elements and a decrease in the longitudinal resistance of these elements, especially of dendritic spines, thereby enhancing the efficacy of their synaptic activation. Such a mechanism could change the relative weights of synaptic inputs and be involved in learning and conditioning, a speculation advanced by Rall (1970) and by Diamond et al. (1970). We have found support for such a glutamate mechanism using a compound, L-proline, which counteracts the effect of glutamate on the Na permeability of the plasma membrane, prevents SD (Van Harreveld and Fifkova, 1973), and suppresses the short-term memory formation (Fifkova, 1973).

The system of perforant fibers synapsing with dendrites of granular cells in the fascia dentata was used as a model for an investigation of the effect of stimulation of presynaptic structures on the postsynaptic dendritic spines. The use of this system seems appropriate since as shown by Lømo (1971a,b) stimulation of the perforant fibers causes a potentiation of the response in granular cells of...
similar duration as the short-term memory.

The perforant fibers were stimulated in the entorhinal area of narcotized rats and several minutes later the head was placed in the guillotine described in the previous abstract, where it was cut at a region which after substitution in acetone with 2% OsO	extsubscript{4} allowed the collection of blocks of the fascia dentata. A number of experiments of this kind have been performed; however, the investigation is not sufficiently advanced to make conclusions about the effect of stimulation on the postsynaptic dendritic spines.

References:

250. TOPICAL APPLICATION OF GLUTAMATE AS A STIMULUS FOR SPREADING DEPRESSION

Investigators: Eva Fifkova, A. Van Harreveld

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

Although several investigators found that glutamate is an effective stimulus for spreading depression when applied topically to the cortical surface of rats and rabbits, Do Carmo and Leão (1972) were unsuccessful with this method of application. We have examined this discrepancy and confirmed our previous observations (Van Harreveld, 1959) that in young rabbits (1.5 to 2 kg) the topical application of L-glutamate in concentrations of 5 to 25 mM (mean 15 mM) causes spreading depression. However, in older rabbits (3 to 3.5 kg) much higher concentrations of glutamate, up to 150 mM, were necessary to produce this phenomenon. In some animals even this high concentration was ineffective, although spreading depression could be elicited in these animals by KCl in the usual concentration (1%). The threshold for D-glutamate was much lower (5 to 20 mM) in these animals. Similar observations were made on rats.

The variability of the stimulating effect of glutamate could be due to the pia glial membrane which covers the cortical surface and may form a barrier against the diffusion of the amino acid into the brain tissue. Uptake of glutamate by cellular structures and metabolism will counteract the building up of the threshold concentration for spreading depression. Furthermore desensitization may decrease the sensitivity of the elements to be affected. The accumulation of glutamate in the cortical tissue has therefore to occur at a certain rate to elicit spreading depression. The pia glial membrane may counteract such a rapid buildup more in older rabbits than in young. It is of interest in this respect that D-glutamate which is not metabolized has a markedly lower threshold than the L form. It is furthermore of interest that stimulation with KCl does not show the variability exhibited by glutamate. This may be due to a difference in the routes of entrance into the cortical tissue. L-glutamate apparently does not affect the glia and its route of entrance may therefore be restricted to the narrow (100 to 200 Å) slits between the overlapping endfeet of glial elements. Gap junctions may even further impede the entrance of glutamate. Since the glial membrane is permeable for K ions the application of KCl to the cortical surface may have a much wider avenue of entrance to the brain tissue than glutamate.

References:
PUBLICATIONS


Our research efforts this year have again centered on the properties shown by individual neurons of the visual system of crustaceans. The most unexpected result concerns the location of the memory mechanism for the visual background, as obtained through the reactions of the optomotor system. It is now clear that, at least in its main aspect, memory is located at an early stage of the information transfer chain between the retina and the motor axons. The direct proof for this statement comes from the discovery of an optical illusion, which results in movement of the eyecup opposite to that in which the background moves as a whole. This illusion occurs when the majority of the background components have apparently been displaced in the opposite direction from the background as a whole. For the memory type of reaction shown by the rock lobster's seeing fibers a similar distribution of independently acting local phenomena is also evident. The entrainment shown by the crayfish's jittery movement fibers to the flicker frequency of light flashes is another example of the fact that complex reactivity occurs in neuronal networks of relatively few elements. For the future this raises the possibility that such phenomena, which have generally been considered to require the participation of very complex neuronal arrangements, such as the mammalian brain, may be located at unitary synaptic levels. This would make such phenomena more available to the type of approach now being applied to the crayfish sustaining fibers, i.e., white-noise stimulation with computer analysis of the data. That peripheral elements may have a profound effect on visual reaction is also shown by the functional and structural abnormalities caused by light deprivation in the retinular cells of the crayfish.

251. THE ORGANIZATION OF OPTOMOTOR MEMORY

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

Decapod crustaceans remember the position of the visual surround as reported in Biology 1972. The animal is placed within a striped drum and allowed to view it for some time. The drum is then rotated to a new position unseen in darkness. Upon reillumination a memory response which can be easily observed by recording the activity of optomotor neurons occurs as the animal moves its eyes.

The memory response results from a comparison of drum position before and after movement with the size of the neuronal response being a function of displacement. Thus by systematically varying different stimulus parameters and observing orderly variation in the size of the response it is possible to determine what information is compared and thus remembered.

In one set of experiments the size of the memory response was found to be directly proportional to the number of stripes present for as many as 6 stripes occupying an area of 132°. This indicated that each stripe contributes the same amount to the memory response as the others and that all parts of the retina can contribute equally to memory. The latter conclusion was confirmed in experiments in which one or two stripes were presented either to the front, middle, or rear third of the eye.

Since the separation between stripes was constant in the preceding experiment the area occupied by a block of stripes increased as the number of stripes increased. As a control the responses to two stripes with different separations were compared. To our surprise the strength of the memory response increased proportionally as the separation between stripes was increased up to 60°.
As the separation increased further, no further change was observed. Subsequent experimentation revealed that the response for 2 stripes occupying a given area was equivalent to that for a greater number occupying the same area, for angles of less than 60°.

To test if the stripes in the middle of the pattern were suppressed by those at the ends, two stripes in the middle of a block of four were rotated while the outer ones remained stationary in a device consisting of a drum within a drum. The outer drum was stationary and bore the stripes at the ends of the pattern. The inner drum rotated and bore the middle stripes between windows through which each of the outer stripes could be viewed. Small but definite memory responses were observed.

To test whether the response was proportional to the size of background area in which the stripe was located, the responses for patterns of 7 narrow and 7 wide stripes were compared. The resulting ratio was approximately equal to that for the areas occupied by the two patterns.

These experiments show that each element of the background is remembered individually and that the contribution of each element to the response is proportional to the area of the visual field represented by the element.

252. FACTORS AFFECTING THE RELATIONSHIPS BETWEEN DISPLACEMENT AND THE SIGN AND STRENGTH OF THE MEMORY RESPONSE

Investigators: Richard L. Hirsh, C. A. G. Wiersma, Daniel B. Diner*

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

As reported previously the size of the memory response increases as displacement is increased to 3° when unequal black and white stripes of 12° are rotated (Biology 1973). As stripe movement is further increased the size of the memory response declines at first slowly and then rapidly with the sign of the response changing when rotation exceeds 12° which in this case is half of the periodic interval. The question whether the location of the middle of the stripe cycle affects the occurrence of the maximum at 3°, the rate of the subsequent decline, or only the change in the sign of the response, was asked. To this end 3 different patterns consisting of 6° stripes separated by 7.5, 12, or 18° were rotated by various amounts.

For all three patterns maximum responses occurred after 3° rotations with slightly weaker responses occurring after 6° turns. No significant difference in the rates at which response strength declined as rotation increased were observed until displacement exceeded half the periodic length, at which time the sign of the response changed. These results indicate that the location of the midcycle point affects only the sign of the response.

In another set of experiments the width of the stripes was varied while the periodic interval remained constant. Black stripes of 6 or 18° separated by 18 or 6° respectively were rotated various amounts. Although in both cases the sign of the response reversed when displacement exceeded 12°, the subsequent decline was slower for the wide black stripes than it was for the narrow ones during its initial phases. Thus stripe width is indicated as a factor determining the reduction in response strength after relatively large displacements.

*Division of Engineering and Applied Science, California Institute of Technology.
253. AN OPTICAL ILLUSION OF THE CRAB

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

The optomotor memory response results from a comparison of the position of the background before and after movement. As in all comparisons, points of reference are required. In these experiments the nature of these reference points was studied. Three hypotheses were tested. One held that the memory responses result from the maintenance of a fixation upon a particular part of the striped pattern. Another held that the comparison involves the position of the pattern as a whole relative to a fixed external point or frame of reference. The third hypothesis was that in each part of the eye on which the image of an individual object falls, its position before and after movement is independently compared.

To test these hypotheses a pattern of 4 black stripes, each 12° and separated by 12°, was rotated 27° and 21° in the direction in which the fiber being observed caused the eye to move (positive) and 21° in the opposite (negative) direction. The resulting responses were examined. Both the fixation and external reference hypotheses predict that the responses following positive turns should be excitatory while those to negative turn displacements should be inhibitory. Piecemeal analysis, however, predicts the appearance of what is in effect an optical illusion. Although after positive turns of 21° the leading stripe is greatly displaced in the direction of rotation, the other three appear to have been displaced 3° in the negative direction. The response should be inhibitory, assuming that all elements contribute at least equally. The converse is true for negative turns of 21°. These latter predictions were confirmed.

The responses to positive turns of 27° and negative turns of 21° were compared quantitatively. In both cases three of the stripes appeared to have moved 3° in the positive direction, although the fourth is greatly displaced in the direction of actual rotation after 27° displacement, and somewhat less in the negative direction after the 21° rotation. No distinguishable difference in the strength of the responses to the two turns was observed. When rotation was increased so that only two stripes overlapped former stripe positions this equivalence was maintained although the responses were commensurately smaller.

These results conclusively show that the position of each of the background elements is remembered in terms of the part of the eye on which its image fell prior to movement, and that the memory response is not the result of the maintenance of a fixation upon a particular feature of the pattern.

254. THE ROLE OF THE EDGES IN DETERMINING THE MEMORY RESPONSES

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

In an effort to find on which features of a striped pattern the memory response depends, only one set of edges of the stripes was moved while the other remained stationary. A double drum (see Biology 1973, No. 18) was employed. Two types of edges, leading and trailing, may be distinguished. Leading edges are defined as those in which the transition from black to white is in the direction in which the observed fibers cause the eye to move. For trailing edges the transition is in the opposite direction.

As displacement of the leading edges increases the size of the memory response increases monotonically although increments in response strength for displacements of more than 3° are relatively small. For displacements of only the
trailing edges the responses are considerably weaker than those for the leading edges, but always also positive. Response strength reaches a maximum at 3° and then undergoes marked decline. The significance of these results is not yet clear. It appears that the response to stripe rotation is mainly if not exclusively caused by leading edge displacement and that the trailing edge, through its involvement in determining stripe width, has an auxiliary function.

255. THE MEMORY RESPONSE OF A VISUAL INTERNEURON

Investigators: Thomas Labhart, C. A. G. Wiersma
Support: National Science Foundation
National Institutes of Health, Public Health Service

The so-called seeing fibers in the optic nerve of the rock lobster described by Wiersma and York (1972) are units which react with a continuous discharge to targets moved in the visual field. They also exhibit a special property, which can be described as memory, shown as follows: an animal is placed in a homogeneously gray drum illuminated by dim light. The light is turned off a short time during which a black target is introduced in the drum within the fiber's visual field. After turning the light on, seeing fibers will react with a prolonged burst. They will not react, however, if the target is already present for some time before the dark period, or the drum is left empty, i.e. if the visual cues are the same before and after the dark period. The strength of the response decreases with increased dark time; memory seems to disappear after 30 seconds. So far, such a memory response could be found for each type of seeing fiber tested.

A memory reaction was also found when the target was already present before the dark time, but was shifted within the visual field of the unit during that time. Obviously seeing fibers can remember visual cues and send a message, if the new visual stimulus is different from the previous one.

Further properties concerning the memory of the seeing fibers are being tested. When memory tests are performed immediately following each other, the response decreases strongly. This is probably due to local habituation to the presented target, for when the target is placed in another unstimulated part of the fiber's visual field, the response is as strong as the initial one.

The memory performance seems also to depend considerably on the condition of the animals. For instance, it may deteriorate during a session for reasons still unknown, though the reactivity to moving objects remains excellent. Especially intriguing are seeing fibers with binocular input. They may allow the study of problems such as memory transfer.

Reference:

256. THE RESPONSE ENTRAINMENT OF JITTERY MOVEMENT FIBERS OF THE CRAYFISH

Investigator: Tateo Shimozawa (by C. A. G. Wiersma)
Support: National Institutes of Health, Public Health Service
National Science Foundation

As reported in Biology 1973, the jittery movement fibers respond with an off-discharge whose time of occurrence is, within certain limits of the parameters, proportional to the frequency of the light flickers presented to the eye. This phenomenon can be described as due to the wait time for the onset of a missing flash, to which about another 50 msec are added before the response occurs. The latency of the "off" response was constant at 50 msec, when flashes longer than ¾ second were given, whereas it became much longer, up to 250 msec, for flash duration shorter than 50 msec down to 5 msec. Between 50 and 250 msec flash duration, off responses were rare or absent. Different fibers showed variation in the number of flashes required to obtain a constant response to a missing flash, but
in most cases 10 flashes at constant frequency were sufficient to obtain good entrainment. Sustaining fibers similarly investigated showed completely different properties.

257. WHITE-NOISE STIMULATION OF A CRUSTACEAN OPTIC INTERNEURON

Investigators: Gilbert D. McCann*, Tateo Shimozawa, C. A. G. Wiersma
Support: National Science Foundation
National Institutes of Health, Public Health Service

In previous investigations it has been found that the reactivity of crayfish sustaining fibers offers some interesting properties with regard to various parameters of the stimulus situation. Because their signals can be very large and be kept over long time periods, it was decided to submit their responses to computer analysis, with the use of white-noise modulated light intensity as the stimulus. When applied through a single spot light in the middle of their excitatory field, very clear first- and second-order kernels are obtained, which are not materially shifted when changes in excited state occur. This indicates that such changes are equivalent in their effects to those in light intensity. The discharges are distinguished in that there is a dynamic and a static component, a “burst” followed by a sustained discharge. Investigations as to their relationship are being pursued by specially developed white-noise programs in which very low frequencies are present. This also involves a single experiment running for several hours without a significant change in any of its properties, which for this type of preparation is quite possible. The interactions from two source levels, either both in the excitatory field, or one there and the other in an inhibitory location, are also studied.

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

258. INFLUENCE OF DARKNESS ON CRAYFISH PHOTORECEPTORS

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

Photoreceptor cells of the compound eye were studied electron microscopically in newly hatched crayfish Procambarus clarki that had been grown in complete darkness for 11 weeks. Since the microvilli of recently differentiated retinula cells appeared uniform in size and were regularly oriented, light is not necessary for their development. Variability in size due to microvilli enlargement and the disruption of their normal orientation became progressively greater with the age of the cell; therefore, light is needed for maintenance of normal features and apparently of function. Behavioral experiments with two older light-deprived animals indicated that they were blind.

259. UNITS IN THE OPTIC NERVE OF THE CRAB, PACHYGRAPSUS

Investigators: Li-Ho Raymond Hou*, C. A. G. Wiersma
Support: National Science Foundation
National Institutes of Health, Public Health Service

A renewed trial (see Biology 1972) was made to obtain information about the properties of the optic fibers of this shore crab, especially by the use of so-called central leads. It was again obvious that the methods used so successfully in crayfish and rock lobster are much less so here, though the needles used were very selective. The number of established fibers increased relatively little. One reason appears
to be that the total number of larger fibers in the optic is small, whereas there are many fine fibers. Only 9 fibers were obtained often enough to be established as compared to more than 80 in crayfish and about 150 in rock lobster. The difficulty of obtaining sustaining and dimming fibers in this crab was confirmed. Jittery movement fibers were most often encountered, of which 17 with different visual fields were found and 5 established. One of the latter is space-constant, i.e. it changes its field as the position of the body of the crab is changed in space. Next most common are medium movement fibers; 12 were found, of which 3 are established, one of which is space-constant. Three fast movement fibers were seen, of which again one was space-constant. Only a single fiber was so far discovered which showed more complex visual-properties, but these were unlike the properties of the lobster’s seeing fibers. A single multimodal fiber with jittery input from the eye and mechanical input from all hairs on the ipsilateral body side was present several times. Several purely mechanoreceptive fibers were also found.

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

PUBLICATIONS


Our research is concerned with the general question of how genes control the formation of biological ultrastructure. We have focused on bacterial virus morphogenesis as a model system, using bacteriophages T4 and T7 to study the structure, function, and assembly of phage components by genetic and biochemical methods.

During the past year, we have continued work on the assembly of phage T4 tail fibers, the organelles that initiate adsorption to the bacterial host cell surface. The goal of this study is to understand the molecular roles of all of the proteins involved in tail fiber assembly and attachment. The fiber itself is a relatively simple structure consisting of four proteins (see Biology 1973, page 172, Figure 1). However, four additional proteins are now known to be required in accessory roles for its assembly and attachment to the phage. Three of these proteins are not present in the finished virus. They are, therefore, candidates for catalytic roles in assembly, and their functions are of great interest. Two of the four proteins somehow promote the folding of the major tail fiber structural protein that determines host range, and the other two promote the attachment of finished tail fibers to the phage particles.

We also have continued to investigate the role of host cell factors in phage morphogenesis, through further characterization of bacterial mutants unable to support T4 morphogenesis and phage mutants that can overcome these host defects (see Biology 1972, Nos. 8 and 9, and Biology 1973, Nos. 231-234). Bacterial mutations causing phage-host defects (phd mutations) that block T4 morphogenesis at an early stage in head assembly have been shown to map at three distinct loci on the bacterial chromosome. Phage mutations that overcome these defects have been shown to map in two phage genes that control head assembly. In other work, characterization of capsid-related structures and of capsid-DNA complexes formed in the course of phage T7 infection has led to a novel proposal for the mechanism of DNA packaging in viral maturation.

In the following reports, defective extracts prepared from nonpermissive (su-) 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 shown in Biology 1972 (page 14). Sodium dodecyl sulfate is abbreviated SDS.

General References:
260. ROLE OF THE WHISKERS IN T4 TAIL FIBER ATTACHMENT

Investigators: William B. Wood, M. Patricia Conley, Ann E. Orel*

Support: National Institutes of Health, Public Health Service

The whiskers of bacteriophage T4 are 20 x 400 Å filaments that extend outward from the collar and play a role in regulating retraction and deployment of the tail fibers (see Biology 1973, No. 229). We have shown now that they are required also for efficient tail fiber attachment during phage assembly. In previous experiments (ibid) infected-cell extracts containing tail fibers and whisker antigen activated ordinary (wac") and whiskerless (wac) tail-fiberless particles equally well. However, extracts containing tail fibers but no whisker antigen activated wac particles about 10 times more slowly than wac" particles, which are activated at the normal rate. The whiskers are not essential for phage growth in vivo, since wac mutant phage can produce plaques; however, one-step growth experiments showed that the mutation causes a delay in the appearance of intracellular phage following infection and a 5-fold decrease in the yield of infectious phage produced.

The following observations support the view that the effect of whiskers on tail fiber attachment is due to a direct interaction between the whisker and the distal half of the tail fiber, similar if not identical to the interaction controlling tail fiber retraction in complete phage. A slow rate of in vitro fiber attachment similar to that described above is seen with wac" particles when the tail fibers carry a mutational alteration in P36, a structural protein of the distal half-fiber near the knee joint (see Biology 1973, page 172), or when the wac" particles are pretreated with anti-whisker serum (Biology 1973, No. 229). Lack of whiskers does not affect the slow rate of attachment of proximal half-fibers to the baseplate of fiberless particles (Biology 1972, No. 7), but lack of whiskers greatly decreases the rate at which particles with attached proximal half-fibers are activated by addition of distal half-fibers.

Since whiskers normally are attached to the phage only after head-tail union (Coombs, 1974), these findings explain why tail fibers do not attach efficiently to the baseplates of free tails. The whisker interaction also probably accounts for a previous observation that sites on the phage head appeared to be involved in tail fiber attachment (Biology 1970, No. 91).

In summary, the whiskers appear to promote tail fiber attachment through a transient interaction with the distal half-fiber. Assembly and attachment of the T4 tail fiber, a simple structure consisting of four proteins, therefore requires the participation of four additional proteins: P38, P57, P63, and Pwac. These findings suggest that accessory proteins promoting the assembly of supramolecular structures may be a general phenomenon in biological systems.

Reference:

*Undergraduate, California Institute of Technology.

261. FURTHER EVIDENCE ON THE ROLE OF P38 IN T4 TAIL FIBER ASSEMBLY

Investigator: Robert J. Bishop

Support: National Institutes of Health, Public Health Service

P38 is a nonstructural viral protein required for the assembly of the distal half-fiber (BC') of bacteriophage T4 (see Biology 1973, page 172). In the absence of P38 function, P37, the major structural protein of the BC' half-fiber, is synthesized in normal amounts but does not dimerize into half-fibers or exhibit the C antigens normally associated with the completed distal half-fiber. The isolation of a mutant phage with altered P37 which produces active BC' half-fibers in the absence of P38 function (Biology 1973, No. 224) supports the possibility that P38 acts by inducing a conformational change in P37 without altering its covalent structure.

During the past year I have isolated additional temperature-sensitive (ts) second-site revertants of a
38-defective mutant in order to determine whether all such second-site mutations map in gene 37 and if so, whether all map in the same region of gene 37. Phage carrying two am mutations in gene 38 were plated on nonpermissive host cells. Plaque-forming particles were found at a frequency of about 10^{-7}. Among 7746 such revertants, 1262 were ts. Since most of the revertants grew poorly, the ts revertants were further screened for good growth on the nonpermissive (su) host, and four were selected for further characterization. These revertants were shown to retain the original am mutations as well as a ts mutation at a second site. The new ts mutations do not map in gene 37, and do not appear to be located in any other previously identified T4 gene. Two of the mutations map between genes 38 and 52 and two map between genes e and 57 (see T4 map, Biology 1972, page 14). Experiments are in progress to establish whether the new genes defined by the ts mutations play a direct role in tail fiber assembly and if so, to elucidate their functions.

Additional evidence on the role of P38 comes from experiments with hybrids of T4 and the related phage T2. All the gene products required for the assembly of active T4 tail fibers are interchangeable with their T2 homologues except that P38 and the C-terminus of P37 must be derived from the same phage. Antiserum specific for the C antigens of the T4 distal half-fiber shows little cross-reaction with the T2 distal half-fiber (see Biology 1972, No. 5).

Hybrid phage were constructed with part of gene 37 from T4, part from T2, and an am mutation in gene 38. Two types of hybrids were obtained, one producing P37 with the N-terminus from T2 and the C-terminus from T4 (N_2-C_4), and another with the N-terminus from T4 and the C-terminus from T2 (N_4-C_2). Although neither of these phage produces active half-fibers in the nonpermissive host due to the gene 38 am mutation, the N_4-C_2 hybrid produces the same amount of C antigen on nonpermissive and permissive hosts. By contrast, the N_2-C_4 hybrid produces normal levels of C antigen on the permissive host but little or no C antigen on the nonpermissive host. It therefore appears that P37 in the N_4-C_2 hybrid can dimerize (as measured by the appearance of C antigen) in the absence of P38 function, although this dimerization is not sufficient to produce an active half-fiber. These results suggest that P38 from T2 is not required for the dimerization of T2 P37 but plays some other role in producing an active C half-fiber. Thus T4 P38 also may have additional functions besides simply facilitating the dimerization of T4 P37.

262. EXPERIMENTS ON THE ROLE OF P57 IN T4 TAIL FIBER ASSEMBLY

Investigator: Richard O. Herrmann
Support: Deutsche Forschungsgemeinschaft
National Institutes of Health, Public Health Service

P57 is a nonstructural phage T4 gene product that acts at three different points in T4 morphogenesis. It is required for the dimerization of P34 and P37 into the rod-like precursors of the proximal and distal halves of the tail fiber, respectively, and for the incorporation of P12 into the phage baseplate, but its role in these processes is not yet understood. I have undertaken two approaches to the problem of P57 function: characterization of the protein with regard to its location in the cell, using polyacrylamide gel electrophoresis in the presence of SDS to assay its presence in cell fractions, and characterization of bacterial mutants that specifically allow a bypass of the normal requirement for P57 in T4 assembly (Biology 1973, No. 225). The following results have been obtained so far.

P57 can be identified on SDS-gel electropherograms of infected-cell extracts by its absence from 57-defective extracts, as shown by Vanderslice and Yegian (1974). On SDS gradient gels of 10 to 20% polyacrylamide, it migrates as a sharp band with the same mobility as myoglobin, indicating a molecular weight of about 18,000. P57 is synthesized throughout the latent period of T4, as shown earlier using other techniques (Hosoda and Levinthal, 1968).

When the bacterial bypass mutants are infected with 57-defective am mutants in liquid culture, the phage yield is 2 to 10% of that obtained in control infections with T4++, whereas infection with a gene 42-defective am mutant gives less than 0.1% of the control yield. The host bypass phenotype is temperature-dependent in some of the mutant
bacteria. The P37 in 57-defective extracts made with normal host cells travels with the fast-sedimenting bacterial-envelope fraction in sucrose gradient sedimentation experiments, whereas the P37 in 57' extracts is present primarily as slowly sedimenting half-fibers. Most of the P37 in 57-defective extracts made with bypass mutant cells also sediments at the slow rate characteristic of half-fibers.

Analysis of the protein composition of the cell envelope in six of the bypass mutants by SDS gel electrophoresis has shown that one of the mutants overproduces two proteins relative to normal cells. When the envelope of this latter mutant is separated into cytoplasmic membrane and outer cell wall fractions by equilibrium sedimentation in a sucrose density gradient, these proteins are found in the outer cell wall fraction. Revertants of this bypass mutant are now being isolated to determine whether the overproduction of cell wall proteins is related to the bypass phenotype.

References:

### 263. DISTRIBUTION OF ANTIGENIC SITES ALONG THE PROXIMAL HALF OF THE T-EVEN PHAGE TAIL FIBER

**Investigator:** Brian S. Seed  
**Support:** National Institutes of Health, Public Health Service

RB phages, a class of T4-related bacteriophages (Russell, 1967), are neutralized by anti-T4 antiserum. When antiserum directed against the proximal (A) half of the T4 tail fiber (anti-A serum) was allowed to react with an excess of any one of 9 RB phages, the antiserum retained some of its ability to neutralize T4 phage. This indicates that none of the 9 RB phages have all the antigens present on the T4 A half-fiber. Similar experiments, in which anti-A serum was incubated with various pairs of RB phages demonstrated a class of A antigens common to all RB phages and T4. Some RB phages share only these antigens with T4. Electron microscopy of all RB phages treated with anti-A serum showed antibody molecules uniformly attached along the entire length of the A half-fiber. These results suggest the possibility that in contrast to the distal half-fiber (Biology 1972, No. 5; Beckendorf, 1973) the proximal half-fiber may exhibit a repeating pattern of antigenic determinants along its length. Experiments to test this hypothesis are in progress.

**References:**  

### 264. USE OF T4 go MUTANT PHAGES TO SELECT NEW CLASSES OF PHAGE HOST-DEFECTIVE (phd) BACTERIAL MUTANTS*

**Investigators:** Helen R. Revel, Barbara L. Stitt  
**Support:** National Institutes of Health, Public Health Service

We have previously isolated about 300 bacterial mutants of *E. coli* strain SKB178 that will not support the growth of wild-type T4 (hd mutants, *Biology* 1972, No. 8). The plating properties of a set of 5 go phage mutants (go A-1, go D-1, go D-3, go C and go F), each selected for ability to overcome the block to T4 development in a particular phd host, defined at least 6 phenotypic classes of phd mutants designated A, A-D, D, B, C, and F. T4 infection of phd classes A, A-D, D and B mutant bacteria leads to normal lysis, but no progeny phage are produced, suggesting a block in phage assembly. Analysis revealed that in all these phd strains, T4 development is arrested early in T4 head morphogenesis at the level of gene 31 function (*Biology* 1973, No. 231). T4 infection of phd mutant classes C and F is characterized by failure to lyse and defective phage DNA synthesis (*Biology* 1972, No. 8).

In order to isolate other classes of phd mutants
blocked in T4 morphogenesis we again have mutagenized SKB178 bacteria and used go mutant phages as selecting agents to eliminate previously isolated classes. As shown in Table I, selection with T4goA-1 + T6 at 30°C or 37°C eliminated all phd mutants of classes A-D and A, and gave 7 isolates ("other" column) that failed to grow T4 or any standard go phage. Selection at 37°C with a recombinant phage T4goD-1, F-1 + T6 eliminated phd classes D and F, drastically reduced the frequency of the A-D class, and gave 64 isolates that failed to grow any standard go phage. At 42°C selection with T4goD-1, F-1 + T6 was not successful because go D-1 does not grow on phd class A-D at this temperature; thus the majority of survivors were A-D. At 30°C this selection yielded few new phd strains. The majority of the surviving colonies contained cells which were quite leaky with respect to wild-type T4 growth but proved to be defective to varying degrees to either go A-1, go D-1 or go D-3.

Lysis following T4 infection was tested for 23 phd mutants from the "other" class (all probably independent). 13 lyse at the normal time, suggesting that their host defect blocks phage assembly. In vitro complementation tests indicate that all 13 are blocked in T4 head formation. go phage mutants have been isolated by plating T4 on some of these new phd strains. One of these go phage has been characterized: it grows on all 13 of the new phd strains and shows plating properties different from any of the standard go phages, but like some of them carries a mutation in or near gene 23. Two of the new phd mutations have been crudely mapped using the F'117 and F'118 episomes, and in both cases the new phd mutation has been located in the same region of the chromosome as the original phd mutations that block T4 head morphogenesis.

In summary, most if not all of the host-defective mutants isolated from SKB178 that block T4 morphogenesis appear to do so at an early step in head formation. Since Pulitzer and Yanagida have found evidence for a bacterial role in the formation of tail fibers in another bacterial strain, W3350, it is likely that the frequencies of different classes of host-defective mutants vary in different bacterial strains.

Reference:


*The symbol phd (phage host defective) replaces the previous designation hd (host defective) to conform with standard bacterial genetic notation. Individual strains are designated HD followed by the isolation number. Phenotypic classification of phd isolates, determined by the plating properties of T4 go mutant phages, is given by a capital letter (A, B, C, etc.) following the isolation number.

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<tr>
<th>Exp.</th>
<th>Selecting phage ( (T) )</th>
<th>Clones</th>
<th>Mutants</th>
<th>phd class, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T4 + T6 ( (37°, 42°) )</td>
<td>25</td>
<td>250</td>
<td>A-D  A  D  B  C  F  Other</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>90  1  4  1  1  1  2</td>
</tr>
<tr>
<td></td>
<td>T4goA-1 + T6 ( (30°, 37°) )</td>
<td>1</td>
<td>26</td>
<td>0  0  42  0  4  27  27</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>0  0  42  0  4  27  27</td>
</tr>
<tr>
<td></td>
<td>T4goD-1, F-1 + T6 ( (42°) )</td>
<td>10</td>
<td>100</td>
<td>48  1  0  0  34  0  17</td>
</tr>
<tr>
<td></td>
<td>( (37°) )</td>
<td></td>
<td></td>
<td>1  4  0  5  26  0  64</td>
</tr>
<tr>
<td></td>
<td>( (30°) )</td>
<td></td>
<td></td>
<td>(see text)</td>
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<td>3</td>
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265. THE GENETIC LOCI OF phd MUTATIONS IN E. COLI

Investigators: Helen R. Revel, Ilga Lielausis
Support: National Institutes of Health, Public Health Service

It was reported previously (Biology 1973, No. 234) that mutations in 7 of 10 phage host-defective (phd) strains in which T4 development is blocked early in phage head assembly are co-transduced with purA (as are similar mutants isolated by others: groEA44, Georgopoulos et al., 1972; mop, Takano and Kakefuda, 1972; groE-1, Sternberg, 1973; tab-2, Coppo et al., 1973). The three phd mutants that failed to show co-transduction with purA, mutants HD71D, HD365DF and HD45B, have been investigated further. All three strains were cold sensitive (cs) for bacterial growth when originally isolated. F117, an episome which extends from 81 to 89 minutes on the E. coli map and includes the purA region, converts HD71D and HD45B to phd+ and cs+. These results show that the phd+ character is dominant and that these phd mutations are close to, but probably not in the same gene as, phd mutations which are co-transduced with purA. This experiment could not be carried out with HD365DF because the stab culture reverted to cs and phd, suggesting that the phd character and the cs phenotype may have been the result of a single mutation. P1 transduction experiments to pinpoint the HD71D and HD45B mutations are in progress. Newly isolated phd mutants, blocked in phage head assembly, map in the same area of the bacterial genome (see Biology 1974, No. 264).

The genetic locus of phd class F mutations, which cause defects in T4 DNA synthesis, is being investigated. The experimental approach is to introduce bacterial episomes carrying various known bacterial dna+ genes which have been shown to be dominant to the corresponding dna alleles and determine if the heterogenotes have become phd+. Such experiments have shown that phdF is not near dnaB, the gene for a host protein involved in λ DNA synthesis.

References:

266. THE GENETIC LOCI OF goD-3 AND goF-1 IN T4

Investigators: Helen R. Revel, Ilga Lielausis
Support: National Institutes of Health, Public Health Service

The T4 mutant goD-3 grows on host-defective bacterial mutants HD36D in which wild-type T4 development appears to be blocked in head assembly at the stage mediated by P31, an accessory protein of unknown function that is required for the organization of the major head protein (P23) into head structures. These phd mutations can be overcome by go mutations in T4 gene 31 (Biology 1972, No. 8). However, the goD-3 mutation has now been shown by complementation tests and two- and three-factor crosses to be located within gene 23. The ability of T4 to overcome the same phd mutations by changes in either P31 or P23 suggests that the host defect in these phd mutants acts fairly nonspecifically to block head assembly. The goD-3 mutation does not bypass the requirement for P31 function entirely since the double mutant goD-3 amN54(gene 31) fails to grow on HD36D, which lacks a suppressor. It is possible, however, that the altered P23 has a less stringent requirement for P31 than does the wild-type head protein.

The T4 mutant goF-1 grows on host-defective bacterial mutants of the phdF class, in which T4 development is characterized by defective DNA synthesis and failure to lyse. The goF-1 mutation had been tentatively mapped close to gene 39, a DNA-delay gene (Biology 1972, No. 8). Complementation tests and two- and three-factor crosses of goF-1 with known am and deletion mutants, have established that goF-1 is not in gene 39 but is located in a nonessential region of the T4 genome between genes 39 and 56. The goF-1 function...
mutation does not represent a loss of function since deletion mutants lacking the goF-1 mutant site fail to grow on phdF strains, although they grow normally on the phd+ parent. Thus in phdF bacterial hosts a nonessential part of the T4 genome has become essential, but it is functional only in a mutated form.

267. ANALYSIS OF EMPTY PHAGE T7 CAPSIDS AND CAPSID-DNA COMPLEXES

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

Two kinds of phage capsid, both of which are probably free of DNA, can be isolated from lysates of T7-infected E. coli. The first capsid (referred to as capsid I) contains large amounts of the protein coded for by T7 gene 9 (P9), a protein reported not to be present in the second capsid (capsid II) or mature phage (Studier, 1972). There is evidence that capsid I has never interacted with DNA and that capsid I is converted into capsid II during an abortive attempt to package DNA (Studier, 1972). Also present in lysates of T7-infected E. coli are capsids bound to DNA that is outside of the phage head (T7 “in vivo” capsid-DNA complexes). Most of the DNA molecules in these complexes are monomer T7 length but some are longer (Serwer, 1974b). To obtain information about phage T7 DNA packaging the above capsids are being characterized.

Using SDS polyacrylamide gel electrophoresis it has been shown that a small amount of a second protein is present in capsid I, but not capsid II or phage, and that this protein is probably P19. Gene 19 is involved in the sizing of T7 DNA (Hausman and La Rue, 1969) and is closer to the right end of the T7 genome than any gene yet discovered (Studier, 1972). The right end of T7 DNA is also where capsids bind in the “in vivo” T7 capsid-DNA complexes. In addition it was found that capsid II and T7 phage contain small amounts of a protein that coelectrophoreses with P9.

The capsid component of the “in vivo” capsid-DNA complexes (capsid III) does not contain large amounts of P9 and resembles capsid II in its protein composition and sedimentation rate. This suggests that the DNA of these complexes had been packaged, but has subsequently left the phage head. Capsid III-like structures also appear to be bound to 100S T7 DNA, a fast-sedimenting replicative form of T7 DNA (Serwer, 1974a).

Capsids I and II were prepared for electron microscopy using the negative staining technique. Capsid I appears to be spherical, has a diameter of 420 Å, an outer shell 40 to 80 Å thick and an inner core which by stereo electron microscopy is cylindrical or helical with a hollow center. Capsid II has an angular profile, a diameter of 480 to 550 Å, an outer shell 15 to 35 Å thick, and an inner core with a diffuse appearance. These data suggest that capsid I has an outer envelope which consists of a shell of P9 inside or outside of a shell of the major head component of phage, P10. The fact that capsid I is smaller than capsid II or phage suggests the possibility that the phage head expands during DNA packaging.

To account for the above data it is proposed that the steps in T7 DNA packaging are: (1) Capsid I binds to T7 DNA concatemers (which are part of a larger DNA complex); (2) A mature length of DNA is packaged and most P9 leaves the phage head; (3) A double-stranded cut is made at each of the two terminal sequences of T7 DNA. It is implicit in this hypothesis that some T7 DNA is discarded during DNA packaging. The force pulling T7 DNA into the phage head may be a difference in pressure between the inside and outside of the phage head created by expansion of the head.

References:

PUBLICATIONS


WILLIAM G. KERCKHOFF MARINE
BIOLOGICAL LABORATORY

Charles J. Brokaw
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. A control mechanism is also needed, to generate rhythmic bending and the phase differences between the bending of different regions, which are required for propagated bending waves. The control mechanism may involve a simple feedback process, whereby the active sliding process at a particular point on a flagellum is regulated by the curvature of the flagellum at that point. We have shown that this mechanism is sufficient to explain the initiation and propagation of flagellar bending waves.

Our work is now directed at obtaining evidence which can identify the control mechanisms which actually exist in flagella and cilia, and at understanding the control of movement parameters such as wavelength, bend amplitude, and beat frequency. This understanding can then be used to obtain new information about the active process which generates sliding in flagella and in muscle.

We are making extensive use of computer programs which simulate the movement of model flagella, in order to test hypotheses about mechanisms in flagella. Last year, we learned how to control the symmetry of the ATP-reactivated beating of demembranated sea urchin spermatozoa, by maintaining appropriate concentrations of Ca\(^{2+}\) ions in the demembranation and reactivation solutions. Much of this year’s work has taken advantage of the improved preparations which can be obtained as a result of this discovery.

268. EFFECTS OF VISCOSITY ON LOW-FREQUENCY MOVEMENTS OF REACTIVATED SPERM FLAGELLA

Investigators: Charles J. Brokaw, Thomas F. Simonick, Donald D. Rintala

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

Spermatozoa from the sea urchin, *Lytechinus pictus*, were demembranated with solutions containing Triton X-100 and 5 mM CaCl\(_2\) and reactivated in ATP solutions containing low concentrations (10\(^{-9}\) M) of free Ca\(^{2+}\) ion, in order to obtain symmetrical bending wave movements. At ATP concentrations of 0.01 to 0.02 mM, the spermatozoa have beat frequencies near 1 Hz, normal bend angles, and wavelengths about 50% longer than normal. Addition of methyl cellulose to the reactivation solutions to increase the viscosity (up to 40 cpm) has a negligible effect on the beat frequencies, in contrast to the decrease in beat frequency which is caused by increased viscosity at normal beat frequencies. The effect of viscosity on wavelength is the same at high and low frequencies.

At low ATP concentrations, beat frequency appears to be determined by an intrinsic rate-limiting process, in contrast to the viscosity-limited behavior at high beat frequencies. The rate-limiting process at low ATP concentrations might be the velocity of sliding between filaments, but examination of the relationship between bend angle and beat frequency failed to support this idea. Alternatively, the movement at low beat frequencies may be dominated by elastic resistances, and the beat frequency may be determined by a time delay which causes the active bending moment to be in phase with the bending moment resulting from elastic resistances. The behavior at low ATP concentrations can be simulated with the computer model which contains an explicit time delay, but we have not been able to simulate this behavior with models in which the active bending moment is derived from properties of the cross-bridges between filaments.
269. MORPHOLOGY OF DEVELOPING BENDS IN SPERM FLAGELLA

Investigators: Stuart F. Goldstein*, Charles J. Brokaw

Support: National Science Foundation
National Institutes of Health, Public Health Service
University of Minnesota Graduate School

A new photographic technique was developed to obtain multiple-exposure photographs of reactivated spermatozoa from the sea urchin, *Lytechinus pictus*. These photographs provide a more complete record of the development of bends near the base of a flagellum than has previously been available.

Bends in these flagella can usually be approximated well by circular arcs. A bend begins to form at the base, in a region no longer than a few µm. As it grows, the length, radius of curvature, and angle subtended all increase. When a bend has grown to about half its final bend angle, and a straight region of about 2 or 3 µm has developed between it and the base, the next bend begins to form in the opposite direction. The angles subtended by these two bends grow at the same rate, so that the angle between the base and the straight region just distal to these two bends remains constant. This implies that there is no sliding in any straight region distal to these bends, indicating that the forces for generating and propagating a bend are localized within the bend itself. On the other hand, the straight region between the two growing bends is a region of intense sliding.

In addition, a small amount of synchronous sliding is superimposed on the pattern just described. Synchronous sliding does not occur in the computer models we have examined, and its significance is not yet clear.

*Department of Zoology, University of Minnesota, Minneapolis, Minnesota.

270. MECHANOCHEMICAL COUPLING IN FLAGELLA

Investigators: Charles J. Brokaw, Donald D. Rintala, Thomas F. Simonick

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

In previous work a decrease in ATP turnover by spermatozoa was observed when their movements were inhibited by increasing the viscosity of the fluid in which they were swimming. The decrease in ATP turnover was approximately the same as the decrease in beat frequency, suggesting a stoichiometric coupling in which 1 or 2 ATP molecules were dephosphorylated in each beat cycle by each molecule of dynein, the flagellar ATPase. However, in those experiments it was not really possible to decide whether beat frequency, or one of the other movement parameters which decreased at increased viscosities, was the relevant variable. We attempted to resolve this question by measuring the metabolism of *Chaetopterus* spermatozoa, which show an increase in bend amplitude and a decrease in beat frequency, at increased viscosities. We had to conclude that there was no coupling between metabolism and motility in these spermatozoa.

Improved techniques for reactivation of the movements of demembranated spermatozoa have now made possible direct measurements of ATP dephosphorylation by sea urchin sperm flagella to discriminate between the effects of changes in beat frequency and bend amplitude. Our results indicate that ATP turnover is related to amplitude, as measured by bend angle, rather than beat frequency, when these parameters are altered by changes in viscosity. This result agrees with predictions of an earlier theoretical model (Brokaw and Rintala, 1974) in which curvature of a flagellum controls the rate of formation of cross-bridges between flagellar filaments.

Reference:
PUBLICATIONS


THE FOLLOWING REPORTS ARE BY GRADUATE STUDENTS IN THE DIVISION OF BIOLOGY WHO ELECTED TO DO THEIR THESIS WORK IN THE DIVISION OF GEOLOGICAL AND PLANETARY SCIENCES AND THE DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING.

271. VARIATION OF CARBON ISOTOPIC COMPOSITION IN GRASSHOPPERS AND MICE

**Investigator:** Michael J. DeNiro*

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

The use of stable isotopes in biology has been restricted primarily to nonradioactive tracer experiments and structural studies; there have been few attempts, outside the area of plant physiology, to make use of the variation of ratios of the stable isotopes of such elements as carbon, oxygen, hydrogen, and nitrogen for the study of biological processes. By use of the modified Neir double collecting mass spectrometer (McKinney et al., 1950), variations of $^{18}O/^{16}O$ and $^{13}C/^{12}C$ ratios of .01% can be measured. Since some enzymatic steps are known to change these ratios by 2% from substrate to product, and physical processes such as diffusion to and across membranes affect the ratios to the same degree (Park and Epstein, 1960), the study of the variation of isotopic ratios could afford a powerful tool in the fields of animal biochemistry and physiology.

The present area of interest is development of a total carbon balance for a herbivore. Grasshoppers have been raised from the egg to the adult stage, fed exclusively on one of two plants, one depleted in $^{13}C$ relative to the other. In both cases, the animal's carbon as well as its excretory products are enriched in $^{13}C$, and its respiratory $CO_2$ is depleted in $^{13}C$ relative to the overall carbon of the food plant.

Methods are currently being developed to allow for extraction of biochemical fractions from the plants and animals to separate various effects such as assimilation of some fraction of plant carbon that differs in isotopic composition from the whole plant, utilization of different carbon sources in the animal for different functions, and isotopic fractionation associated with metabolic pathways. Preliminary work with mice indicates a trend in the opposite direction, with the isotopic composition of various organs showing a depletion in $^{13}C$ relative to the composition of the food. Future research with mice will be directed toward defining the causes of variation of isotopic composition of various organs and biochemical fractions with an eye toward potential utilization of these methods in medical studies.

**References:**

*Working with Professor Samuel Epstein, Division of Geological and Planetary Sciences, California Institute of Technology.

272. STEROID EFFECTS ON MEMBRANE STRUCTURE

**Investigators:** Jay Edelman, S. I. Chan*

**Support:** Earle C. Anthony Fellowship

Although cholesterol is found in many biological membranes, its role in membrane functions is largely unknown. It is well established that cholesterol decreases the temperature dependence of many membrane properties—"fluidizing" them below their phase transition temperature, and "condensing" them above it. The details of how this occurs are unclear; further work is needed on the arrangement and mobility of steroids in membranes, the microscopic interactions between phospholipids and steroids, changes in phospholipid mobility produced by steroids. Previous work has shown that NMR linewidths and relaxation rates are sensitive to the details of hydrocarbon rotational motions in
membranes. Although cholesterol produces striking changes in the $^1$H-NMR spectrum of lecithin, present day theory of NMR lineshapes is insufficient for analyzing these changes. Present work is directed towards the development of statistical mechanical models of steroid-containing membranes. This, combined with a dispersion theory analysis of the stochastic Liouville equation (describing NMR relaxation and lineshape), should eventually allow one to relate the NMR effects to structural properties of membranes.

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

GRADUATES

FINANCIAL SUPPORT
VISITING LECTURERS


E. Haber, Harvard Medical School and Massachusetts General Hospital: The Value of Antibodies in Diagnosis and Therapy.

B. Rasmuson, University of Sweden: Effects of Some Suppressors on the GTP Cyclohydrolase Activity in Drosophila melanogaster.

R. Pollack, Cold Spring Harbor Laboratory: Is There a Need for Persistent Viral Gene Function to Maintain the Phenotype of SV40 Transformation?

H. F. Lodish, Massachusetts Institute of Technology: Transcription and Structure of the Genome of the Eukaryotic Cellular Slime Mold Dictyostelium discoideum.

T. D. Gelehrter, Yale University School of Medicine: Regulation of Gene Expression in Cultured Hepatoma Cells by Glucocorticoids and Insulin.

L. D. Smith, Purdue University: Molecular Interactions During Oocyte Maturation.

J. C. Bennett, University of Alabama Medical Center, Birmingham: Structure-Function Relationships in Polymeric Immunoglobulins.

J. Kyte, University of California, San Diego: Active Transport of Sodium and Potassium in Mammalian Cells.

G. Nicholson, Salk Institute of Biological Sciences, La Jolla: The Dynamic Organization of Biological Membranes and Its Role in Cell Recognition, Cell Contact and Escape from Cytotoxic Pressure.

D. Shreffler, University of Michigan Medical School: Fine Structure of the H-2 Histocompatibility Gene Complex in Mice.

H. Berg, University of Colorado: Bacteria Swim by Rotating Their Flagella.

G. Pappas, Albert Einstein College of Medicine: Structure and Function in Electrical and Chemical Synapses Work in Progress.

L. A. Culp, Western Reserve University: Surface Properties of Normal SV40 Transformed and Revertant Mouse Fibroblasts.


J. Diamond, McMaster University, Hamilton, Ontario, Canada: Axoplasmic Factors and the Control of Nerve Territory.

B. W. Knight, Rockefeller University: On the Dynamics of Limulus Eyes.

E. Azmitia Jr., National Institutes of Health: Adrenal Steroid Regulation of Serotonin Biosynthesis in the Rat Brain.

N. Warner, Walter and Eliza Hicks Institute, Melbourne, Australia: Current Topics in Immunology.

W. Ostertag, Max Planck Institut für Experimentelle Medizin, Göttingen, Germany: Friend Virus (SFFV) Transformed Erythroleukemic Cells as a Model System for Studying Differentiation.


G. Tomkins, University of California School of Medicine, San Francisco: Genetic Analysis of Gene Regulations.

R. Davidson, Harvard Medical School: Somatic Cell Genetics, BUdR-Dependence in Mammalian Cells, and Gene Transfer by Herpes Virus.

N. Maizels, Harvard University: Sequences of Controlling Regions of the Lactose Operon of *E. coli*.

J. Sheppard, University of Minnesota: Cyclic AMP and Cell Division.

R. C. Leif, Papanicolaou Cancer Research Institute, Miami, Florida: Cytophysical Investigation of Cells.

S. J. Singer, University of California, San Diego: Membrane Asymmetry and Membrane Function.

F. Kafatos, Harvard University: Specific Protein Synthesis and Cell Differentiation.

T. Kindt, Rockefeller University: Inheritance of Rabbit Anti-Streptococcal Antibodies.

H. G. Heinrich, Max Planck Institut für Biochemie, Munich, Germany: The Technique of Continuous Free Flow Electrophoresis for the Separation and Characterization of Cell Organelles and Membranous Components.

G. Brewer, Massachusetts Institute of Technology: Membrane-Mediated Macroregulatory Effects of Colicin K on *E. coli*.


M. Steinberg, Princeton University: How Cells Self-Assemble into Tissues and Organs.

M. C. Niu, Temple University: Functional Differences Between Somatic Tissue and Germ Cell mRNAs.

M. Achtman, Max Planck Institut für Molekulare Genetik, Berlin, Germany: The Operon Structure of Tra Cistrons on the Flac Factor.

M. Edidin, Johns Hopkins University: Arrangement and Rearrangement of Antigens in the Plasma Membrane.


E. Schwartz, University of California, Los Angeles: Responses of Vertebrate Photoreceptors.

R. H. Reeder, Carnegie Institution of Washington: Fidelity of Transcription with Bacterial and Eukaryotic Polymerases.

T. Dobzhansky, University of California, Davis: Intra-Specific Sterility in *Drosophila pseudoobscura*.

M. Day, St. Thomas's Hospital Medical School, London, England: Locomotor Patterns and Manipulatory Ability in Early Hominids with Special Reference to Recent Finds in East Africa.


C. Kung, University of California, Santa Barbara: Genetic Dissection of the Excitable Membrane of *Paramecium aurelia*.


R. Pertel, National Institute of Allergy and Infectious Diseases: Post-Embryonic Developmental Genetics of a Free-Living Nematode.

D. Jenden, University of California, Los Angeles: Studies on the Kinetics of Brain Acetylcholine Metabolism Using GC/MS.


W. Rutter, University of California Medical School, San Francisco: Regulation of Cytodifferentiation via Extracellular Factors.

D. Pettijohn, University of Colorado Medical Center: RNA-DNA Interactions Stabilizing DNA Folds and Segregating Domains of Supercolling in Isolated Bacterial Nucleoids.

Z. Hall, Harvard Medical School: Acetylcholine Receptors in Normal and Innovated Muscle.

T. Woolsey, Washington University School of Medicine: Anatomical Studies of the Mouse SmI Neocortex.
T. Honjo, National Institutes of Health: Organization of Immunoglobin Genes.
R. Barth, University of Kansas School of Medicine: Recent Work Using Technetium-99m in Cellular Studies of Immune Systems.
L. I. Rothfield, University of Connecticut: Reassembly of Purified Bacterial Membrane Components.
J. Trinkhaus, Yale University: Dynamics of Cell Surface Interactions in Morphogenesis.
R. Wehner, University of Zurich, Switzerland: Visual Pattern Recognition in Insects.
M. Konishi, Princeton University: Neurophysiological Studies of Auditory Communication in Birds.
J. J. Marchalonis, Walter and Eliza Hall Institute of Medical Research, Victoria, Australia: Cell Surface Molecules on Lymphocytes.
S. Tonegawa, Basel Institute for Immunology, Basel, Switzerland: Hybridization Studies to Count Immunoglobulin Genes.
H. Küntzel, Max Planck Institut für Experimentelle Medizin, Göttingen, Germany: Mitochondrial Gene Products in Wild Type and Mutants of Neurospora crassa.
J. H. Wilson, Baylor University School of Medicine: Host Cell Participation in SV40 Infection.
R. Konopka, Stanford University: Dissecting the Biological Clock.
L. Dure, University of Georgia: Molecular Biology of Cottonseed Germination.
S. Tonegawa, Basel Institute for Immunology, Basel, Switzerland: Reiteration Frequency of Antibody Genes.
H. Kühn, Institute for Neurobiology, Jülich, Germany: Reversible Phosphorylation of Rhodopsin in Living Frogs.
D. W. Cooper, Macquarie University, North Ryde, Australia: The Kangaroo as a Mealybug.
E. Raviola, Harvard Medical School: Intramembranous Specializations in Retinal Synapses.
E. Cerdá-Olmedo, University of Seville, Seville, Spain: Mechanism of Nitrosoguanidine Mutagenesis.
B. Bowman, University of Texas Medical Branch, Galveston: Cystic Fibrosis: Secrets of the Humble Oyster.
G. Winkelmann, University of Tübingen, Tübingen, Germany: Kinetic Studies of Iron Transport in Neurospora crassa.
G. Hoyle, University of Oregon: Fixity and Plasticity in the Functioning of Identified Insect Neurons.
L. B. Cohen, Yale University Medical School: Optical Studies of Neurone Function and Organization.
M. Stroun, University of Geneva, Geneva, Switzerland: Circulating DNA.
P. Lengyel, Yale University: Studies on the Interferon System.
T. Hall, University of Southern California Medical Center: Blocked Ontogeny in Human Cancer.
J. Palka, University of Washington: Precision and Plasticity in a Regenerating Sensory System.
Y. Hatefi, Scripps Clinic and Research Foundation, La Jolla: Structure and Function of the Mitochondrial Electron Transport and Oxidative Phosphorylation System.

A. G. Motulsky, University of Washington School of Medicine: Current Status and Future Trends in Human and Medical Genetics.


H. Rapp, National Cancer Institute: Immunotherapy of Experimental Cancer and Its Significance to Clinical Oncology.

G. Gutman, Stanford University School of Medicine: Tissue Localization and Functional Characterization of T-Cells and B-Cells in Mice.

J. Majkowski, Research and Didactic Center of Medical Academy, Warsaw, Poland: Evolution of Auditory Evoked Potentials During Conditioning Before and After Brain-Stem Lesions.

T. Rasmuson, University of Umea, Umea, Sweden: An Inducible Antibacterial Activity in Insect Hemolymph.

L. S. Rodkey, Kansas State University: Antibodies Regulate Their Own Synthesis.
GRADUATES

Fifty-two students in Biology were awarded the B.S., M.S. or Ph.D. degree in June, 1974. The names, degrees conferred, the titles of doctoral theses, and the plans of the recipients for future work are as follows:

Bachelor of Science

Phyllis Jean Anwyl, B.S.
Dale Eric Bredesen, B.S. with honor.
Donald R. Carrigan, B.S. with honor. Graduate study, University of California, San Diego.
Andrew Fort Cockburn, B.S. with honor. Graduate study, Department of Genetics, Stanford Medical School.
Debra Valorie Dison, B.S. Research Assistant, California Institute of Technology.
Karen Sue Eaton, B.S. with honor.
Gary Alan Frankel, B.S. with honor. Graduate study, University of California, San Diego.
Rene Charles Gandolfi, B.S. with honor.
Mark Andrew Grunwald, B.S. with honor. Medical student, Washington University Medical School, St. Louis.
Christina Anne-Marie Harrington, B.S. Research Assistant, California Institute of Technology.
Edward Monroe Hedgecock, B.S. with honor.
David Da-i Ho, B.S. with honor. Medical student, Harvard Medical School.
Nelson Daniell Johnson, B.S. with honor. Graduate study, California Institute of Technology.
David Louis Kazan, B.S. with honor. Research Assistant, Chemistry Department, Stanford University.
John Lehmann, B.S. with honor.
Anita Crafts Lighty, B.S. with honor. Graduate study, Cambridge University.
James Matthew Loddengaard, B.S. with honor. Medical student, UCLA Medical School.
Michael John Mariani, B.S. with honor.
Donald Lee Miller, B.S.
Stanley Kazuo Nakamoto, B.S. with honor.
Gregory Alan Nelson, B.S. with honor. Graduate study, Harvard University.
Carol Nottenburg, B.S. with honor. Graduate study in genetics, Stanford University.
Edward Burner Pontius, B.S. with honor. Research Assistant, California Institute of Technology.
James William Posakony, B.S. with honor. Graduate study, University of California, San Diego.
Karen Verna Roberts, B.S.
Haywood James Robinson, B.S. Medical student, University of California Irvine Medical School.
Charles Joseph Romeo Jr., B.S. Research Assistant, California Institute of Technology.
Thomas Peter St. John, B.S. with honor. Graduate study in biochemistry, Stanford Medical School.
Jacqueline Siegel, B.S. with honor. Medical student, University of Southern California Medical School.
Vanessa Roberta Skedzeleski, B.S. with honor. Graduate study, University of Wisconsin.
Paul Arthur Thomas, B.S. Graduate study, University of California, San Diego.
W. Scott Thompson, B.S.
William Douglas Trotter, B.S. with honor. Medical student, University of Washington Medical School.
James Charles Vibber, B.S. with honor.
Janet Christine Wainwright, B.S.
Thomas Wai Yee, B.S.

Master of Science

Christopher Scott Amenson, M.S. Science instructor, Mayfield School, Pasadena.
Galina Dmitriyevna Moller, M.S. Graduate student, California Institute of Technology.

Doctor of Philosophy

Tommy Charles Douglas, Ph.D. Thesis: The Theta Antigen of Mice and Its Analog in Rats. Postdoctoral fellow, Basel Institute for Immunology, Switzerland.
William Jack Driskell, Ph.D. Thesis: The Role of Tyrosine in the Sclerotization and Tanning of the Puparium of Drosophila melanogaster. Member of staff, City of Hope National Medical Center, Duarte, California.
Jeffrey Lewis Ram, Ph.D. Thesis: Effects of High K⁺ Media on Leucine Incorporation into Aplysia Nervous Tissue. Postdoctoral fellow, University of California, Santa Cruz.
Jessica Tuchman, Ph.D. Thesis: The Developmental Role of Membrane in the Cellular Slime Mold, Dictyostelium discoideum.
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Research, particularly as related to the brain
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Research in chemical biology
General support
General support
Cancer research
Plant growth research
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Cancer research
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Arthritis research
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BACK COVER PHOTOS

Top: The infection cycle of a virus begins when the virus adsorbs to a specific receptor on the surface of a susceptible cell. This electron micrograph (at a magnification of 15,000) shows a surface replica of two chick embryo fibroblasts with adsorbed Sindbis virus. One cell has adsorbed 96 virus particles per square micron, the other 46.

Bottom: The genetic map of ΦX174 superimposed on a ΦX174 RF II molecule containing two insert loops produced by specific ΦX174 DNA fragments.