CALTECH Biology Annual Report 1979

FRONT COVER

A series of facial expressions used by macaque monkeys: top left, a relaxed face during grooming; top right, an excited play face during rough and tumble play; center left, pouches in the buccinator muscle extended as the monkey stuffs banana into them; center right, a threat; bottom left, mild fear; bottom center, intense fear. The artist was Leslie Wolcott.

Drs. McGuinness and Allman are seeking to determine the neural mechanisms responsible for the control of the muscles of facial expression in primates. The most commonly elicited facial muscle movements in motor cortex are in the muscle zygomaticus, which retracts the corner of the mouth and is a major component in the expressions of threat, intense fear, and play (see abstract 170). We hope to work back from the control of simple muscle movement to more complicated integrated facial expressions.

BACK COVER

Scanning electron micrograph (X4410) of an isolated photoreceptor cell, in primary cell culture, from the eye of Aplysia californica. The bush at the apical portion of the cell is produced by dense microvillous processes which contain photic vesicles. The eye of Aplysia is of interest because it contains a neuronal circadian oscillator which operates, in vitro, for up to two weeks. Intracellular recordings from isolated photoreceptors indicate high light sensitivity but no circadian fluctuation. The circadian oscillator function resides, perhaps, in one of the remaining cell types in this eye (F. Strumwasser, D. P. Viele and J. M. Scotese).

A REPORT FOR THE YEAR 1978-79
ON THE RESEARCH AND OTHER ACTIVITIES
OF THE
DIVISION OF BIOLOGY
AT THE
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

STAFF OF BIOLOGY 1979

Bernita K. Larsh: Compilation and typing.

Elizabeth T. Hanson: Copy editing.

The helpful assistance of Barbara Cathey, JoAnn Chikahiro, Geraldine Cranmer, and Lody Kempees is greatly appreciated.

Pasteup: Caltech Graphic Arts.

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 1978-June 1979, are listed separately, at the end of the research reports of each group.

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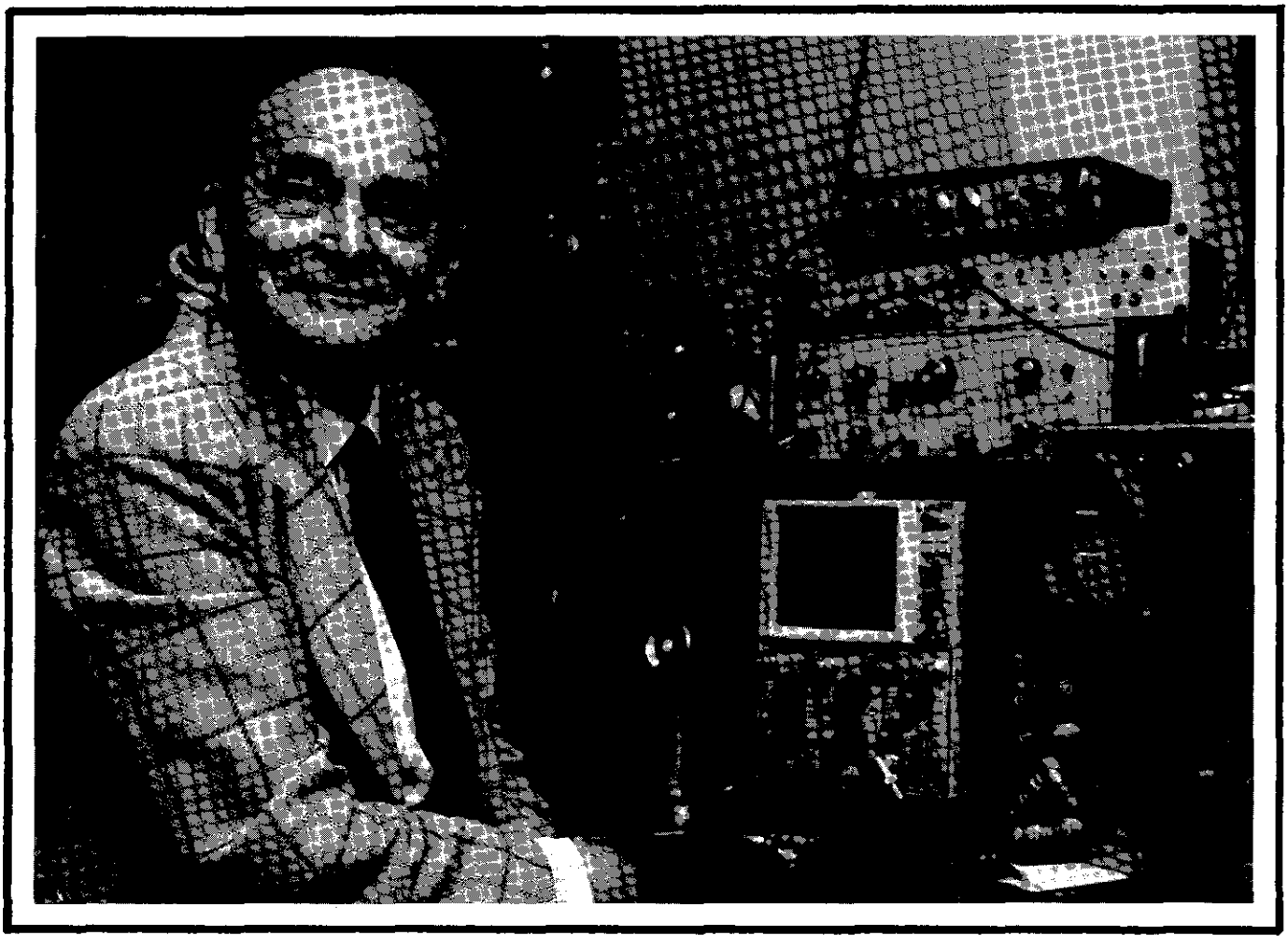


Professor Cornelis A. G. Wiersma, d. May 19, 1979

THE FACULTY

We are saddened to report the death of Cornelis A. G. Wiersma, Professor Emeritus of Biology, on May 19, 1979. Professor Wiersma, or "Kees" as he was known to his friends, came to Caltech from his native Holland in 1933 and had been here ever since. He had an important role in the development of the Biology Division, especially in connection with the decision to expand neuroscience. Although officially retired, he was working on a book entitled "The Nervous System of the Crayfish and Related Species," with the support of a grant from the National Science Foundation, at the time of his death. He was elected a Foreign Associate of the National Academy of Sciences a month before he died. A Memorial Service for Kees will be held in early fall.

Dr. Elliot Meyerowitz, presently a postdoctoral fellow at Stanford University, will join the faculty in January, 1980 as Assistant Professor of Biology. Dr. Meyerowitz works on the molecular genetics of Drosophila.

Honors and Appointments

Professor Seymour Benzer was awarded the Dickson Prize from Carnegie-Mellon University. He also was the Grass Foundation Lecturer of the Society for Neuroscience.

Professor Max Delbrück was elected a Foreign Associate of the Académie des Sciences of France.

Professor Norman Horowitz was appointed to the Committee on Science and Public Policy of the National Academy of Sciences.

Professor Mark Konishi was elected to membership in the American Academy of Arts and Sciences.

Professors Mark Konishi and Jack Pettigrew, and Dr. Eric Knudsen shared the Newcomb Cleveland Prize Award of the American Association for the Advancement of Science for two scientific papers which appeared in Science (Knudsen, Konishi and Pettigrew, Receptive fields of auditory neurons in the owl, Science **198:** 1278-1280, 1977; Knudsen and Konishi, A neural map of auditory space in the owl, Science **200:** 795-797, 1978).

Professor Elias Lazarides was a Haverford Distinguished Lecturer at Haverford College in April.

Professor Ray Owen was elected an Honorary Fellow of the American Academy of Allergy. He was also awarded an honorary Sc.D. degree from the University of Wisconsin.

Professor Roger Sperry received an honorary Sc.D. degree from Kenyon College.

Professor Cornelis Wiersma was elected a Foreign Associate of the National Academy of Sciences.

BIRTHDAY PARTY

In celebration of the 50th Anniversary of the founding of the Division of Biology, a symposium entitled "Genes, Cells, and Behavior: A View of Biology Fifty Years Later," was held in Beckman Auditorium on November 1-3, 1978. Eighteen papers, covering topics ranging from the molecular genetics of bacteriophage to human behavior, were presented in five sessions. The speakers were all alumni or former members of the Division. Over 700 alumni, students, and friends of the Division attended. A reception and reunion dinner were held in connection with the symposium. At the dinner, Professor James Bonner gave a slide-show talk on the history of the Division. A report on the symposium, including summaries of all the papers, appeared in Engineering and Science, March-April and May-June, 1979. The full symposium will be published as a book by W. H. Freeman and Co.

The 50th Anniversary Symposium was funded in part by:

Boehringer Mannheim Biochemicals
Calbiochem-Behring Corporation
McBain Instruments, Inc.
National Science Foundation
New England Biolabs, Inc.
New England Nuclear
Pierce Chemical Company

The photographs on the next pages were taken by Richard Kee, the Institute photographer, in the course of the 2-1/2-day celebration.

50th ANNIVERSARY

Left to right: Mary Emerson, Ingelore Bonner, James Bonner, Sterling Emerson

Standing: Sterling and Mary Emerson
Others visible: Manny Delbrück, June and Ray Owen, Lisl and Henry Borsook, Phoebe Sturtevant, Maria Haagen-Smit

Left to right: Renato Dulbecco, Ed Carusi, Mildred Goldberger, Marvin Goldberger, Margaret Lieb, Maureen Dulbecco
Background: Don Hawthorne

Left to right: Dale Kaiser (back to camera), Herman Lewis, Burke Judd, Lee Hood

50th ANNIVERSARY

Left to right: Max Delbrück, George Beadle

Left to right: Arnold Beckman, C. C. Tan

Left to right: Noboru Sueoka, Rose Itano, Harvey Itano

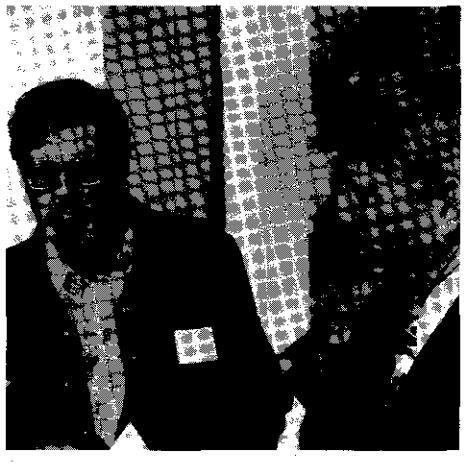
Left to right: Gunther Stent, George Laties, Matt Meselson, Dan Lindsley
Background: Dale Kaiser

50th ANNIVERSARY

Left to right: Renato Dulbecco, George Beadle, Max Delbrück, Howard Temin

Head table: George Beadle, Mildred Goldberger, Marvin Goldberger, Shirley Hufstedler, Robert Sinsheimer, Arnold Beckman, Pearl Horowitz
Others visible: Zoltan Tokes, James Strauss, Ted Young

Left to right: H. M. Kalckar, C. C. Tan, Paul Ts'o, Gordon Sato



Left to right: Rowland Davis, Al Knudson

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American Cancer Society
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Gosney Fund
John A. McCarthy Foundation
National Institutes of Health, USPHS
National Science Foundation

Summary: The study of the organization of the genes in the human mitochondrial DNA and of their mode of expression has been continued by a detailed mapping, structural, and metabolic analysis of the mitochondrial DNA transcripts. RNA-DNA hybridization experiments utilizing a variety of procedures have allowed the identification in the physical map of HeLa cell mitochondrial DNA of the segments coding for almost all of the poly(A)-containing RNA species. A striking result of this analysis is the frequent overlapping of the segments coding for the poly(A)-containing RNA species with the sites of tRNA genes. A fine mapping of the ribosomal DNA region of mitochondrial DNA has not revealed the presence of any intervening sequences in the main body of the 12S and 16S rRNA genes. A combined RNA and DNA sequencing analysis has allowed the alignment of the DNA sequence preceding the origin of mitochondrial DNA heavy strand synthesis with the 5'-end proximal segments of the 12S rRNA (heavy strand transcript) and the RNA #18 (7S RNA), which is the smallest poly(A)-containing RNA species (light strand transcript). Mapping experiments utilizing nascent RNA molecules isolated from transcription complexes have led to the identification of a major site of light strand transcription near the origin of heavy strand synthesis. A study of the metabolic stability of the mitochondrial DNA-coded poly(A)-containing RNA components has revealed that the polysome-associated species have a half-life varying between 1 and 3 hr. A shorter half-life has been measured for the larger poly(A)-containing RNA components which are not associated with polysomes. Among these, a giant light strand transcript with a half-life of about 7 min and extending over more than one-half of the genome has been identified and mapped.

1. CLONING OF HUMAN MITOCHONDRIAL DNA

Investigators: **Monica Mottes, Giuseppe Attardi**

Experiments have been started for the purpose of cloning HeLa cell mitochondrial DNA (mit-DNA). mit-DNA, extensively purified by repeated cycles of CsCl density gradient centrifugation and sucrose gradient centrifugation, has been inserted into the 31 kb cloning fragment (annealed Eco RI end fragments) of the EK2-certified lambda Charon 4A vector (Blattner et al., 1977; Maniatis et al., 1978).

Two methods have been used: in the first approach, HeLa mit-DNA has been cut at its unique Bam HI site and treated with Eco RI methylase to make the Eco RI sites enzyme-resistant; then the Bam I-generated cohesive ends have been filled up by avian myeloblastosis virus reverse transcriptase, and finally the linear molecule has been joined by blunt-end ligation to chemically-synthesized duplex DNA dodecamer linkers containing the Eco RI sequence. After cutting the mit-DNA with Eco RI, recombinant molecules have been generated by joining it with T4 ligase to the Charon 4A arms.

In a more direct approach, HeLa mit-DNA has been subjected to controlled partial digestion with Eco RI (which cuts the genome into three fragments), and the linear monomer forms have been isolated and joined by cohesive-end ligation to the Charon 4A DNA arms. In both cases ligated molecules have been packaged and propagated in DP50 SupF.

The phage products of the packaging reactions are at present under investigation. All cloning experiments have been performed under P2-EK2 containment facilities.

References:

Blattner, F. R., Williams, B. G., Blechl, A. E., Thompson, K., Faber, H. E., Furlong, L. A., Grunwald, D. J., Kiefer, D. O., Moore, D. D., Schumm, J. W., Sheldon, E. L. and Smithies, O. (1977) Science **196:** 161-169.
Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. and Efstratiadis, A. (1978) Cell **15:** 687-701.

2. Hinc II RESTRICTION MAP OF HeLa CELL MITOCHONDRIAL DNA

Investigators: **Deanna K. Ojala, Joy Shaffer*, Bruce Baskir***

For the purpose of providing additional fragments to aid in mapping the numerous transcripts coded by the HeLa cell mitochondrial genome, a physical map of the mit-DNA fragments produced by cleavage with the Hinc II restriction enzyme has been constructed. Hinc II, which recognizes the base sequence GTPyPuAC, cleaves HeLa cell mit-DNA into eleven fragments, ranging in size from ~3700 to 140 base pairs. These fragments were sized, and their relative positions ordered with respect to the Hpa II restriction map (Ojala and Attardi, 1977). Two main techniques were used. The first involved double digests in which either isolated Hinc II fragments were cleaved by restriction enzymes whose cleavage sites had previously been mapped, or isolated Hpa II or Hind III fragments were cleaved by Hinc II. The second technique utilized isolated Hinc II fragments which were labeled to high specific activity by nick-translation (Rigby et al., 1977), and then hybridized to Hpa II + Bam I digests of HeLa cell mit-DNA transferred to nitrocellulose membranes (Southern, 1975).

References:

Ojala, D. and Attardi, G. (1977) Plasmid **1:** 78-105.
Rigby, P., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. **113:** 237-251.
Southern, E. (1975) J. Mol. Biol. **98:** 503-518.

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3. APPARENT OVERLAPPING OF MITOCHONDRIAL DNA SEGMENTS CODING FOR poly(A)+ RNAs AND tRNAs

Investigators: **Deanna K. Ojala, Robert A. Gelfand**

A variety of approaches has been pursued in experiments aimed at identifying the HeLa mitochondrial DNA (mit-DNA) segments coding for poly(A)-containing RNA species. In the approach described here, highly purified mitochondrial RNA species were obtained from cells labeled with [^{32}P]orthophosphate, using nuclease treatment of mitochondria to destroy cytoplasmic RNA contaminants. Individual poly(A)-containing components were resolved by agarose CH_3HgOH gel electrophoresis, eluted, and utilized for hybridization with Hpa II-Bam I digests of HeLa cell mit-DNA transferred to nitrocellulose membranes (Southern, 1975). By using this approach, almost all 18 poly(A)-containing components previously identified in HeLa cell mitochondrial RNA (Amalric et al., 1978) have been mapped on mit-DNA. A number of conclusions can be drawn from the results obtained. First, the sequences coding for the poly(A)-containing RNA species extend over the entire or almost entire length of mit-DNA. Among the species coded for by the heavy strand there is a certain extent of overlap in mapping position. Some of the overlapping species may be related to each other by precursor to product relationship, as judged from considerations of metabolic stability and presence or absence of these species in mitochondrial polysomes. Finally, the most striking feature of the transcription map is the frequent overlapping of the segments coding for poly(A)-containing RNA species and those coding for tRNAs on the heavy or light strand. The poly(A)-containing RNA species in question are very probably mature species, judged by their relatively long half-life (see Biology 1979, No. 9) and their presence in polysomes. Whether the tRNA or anti-tRNA sequences are actually present in the poly(A)-containing RNAs or have, on the contrary, been spliced out is at present being directly investigated.

References:

Amalric, F., Merkel, C., Gelfand, R. and Attardi, G. (1978) J. Mol. Biol. **118:** 1-25.
Southern, E. (1975) J. Mol. Biol. **98:** 503-518.

4. FAILURE TO DETECT INSERTS IN THE MAIN BODY OF THE 12S AND 16S mit-rRNA GENES

Investigator: **Deanna K. Ojala**

Recent work in several laboratories has revealed the occurrence of intervening sequences in the gene coding for the large ribosomal RNA (rRNA) in mitochondrial DNA from yeast (Bos et al., 1978) and Neurospora (Hahn et al., 1979). In mit-DNA from animal cells, including HeLa cells, previous electron microscope analysis of RNA-DNA hybrids had not revealed the presence of obvious discontinuities in the rRNA genes. However, this type of analysis would have not detected discontinuities of 100 nucleotide pairs or less. In order to obtain information on the possible existence of inserts in the mitochondrial rRNA genes of HeLa cells, the methodology developed by Berk and Sharp (1977) has been applied. For this purpose, highly purified rRNA species were hybridized with separated strands or restriction fragments of in vitro or in vivo ^{32}P-labeled DNA, the RNA-DNA hybrids were treated with the single-strand-specific Aspergillus nu-

clease S1 and E. coli exonuclease VII, and the protected DNA sequences were analyzed in denaturing gels. It was found that hybridized 12S RNA protected from both S1 and exonuclease VII digestion a DNA segment about 1000 nucleotides long, close to the electron microscope size estimate of this RNA species. Similarly, hybridized 16S RNA protected from both S1 and exonuclease VII digestion a DNA segment about 1600 nucleotides long, which is only slightly smaller than the size estimated by electron microscopy for this RNA. The above results indicate that there are no obvious intervening sequences in the main body of the 12S and 16S rRNA genes. However, the occurrence of subterminal inserts, possibly related to the 4S RNA genes situated at the 5'-end of the 12S RNA gene, at the 3'-end of the 16S RNA gene, and between the two genes, may have not been detected by this type of analysis.

References:

Berk, A. J. and Sharp, P. A. (1977) Cell **12:** 721-732.
Bos, J. L., Heyting, C., Borst, P., Arnberg, A. C. and Van Bruggen, E. F. J. (1978) Nature **275:** 336-337.
Hahn, U., Lazarus, C. M., Lünsdorf, H. and Küntzel, H. (1979) Cell **17:** 191-200.

5. APPLICATION OF THE RNA TRANSFER TECHNIQUE TO THE MAPPING OF THE TRANSCRIPTS OF MITOCHONDRIAL DNA

Investigator: **Christian G. Merkel**

Evidence from this laboratory has indicated the existence of at least 18 discrete poly(A)-containing RNA components coded for by mitochondrial DNA (mit-DNA). These RNA components have been resolved by agarose slab gel electrophoresis in the presence of methylmercuric hydroxide as a denaturing agent and have molecular weights of 9.3×10^4 to 3.4×10^6 daltons. A detailed physical map of the circular HeLa cell mit-DNA has been constructed by ordering the 21 DNA fragments generated by the endonuclease Hpa II (Ojala and Attardi, 1977). The positions in this map of the sequences complementary to almost all the poly(A)-containing RNA species have been determined by Southern blot hybridizations (see Biology 1979, No. 3). In order to obtain information by an independent approach on the mapping positions of the poly(A)-containing RNA species and to introduce further resolution in the construction of the transcription map, the RNA transfer technique developed by Alwine et al. (1977) has been applied. For this purpose, we have isolated the individual DNA fragments generated by Hpa II and labeled them to high specific activity with [α-^{32}P]deoxynucleoside triphosphates using the nick-translation method of Rigby et al. (1977). Each of the labeled DNA fragments was then hybridized to the mit-RNA which had been run out on methylmercuric hydroxide-agarose gels and transferred to diazobenzyloxymethyl paper using the procedure of Alwine et al. (1977). Following hybridization, the specific RNA bands which hybridized with a given DNA fragment could be visualized by autoradiography. The results obtained by this technique concerning the mapping positions of most of the poly(A)-containing RNA species are in substantial agreement with those obtained by the Southern transfer method; furthermore, it has been possible by this approach to define more precisely in several cases the positions in the Hpa II map of the ends of the RNA species. Further studies are being carried out to determine the 5'-end position in the physical map of a presumptive ribosomal RNA precursor. In order to accomplish this, DNA fragment 8 generated by Hpa II digestion which contains the 5'-end sequences of the 12S rRNA and of the presumptive precursor, will be isolated and further digested with different restriction enzymes, such as Alu I, following which these DNA fragments will be labeled with ^{32}P and hybridized to the RNA, as described above.

References:

Alwine, J. L., Kemp, D. J. and Stark, G. R. (1977) Proc. Nat. Acad. Sci. USA **74:** 5350-5354.
Attardi, G., Cantatore, P., Ching, E., Crews, S., Gelfand, R., Merkel, C. and Ojala, D. (1979) ICN-UCLA Symposium on Extrachromosomal DNA. In press.
Ojala, D. and Attardi, G. (1977) Plasmid **1:** 78-105.
Rigby, P., Dieckman, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. **113:** 237-251.

6. SEQUENCE ANALYSIS OF MITOCHONDRIAL DNA TRANSCRIPTS NEAR THE ORIGIN OF REPLICATION

Investigators: **Stephen T. Crews, Giuseppe Attardi**

A combined RNA and DNA sequencing approach has yielded a detailed physical map of a section of the human mitochondrial genome containing the origin of mit-DNA replication. An 826 nucleotide pair Hpa II fragment (#8) has been sequenced using the Maxam-Gilbert method on various strand-separated Alu I, Mbo I or Hae III subfragments. The H strand origin of replication was previously shown to begin 87 nucleotides (NT) from the Hpa II site between Hpa II fragments 8 and 17 (Crews et al., 1979).

It is known that the 12S rRNA hybridizes to the H strand of Hpa II fragment 8. It is also known, by electron microscopy, that a 4S RNA near the 5'-end of the

12S rRNA is also transcribed from the H strand. Furthermore, a low molecular weight poly(A)-containing RNA (RNA #18) hybridizes to the L strand of fragment 8. In order to precisely localize their 5'-ends, the 12S rRNA, 16S rRNA, and RNA #18 were sequenced by 5'-end labeling of purified RNAs and partial digestion with base-specific nucleases followed by electrophoresis on sequencing gels. The results indicate the 12S rRNA begins 285 NT from the restriction enzyme site between Hpa II fragments 8 and 3. The DNA and RNA sequences are colinear for the 75 NT of the RNA sequenced. RNA #18 begins 304 NT from the junction between Hpa II fragments 8 and 17, leaving 217 NT between the 5'-end of the RNA and the origin of replication. This is approximately the length expected for RNA #18 (∿280 NT) after subtracting the contribution of the 3'-poly(A) tail (∿55 NT). The role of this RNA as a mRNA (a 42 amino acid hydrophobic protein sequence can be derived from the DNA sequence), or possible protagonist in DNA replication, remains to be investigated.

Reference:

Crews, S., Ojala, D., Posakony, J., Nishiguchi, J. and Attardi, G. (1979) Nature **277**: 192-198.

7. A GIANT POLYADENYLATED LIGHT STRAND TRANSCRIPT OF MITOCHONDRIAL DNA WITH SOME INTERESTING PROPERTIES

Investigators: **Robert A. Gelfand, Giuseppe Attardi**

There are at least 18 distinct polyadenylated RNA species produced by the mitochondria of HeLa cells (Amalric et al., 1978). This report concerns an RNA species [poly(A)-containing species 2 of Amalric et al.] with several interesting properties.

This species is approximately 8500 nucleotides in length, which is a little more than half the size of the entire mitochondrial genome. Its half-life, as determined by cordycepin blockage of radioactive precursor incorporation, was found to be approximately 7 min, whereas most of the other polyadenylated species studied had half-lives of 1 to 3 hr (Biology 1979, No. 9).

We have mapped RNA species 2 on the mitochondrial genome. This was accomplished by hybridizing species 2 RNA eluted from agarose-methylmercuric hydroxide gels with mitochondrial DNA (mit-DNA) restriction fragments immobilized on nitrocellulose paper. In this way, the position of the sequences coding for RNA species 2 was determined on the restriction map of HeLa mit-DNA. In addition, in hybridization tests with separated light (L) and heavy (H) strands of mit-DNA, species 2 was found to hybridize to the L strand, unlike most of the other polyadenylated species, which hybridize to the H strand. Interestingly, species 2 maps onto a part of the mitochondrial genome which contains the sequences coding for several of the relatively more stable polyadenylated RNAs which have been found to hybridize to H strand mit-DNA.

Species 2 is therefore a large polyadenylated RNA molecule of short half-life which is transcribed symmetrically (i.e., off the opposite strand) with several relatively more stable polyadenylated RNAs.

The physiological function of RNA species 2 remains unknown. It may be the precursor to several tRNAs which map in the same region of the HeLa mitochondrial genome and are transcribed from the L strand, or it may have some as yet unknown function related to H strand transcription.

Reference:

Amalric, F., Merkel, C., Gelfand, R. and Attardi, G. (1978) J. Mol. Biol. **118**: 1-25.

8. IDENTIFICATION OF A MAJOR SITE OF INITIATION OF LIGHT STRAND TRANSCRIPTION

Investigator: **Palmiro Cantatore**

The study of the location of promoter sites on HeLa cell mitochondrial DNA (mit-DNA) has been pursued. In particular, the Berk and Sharp (1977) procedure has been adapted to the mapping of nascent RNA molecules isolated from transcription complexes of mit-DNA (Aloni and Attardi, 1972; Carré and Attardi, 1978). The results obtained have pointed to the presence of a major site of initiation of light strand transcription in the region close to the origin of mit-DNA replication. This conclusion is in agreement with observations previously made by a different approach (Biology 1978, No. 4).

References:

Aloni, Y. and Attardi, G. (1972) J. Mol. Biol. **70**: 363-373.
Berk, A. J. and Sharp, P. A. (1977) Cell **12**: 721-732.
Carré, D. and Attardi, G. (1978) Biochemistry **17**: 3263-3273.

9. STABILITY OF MITOCHONDRIAL RNA SPECIES IN HeLa CELLS MEASURED AFTER DRUG INHIBITION OF RNA SYNTHESIS

Investigators: **Robert A. Gelfand, Giuseppe Attardi**

The nucleoside analog cordycepin (3'-deoxyadenosine) is known to inhibit mitochondrial RNA synthesis. Within 5 min after addition of the drug, incorporation of

radioactive precursors into all mitochondrial RNA species is reduced 90% or more, and by 15 min, incorporation is reduced 95 to 100%.

This effective inhibition of mitochondrial RNA synthesis by cordycepin has been utilized to determine the metabolic stability of polyadenylated mitochondrial RNA species. For this purpose, HeLa cells were preincubated with [^{32}P]orthophosphate and then treated with 50 μg/ml of cordycepin in order to block further incorporation of radioactive label. Samples of the cell population were harvested at various times after cordycepin addition. RNA was extracted from each sample and separated into poly(A)-containing and poly(A)-lacking fractions by oligodeoxythymidine-cellulose chromatography. Both portions were then fractionated by electrophoresis under strongly denaturing conditions in agarose-methylmercuric-hydroxide gels. In order to correct for differences in yield, each sample of cells was mixed with an equal volume of non-drug-treated, [^{3}H]uridine-labeled HeLa cells at the time of cell harvesting.

The best semilog curves were fitted to the data under the assumption that the individual RNA species decay with first-order kinetics. The half-lives of individual species were then calculated.

The results indicate that the majority of the polyadenylated species have half-lives of 1 to 3 hr. Some of the larger species appear to be less stable. In particular, a large L strand transcript which is approximately half the size of the total mitochondrial genome has a half-life of about 7 min (see Biology 1979, No. 7). The mitochondrial ribosomal RNAs are more stable than polyadenylated RNAs and their half-lives could not be measured using this method.

10. CHARACTERIZATION OF POLYPEPTIDES SYNTHESIZED ON MITOCHONDRIAL RIBOSOMES

Investigator: **Edwin P. Ching**

By appropriate use of antibiotics specifically affecting translation by cytoplasmic ribosomes, it has long been possible selectively to incorporate radioactively-labeled amino acids into mitochondrially-translated polypeptide species. Upon analysis of these translation products by high resolution SDS polyacrylamide gel electrophoresis, a significantly larger number of discrete polypeptide species have been identified than the ten previously characterized. The possibility of precursor to product relationships has also been studied.

11. STUDIES ON THE IN VITRO TRANSLATION OF MITOCHONDRIAL mRNAs

Investigators: **Antonio Montalvo-Correa, Néstor F. González-Cadavid, Giuseppe Attardi**

In vitro translation experiments have been undertaken with the purpose of identifying the individual mRNA species coding for the three hydrophobic mitochondrially synthesized peptides of cytochrome c oxidase. This identification would provide a means for mapping the corresponding genes in the mitochondrial genome in human cells. The main problem is that so far the organelle mRNA (yeast) could only be translated in E. coli systems without correlation between the in vitro products and the peptides synthesized in vivo by intact cells or isolated mitochondria (Borst and Grivell, 1978). This may be due to the apparent inability of poly(A)+ mitochondrial RNA to bind to eukaryotic ribosomes, possibly reflecting the lack of a terminal cap (Grohmann et al., 1978). A further complication is the possible requirement of membrane fragments for any co- or post-translational processing that may occur.

In the present study we have prepared poly(A)+ and poly(A)- RNAs from either unfractionated mitochondria or from mitochondrial polysomes, after nuclease treatment to remove most of cytoplasmic RNA contaminants from the organelles. The poly(A)+ RNA obtained from the polysome peaks was analyzed by agarose gel electrophoresis and shown to contain the usual mitochondrial species with a minimum amount of cytoplasmic RNA. Most of the components in this profile hybridized with mitochondrial DNA by the Southern blot technique. However, when this RNA fraction was translated in the reticulocyte lysate system, the PAGE bands did not significantly differ from those observed with the cytoplasmic mRNA-directed system. This suggested that the traces of nonmitochondrial RNA species were preferentially bound to the cytoplasmic ribosomes under the standard conditions. By increasing the RNA concentration and decreasing K^+ some new bands were apparent, although their relative intensity was not increased in the presence of a cap donor (S-adenosyl methionine) or an inhibitor of capped mRNA binding (7-methylguanosine-5' monophosphate). No significant immunoprecipitation of the three larger cytochrome c oxidase subunits could be detected, suggesting that the reticulocyte system may not be adequate for the translation of mitochondrial poly(A)+ mRNAs. Other subcellular systems, such as those derived

from wheat germ and E. coli, will be tested, supplemented with membrane fragments and protein synthesis factors. Purified specific antibodies directed against individual subunits of cytochrome oxidase will be employed to increase specificity of detection.

References:
Borst, P. and Grivell, L. A. (1978) Cell **15:** 705-723.
Grohmann, K., Amalric, F., Crews, S. and Attardi, G. (1978) Nucleic Acids Res. **5:** 637-652.

12. MAPPING GENES FOR CYTOCHROME C OXIDASE IN HeLa CELL MITOCHONDRIAL DNA

Investigators: **Anne Chomyn, Edwin P. Ching, Giuseppe Attardi**

Hare et al. (1979) have recently shown that the three largest of the seven subunits of cytochrome c oxidase in HeLa cells are synthesized on mitochondrial polysomes. Furthermore, there is good evidence to indicate that the great majority, if not all, of the mitochondrial mRNAs are mitochondrial DNA (mit-DNA) transcripts. We intend to map in mit-DNA of HeLa cells the genes coding for the mitochondrially synthesized subunits of cytochrome c oxidase.

Antibodies to the three individual subunits will be purified from antiserum directed against the holoenzyme by affinity chromatography using purified subunits. Polysomes synthesizing these subunits will then be isolated by the immunochemical procedure described by Mueller-Lantzch and Fan (1976) and Gough and Adams (1978). Messenger RNA extracted from each class of purified polysomes will be characterized as to its size, and the sequences of mit-DNA which code for this RNA will be identified by gel blot hybridization (Southern, 1975) or by the method described by Berk and Sharp (1977).

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Berk, A. J. and Sharp, P. A. (1977) Cell **12:** 721-732.
Gough, N. M. and Adams, J. M. (1978) Biochemistry **17:** 5560-5566.
Hare, J., Ching, E. and Attardi, G. (1979) Biochemistry. Submitted for publication.
Mueller-Lantzch, N. and Fan, H. (1976) Cell **9:** 579-588.
Southern, E. M. (1975) J. Mol. Biol. **98:** 503-517.

13. MECHANISM OF SYNTHESIS OF NUCLEAR-CODED SUBUNITS OF HUMAN CYTOCHROME C OXIDASE

Investigators: **Néstor F. González-Cadavid, Giuseppe Attardi**

Pulse-chase studies in yeast have shown that the four smaller polypeptides of the cytochrome c oxidase complex are synthesized on cytoplasmic ribosomes as a single precursor that appears to be transferred to the mitochondria post-translationally and then cleaved to the individual subunits inside the organelle (Poyton and McKemmie, 1979a,b). The relative role of membrane-bound and free ribosomes and the precise pathway of transport, together with the mechanism of proteolytic conversion and integration with the mitochondrially made polypeptides, are essential points for understanding the general assembly of the mitochondrial inner membrane (see, e.g., Chua and Schmidt, 1979). A conclusive approach depends on the translation of mRNAs from different ribosomal populations into identifiable cytochrome c oxidase polypeptides amenable to further processing. In the present work we have investigated the [^{35}S]methionine-labeled products of HeLa cell RNA translation in micrococcal nuclease-treated reticulocyte lysates, by the slab gel polyacrylamide electrophoresis/fluorography technique.

RNA was extracted from three fractions of post-mitochondrial ribosomes and from two fractions representing mainly cytoplasmic contaminants associated with mitochondrial preparations. Poly(A)+ and poly(A)- mRNAs were separated and tested independently. No substantial difference was found in the polypeptide pattern between the five poly(A)+ RNA fractions suggesting that essentially the same mRNA species are expressed irrespective of the subcellular location, although the translation efficiency varied considerably. The fractions lacking poly(A) showed more distinctive PAGE profiles. Immunoprecipitation was carried out by IgG adsorption on protein A-rich Staphylococcus aureus cells (Cowan I strain), under conditions which were shown to allow recognition of the seven subunits of [^{125}I]cytochrome c oxidase with antiserum prepared against the whole enzyme. The products of translation of poly(A)+ cytoplasmic RNAs contaminating the mitochondrial fraction gave the higher proportion of radioactivity precipitated by the antiserum, showing a main band in the 55,000 region where the yeast cytochrome c oxidase precursor was found to migrate.

Experiments are in progress to confirm the immunoprecipitation specificity using the double antibody technique and antibodies directed against individual subunits. The translation patterns will be compared to those obtained with the wheat germ system, taking as reference the synthesis of a well-identified single chain mitochondrial protein such as cytochrome c. Membrane-dependent in vitro proteolytic cleavage and fingerprint analysis will

also be used to establish whether the high molecular weight band is in fact a cytoplasmically synthesized precursor of the four smaller subunits of cytochrome c oxidase.

References:

Chua, N.-H. and Schmidt, G. W. (1979) J. Cell Biol. **81:** 461-483.
Poyton, R. O. and McKemmie (1979a) J. Biol. Chem. **254:** No. 12. In press.
Poyton, R. O. and McKemmie (1979b) J. Biol. Chem. **254:** No. 12. In press.

14. MITOCHONDRIAL DNA IN MOUSE-HUMAN CELL HYBRIDS

Investigator: **Laura De Francesco**

A large number of mouse-human cell hybrids constructed by Carlo Croce of the Wistar Institute have been analyzed for the species of mitochondrial DNA (mit-DNA) retained. Among these hybrids, some lose human chromosomes and some lose mouse chromosomes depending on the mouse parent in the cross. Extensive characterization of the karyotype as well as of the expression of nuclear DNA has been done by Croce and his collaborators. Analysis of the mit-DNA from different kinds of hybrid cells was performed by restricting the DNA from the hybrid, blotting onto nitrocellulose paper, and hybridizing it with parental mit-DNA probes. In all types of hybrids, a single species of mit-DNA was detected, that of the species whose nuclear DNA was more stable. This was true even in hybrids in which a copy of each chromosome of the segregating parent still persisted in a large fraction of the cell population. Furthermore, no evidence was seen for recombination between the two species of mit-DNA. A similar situation has been observed by Croce and collaborators in the case of several nuclear coded genes. Hybrids of all types expressed only the ribosomal RNA genes and the genes required for the growth of species-specific viruses of the parent whose nuclear DNA was more stable (Croce et al., 1977; Huebner et al., 1977). The uniparental origin of the mit-DNA found in the hybrids suggests that the genes required for the replication of mit-DNA are among those that are suppressed in hybrid cells.

References:

Croce, C., Talavera, A., Bascilico, C. and Miller, O. (1977) Proc. Nat. Acad. Sci. USA **74:** 694-697.
Huebner, K., Shander, M. and Croce, C. (1977) Cell **11:** 25-33.

15. ISOLATION OF VARIANTS OF HUMAN CELL LINES RESISTANT TO METHOTREXATE

Investigators: **Giuseppe Attardi, Benneta Keeley**

Recent work has shown that resistance to methotrexate, a folic acid analog, in mouse and Chinese hamster cell lines is often the result of overproduction of dihydrofolic acid reductase, the enzyme which is specifically inhibited by this drug (Alt et al., 1978; Nunberg et al., 1978). The observed increase in enzyme level depends on an increased level of dihydrofolate reductase mRNA and a corresponding increase in the number of dihydrofolate reductase genes. This phenomenon of selective gene amplification, besides providing a powerful tool for genetic engineering, raises intriguing questions concerning its mechanism and its possible role in normal development and evolution. Work has been started to investigate the possible occurrence of this selective gene amplification in human cell lines. By exposure to progressively increasing doses of methotrexate, variants have been isolated from several human cell lines which show resistance to a level of the drug 1000 to 2000 higher than the dose killing the sensitive parental cells.

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Summary: We know that the DNA of eukaryotic chromosomes is complexed with the five species of highly conserved histones (Fambrough and Bonner, 1966, 1968). Also, in the transcribed portion of the genome in any specialized cell type the transcribable chromatin differs from the nontranscribable. Firstly, transcribed chromatin is readily attacked by nucleases (Marushige and Bonner, 1971; Billings and Bonner, 1972). Secondly, the histones, although they protect chromosomal DNA from melting in the nontranscribable portion, do so minimally in the transcribable portion of the genome (Gottesfeld et al., 1974). Thirdly, in addition to other alterations, transcribable chromatin (which retains its histones) contains histones which are multiply acetylated in the N-terminal peptides (see, e.g., Davie and Candido, 1978). This modification apparently causes the histones to relax their grip on the DNA and results in alterations in DNA properties as well as the extension of the DNA packing ratio in nucleosomes from about seven in nontranscribable chromatin to about 1 to 1.5 in transcribed chromatin (McKnight et al., 1977).

We have purified a chromosomal acetyltransferase which acetylates the N-terminal peptides in nucleosomes (Wiktorowicz, personal communication). It does so DNA sequence indiscriminately. The secret of gene-specific activation does not lie unaided in this enzyme. Chromatin contains also a histone deacetylase (Riggs et al., 1977) which is inhibited by butyrate and other small fatty acids. The acetylase acetylates all nucleosomes in the presence of an inhibitor of deacetylase (Sealy and Chalkley, 1978; Vidali et al., 1978a,b). We ask, therefore, is the deacetylase sequence-specific? We shall find out.

We study individual genes to seek the missing factor which introduces DNA sequence specificity into chromatin activation. As described below, we have isolated and characterized a gene from the rat genome which codes for serum albumin. This gene is active in liver. We have isolated and partially characterized a gene which is not expressed in normal adult liver, that for α-fetoprotein.

Complementary to these studies we have made a major effort to physiologically reconstitute fragments of DNA into minichromosomes. We use the knowledge that in nature, "assembly factor" causes the assembly of histone octamers and DNA to form nucleosomes at physiological ionic strength (Laskey et al., 1977). When our effort is successful we will manufacture minichromosomes containing the serum albumin gene to find out what element in the rat liver nucleus or cytoplasm determines that this minichromosome shall become active and that for α-fetoprotein not.

We have further mapped the rat ribosomal genes by R-looping and restriction enzyme mapping. The findings concur with those found by R-looping of rDNA purified by affinity chromatography (Stumph et al., 1979), in that the rDNA repeat length is found to be about 38 kb $\pm$ 0.5 kb. In addition, however, the individual rDNA coding sequences are found to be nonidentical in their nonexpressed spacers.

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16. THE ROLE OF HISTONE ACETYLATION IN GENE EXPRESSION

Investigator: **John E. Wiktorowicz**

It is now well established that histones interact with DNA in a complex structure called the nucleosome (Hewish and Burgoyne, 1973; Kornberg and Thomas, 1974). The four histone classes, H2a, H2b, H3, and H4 are thought to interact with DNA by virtue of an electrostatic attraction between the N-terminal lysines of the histones and the phosphate backbone of DNA. Furthermore, the C-terminal regions of these histones interact with each other, forming an inner core which supports the rest of the nucleosome structure. It is apparent that modification of the amino acid side chains involved in the DNA-histone or histone-histone interactions may lead to conformational changes in the nucleosome structure, and may provide the first step in gene derepression. Indeed, chemical modification of template-inactive chromatin with acetic anhydride leads to acetylation of the N-terminal lysines of the histones and an increase in template activity (Marushige, 1976; Wallace et al., 1977). In addition, highly acetylated histones are preferentially associated with template-active chromatin (Davie and Candido, 1978). In order to understand nucleosomal modification, specifically acetylation, and its relationship to gene derepression, we are investigating the properties of histone acetyltransferase, the nuclear enzyme responsible for nucleosome acetylation.

We have succeeded in purifying the enzyme to homogeneity by a combination of salt extraction of chromatin, ammonium sulfate fractionation, ion exchange chromatographies, gel exclusion chromatography, and affinity chromatography. We have determined that the enzyme is an acidic protein (pI = 5.9) and, in crude preparations, may be found tightly bound to histones. Its relatively high molecular weight (90,000) facilitates its separation from the histones (14,000-23,000) via gel exclusion chromatography in high salt. The enzyme is inhibited to a high degree by divalent cations, and requires ammonium ion for stability. In comparatively high concentrations, the enzyme tends to aggregate (MW >200,000) and lose activity. Preliminary studies with highly purified enzyme indicate a K_m for acetyl-CoA of 2.16 μM when mononucleosomes are acetylated, and a K_m of 12.5 μM when total histone is acetylated.

We also have obtained antisera against a highly purified preparation of enzyme and further investigations using this antiserum are in progress.

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17. NONHISTONE PROTEIN-BOUND DNA SEQUENCES OF THE RAT GENOME

Investigators: **Linda L. Jagodzinski, J. Sanders Sevall*, James Bonner**

Chromosomal nonhistone protein-bound DNA fragments 3400 bp in length were isolated by nitrocellulose membrane filtration. The nonhistone-bound DNA sequences were labeled with ^{32}P in vitro by nick-translation and used as a probe in Southern blots of the serum albumin, α-fetoprotein, and ribosomal genes in the rat genome. Hybridization was observed in all cases, indicating that the nonhistone proteins do bind to repetitive DNA sequences which are located in transcribed genes.

The nonhistone-bound DNA sequences were tailed with [α-^{32}P]dGTP and inserted in the Pst I site of the ampicillin-resistant gene of plasmid pBR322. Nineteen clones were obtained. The repetitiveness of these clones was checked by Southern blot analysis. Thirteen of the bound DNA clones are highly repeated. The remainder are repeated a few times or not at all in the rat genome. These cloned DNA fragments can be used to make DNA cellulose columns. By use of these columns, DNA-binding

nonhistones which bind to highly and moderately repeated DNA sequences can be isolated and studied.

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18. THE RAT SERUM ALBUMIN GENE: ANALYSIS OF CLONED SEQUENCES

Investigators: **Thomas D. Sargent, Jung-Rung Wu, José M. Sala-Trepat, R. Bruce Wallace, Antonio A. Reyes, James Bonner**

We have isolated the rat serum albumin gene by recombinant DNA techniques (Sargent et al., 1979; Biology 1978, No. 11). Electron microscopic examination of "R-loops" formed when albumin mRNA was hybridized to the isolated clone DNA revealed that this gene is divided into 14 structural sequences (exons) by 13 intervening sequences (introns). The overall length of the gene is 14,500 bp, which is presumably transcribed and subsequently cleaved and ligated to generate the 2200 nucleotide mRNA.

The genomic clones have been cleaved with restriction endonucleases to generate fragments of average length 1500 bp. These have been transplanted ("subcloned") into the genome of the single-stranded DNA bacteriophage M13. These small subclones will be extremely useful for determination of nucleotide sequences and analysis of the metabolism of albumin mRNA and its biosynthetic precursors in rat liver nuclear RNA.

We are also investigating the repetitive sequences located near the albumin gene. Two or more such sequences appear to be contained within the introns and will be of particular interest with regard to the evolution of this gene and of repetitive sequences in general.

Reference:

Sargent, T. D., Wu, J.-R., Sala-Trepat, J. M., Wallace, R. B., Reyes, A. A. and Bonner, J. (1979) Proc. Nat. Acad. Sci. USA. In press.

19. ISOLATION OF RAT α-FETOPROTEIN GENE

Investigators: **Antonio A. Reyes, Thomas D. Sargent, Jung-Rung Wu**

Two cDNA clones, pAFP 65 and pAFP 87, which were prepared from α-fetoprotein (AFP) mRNA have been mapped and found not to overlap. The combined message length of these two clones is around 1800 bases. These two clones were used to screen the rat library. We have isolated three different genome clones, λAFP 6, λAFP 10, and λAFP 22. Clone λAFP 6 haS three rat Eco RI fragments, with molecular weights of 8 kb, 6 kb, and 1.5 kb. Clone λAFP 10 carries only the 8 and 6 kb fragments. The 6 kb and 1.5 kb fragments hybridize to pAFP 87, but not to pAFP 65. Clone λAFP 22 contains two rat Eco RI fragments, with sizes of 13 kb and 3 kb. The 3 kb fragment hybridizes to both pAFP 65 and pAFP 87. It is not clear whether the 1.5 kb and 3 kb fragments are adjacent to each other in the genome. It is possible that one or more Eco RI fragments separate the two. Currently, we are trying to determine whether any such fragments exist.

20. CHROMATIN RECONSTITUTION

Investigators: **Gordon C. Machray, Carlotta A. Glackin**

The basic subunit of eukaryotic chromatin is the nucleosome, a complex of approximately 200 bp of DNA and an octamer of histones (a pair each of histones 2a, 2b, 3, and 4). This structure is repeated regularly along the chromatin fiber. The reconstitution of such a complex from isolated histones and the cloned DNA sequence of a single gene will allow studies of the transcription of that single gene to be undertaken. Previous attempts at reconstitution focused on mixing the dissociated components in solutions of high salt and/or urea concentration followed by a gradual reduction in salt/urea concentration to promote reassociation. More recently assembly of nucleosomes by direct mixing of DNA and histones in physiological salt conditions has been achieved in the presence of cell extracts which contain a putative nucleosome assembly factor. We have confirmed the ability of a cytoplasmic extract from the unfertilized eggs of Xenopus to assemble nucleosomes in 0.15 M KCl (Laskey et al., 1977). We have further sought to determine if such an activity can be demonstrated in an extract of rat liver chromatin.

Rat liver chromatin prepared by standard techniques has been extracted with 0.15 M KCl. The extract was subjected to various treatments removing 80% of the total protein. This partially purified extract when mixed with DNA and histones was able to mediate the assembly of nucleosomes as judged by isokinetic sucrose gradient centrifugation after staphylococcal nuclease digestion. The assembly process is dependent on the stoichiometry of extract to DNA and to histone, the order of mixing of the components, and the time and temperature of incubation. Under the optimal conditions determined thus far an estimated 50% of the input DNA was assembled into

nucleosomes by the crude extract. The regular 200 bp repeat pattern generated by staphylococcal nuclease digestion of native chromatin has not been observed however, suggesting that the assembly process is not cooperative. We are at present engaged in the further purification of the assembly activity from the crude extract in order to effect a more complete reconstitution.

Reference:

Laskey, R. A., Mills, A. D. and Morris, N. R. (1977) Cell **10:** 237-243.

21. ANALYSIS OF CLONED RAT RIBOSOMAL RNA CODING SEQUENCES

Investigators: **Antonio A. Reyes, R. Bruce Wallace, Jung-Rung Wu**

We have taken a closer look at the organization of the ribosomal genes in the rat genome. Previous studies on mammalian ribosomal genes have shown that the 18S and 28S cistrons are arranged in tandem to form a unit which occurs repeatedly in a linear array. In addition to the coding regions, a repeat unit consists of transcribed and nontranscribed spacer regions. These repeat units are known to be heterogeneous. One evidence for this is the presence of several minor bands when restricted genomic DNA is hybridized to nucleic acid probes specific for the 18S and 28S genes. The same technique has been used to determine the length of the major repeat unit. One limitation of this approach is that fragments which are part of the repeat unit but which consist entirely of spacer sequences cannot be detected. Hence, repeat lengths predicted by this method can only be minimum estimates.

We have constructed a restriction map of the DNA region flanking the 18S and 28S genes by hybridizing restriction fragments of whole genomic DNA with 18S and 28S cDNA probes. The map has been confirmed by the analysis of four Charon 4A clones carrying rat ribosomal DNA inserts.

An example of restriction site heterogeneity is apparent from a comparison of the maps of clones 1803, 1804, and 2807. Thus, 2807 contains two Eco RI sites which are absent, in the same region, in 1803 and 1804.

A 4.9 kb Eco RI fragment of clone 2806 has been used to probe Pst I-restricted genomic DNA and identify a spacer fragment. From this result, we have expanded our genome map and estimated the length of the major repeat unit at 38.3 kb. This agrees with the mean length estimated from electron microscopic measurements, which is 37.2 kb (Stumph et al., 1979).

Reference:

Stumph, W. E., Wu, J.-R. and Bonner, J. (1979) Biochemistry **18:** 2864-2871.

22. DEVELOPMENTAL EXPRESSION OF α-FETOPROTEIN AND ALBUMIN GENES

Investigators: **Kelwyn Thomas*, José M. Sala-Trepat, Stewart Sell*, James Bonner**

Labeled cDNA complementary to highly purified poly(A)+ albumin and α-fetoprotein (AFP) mRNA sequences were utilized as molecular hybridization probes to quantitate the number of albumin and AFP mRNA sequences present in fetal liver and yolk sac at various stages of gestation in rats. In yolk sac a continuous high number of AFP mRNA sequences are present up to term whereas the number of albumin mRNA sequences rapidly decreases. In contrast in fetal liver there is a steady decrease in the number of AFP mRNA sequences with a concomitant increase in the number of albumin sequences. In previous studies we have clearly demonstrated that phenotypic expression of these proteins is closely related to their functional mRNA levels indicating that the control mechanisms are at the level of transcription and/or hnRNA processing and transport. Our future studies are aimed at elucidating the specific molecular mechanisms regulating gene expression of these proteins during development and oncogenesis.

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23. MOLECULAR ANALYSIS OF ALBUMIN AND AFP EXPRESSION IN NORMAL RAT LIVER AND RAT HEPATOMAS

Investigators: **José M. Sala-Trepat, Kelwyn Thomas*, Stewart Sell*, James Bonner**

Highly radioactive complementary DNA probes (cDNA) have been synthesized from purified rat albumin and rat α-fetoprotein (AFP) messenger RNAs and used in molecular hybridization studies to quantitate albumin and AFP mRNA molecules during liver postnatal development and in different transplantable rat hepatomas. The number of polysomal AFP mRNA molecules per liver was found to decrease drastically during the first weeks of

postnatal life, concomitant with a decline in the AFP synthetic capacity of the liver and in the serum concentration of AFP. During this period, the concentration of albumin mRNA molecules per cell in the liver remained at high, approximately constant, levels. In the different hepatomas studied a good correlation was found between the concentrations of albumin and of AFP and the concentrations of the two kinds of mRNA sequences in the polysomes. From these results we conclude that the level of expression of albumin and AFP in normal rat liver and in rat hepatomas is mainly regulated by modulating the steady-state concentration of the corresponding functional mRNAs. Further studies involving subcellular distribution experiments have shown that in adult rat liver and in rat hepatoma tissues more than 95% of the albumin and AFP mRNA sequences are located in the heavy polysome fraction. Only minimum amounts (<0.5%) of these mRNA sequences are found in the nucleus and in the post-polysomal cytoplasmic fraction. These results together with previous experiments concerning the number of albumin and AFP genes in normal rat liver and Morris hepatoma 7777 (Sala-Trepat et al., 1979) suggest that the changes in expression of albumin and AFP genes associated with liver carcinogenesis are under transcriptional control.

Reference:

Sala-Trepat, J. M., Sargent, T. D., Sell, S. and Bonner, J. (1979) Proc. Nat. Acad. Sci. USA **76:** 695-699.

24. IDENTIFICATION OF RNA PRECURSORS FOR ALBUMIN mRNA AND α-FETOPROTEIN mRNA

Investigator: **Murray F. Teitell**

The messenger RNA (mRNA) for certain animal virus genes (adenovirus, SV40) and certain high abundance genes from mammals (ovalbumin, β-globin, and the immunoglobulins) is derived from initial transcripts that are an order of magnitude larger than the mRNA. The initial transcripts are processed in discrete steps that excise sequences homologous to the introns (intervening sequences) of the gene and splice together sequences homologous to the exons (coding sequences) of the gene.

Albumin and α-fetoprotein (AFP) are two serum proteins synthesized in the mammalian liver in different developmental stages. To study the transcription of the genes for these proteins, nuclear and whole cell RNA was isolated from adult and fetal rat liver, Morris 7777 hepatoma, and cultured rat hepatocytes. The RNA was characterized by electrophoresis in agarose gels containing methylmercury.

To identify the transcription processing steps, this RNA is hybridized to cloned cDNA and cloned genomic DNA sequences for albumin and AFP under conditions where either the RNA or DNA is in vitro-labeled with ^{32}P and either the RNA is covalently bound to a filter or the DNA is filter-bound. We also use in vitro-labeled whole nuclei from which one can, we believe, obtain the initial transcripts for albumin and AFP with minimum degradation. We have not as yet found any nuclear RNA containing serum albumin or AFP sequences which is substantially longer than the final message.

There is no evidence thus far for transcripts of the AFP gene in the nuclear RNA of normal rat liver.

25. PROPERTIES OF NEWLY-REPLICATED CHROMATIN

Investigators: **Robert F. Murphy, R. Bruce Wallace**

Our previous investigations of the properties of chromatin containing newly-synthesized DNA have been extended (Murphy et al., 1978; Biology 1978, No. 22). Nuclease digestion rates suggest that histones are segregated conservatively at the replication fork. A fraction of soluble nucleosomes enriched in newly-replicated DNA has been isolated by means of its increased density in metrizamide relative to bulk chromatin. This fraction is shown to be packaged into nucleosomes, but at an interval of approximately 160 base pairs, as opposed to the 200 base pair repeat seen for total chromatin. We have obtained evidence that the density difference is due to this altered spacing. While pulse-labeled DNA is present in the dense fraction, nucleosomes labeled with short pulses of arginine or acetate are of normal density. The data are consistent with the conclusion that newly-replicated DNA is associated with preexisting histones in a short-lived, compact structure, while newly-synthesized histones are deposited at normal spacing some distance from the replication fork.

Reference:

Murphy, R. F., Wallace, R. B. and Bonner, J. (1978) Proc. Nat. Acad. Sci. USA **75:** 5903-5907.

26. COMPUTER PROGRAMS FOR ANALYSIS OF BIOCHEMICAL DATA

Investigator: **Robert F. Murphy**

We have developed a set of flexible, interactive programs for the analysis and storage of biochemical data. The ability of the programs to accept English commands and prompt the operator for needed information allows even an inexperienced computer user to analyze a reassociation curve or gel profile in under an hour. In addition to the analysis of data from nucleic acid hybridization, thermal denaturation, and gel electrophoresis (Biology 1978, No. 25), the programs may be used for a number of other applications, such as resolution of components in velocity and equilibrium density gradients and the determination of rate constants for enzyme reactions. The programs also provide a framework for the development of other data analysis systems. The programs described are available from the authors on a variety of machine-readable media.

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Summary: The work of this laboratory is concerned with genomic sequence organization, the regulation of gene expression, and gene control during development. Recombinant DNAs are used in many of the experiments. Problems which are central to our research include the structure, function, and evolution of repetitive DNA families. Questions of evolutionary changes are being examined by extensive comparison of DNA sequences of several species of sea urchins. Other studies involve analysis of the sequences expressed in RNAs of oocytes, embryos, and adult tissues.

27. DNA SEQUENCE ORGANIZATION AROUND mRNAs IN THE SEA URCHIN

Investigators: **Terry L. Thomas, Eric H. Davidson**

We have cloned two DNA sequences, Sp34 and Sp88, which have been shown to code for rare mRNAs. These clones display a complex DNA sequence organization. Thus in each cloned fragment there exist several non-repetitive DNA sequence elements interspersed with different repetitive DNA sequence elements. We have also shown that the number of Sp88 and Sp34 mRNAs change drastically during sea urchin development (Biology 1979, No. 40). The length of Sp88 transcripts changes during development (Biology 1979, No. 41).

The Sp88 and Sp34 DNA fragments contain only flanking sequences which are located 3' to the coding region. Consequently, we are now attempting to isolate longer DNA sequences containing the Sp88 and Sp34 structural genes. This is being done by screening bacteriophage λ libraries, containing ~15 kb sea urchin DNA inserts, with labeled DNA probes made from the codogenic regions of Sp88 and Sp34. With these λ recombinants, we will determine the longer range sequence organization surrounding Sp88 and Sp34 coding regions, i.e., we will define with respect to the coding region the location of repetitive sequence elements and other codogenic and noncodogenic single copy DNA sequences. We will also use these clones to further examine the interesting and probably complex relationship between the primary transcripts of Sp88 and Sp34 and their respective mRNAs. Sp88 and Sp34 λ recombinants also will be invaluable in testing two aspects of the Britten-Davidson model of gene regulation. In particular, we can ask whether there are other structural genes adjacent to the same repetitive sequence family members as found in Sp88 or Sp34, i.e., do certain repetitive sequence families define gene batteries? We can also ask what is the relationship between repetitive sequences adjacent to expressed structural genes and repetitive sequence transcripts in the nuclear RNA of various developmental stages.

28. SEQUENCE ORGANIZATION OF REPETITIVE DNA SEQUENCE FAMILY MEMBERS

Investigators: **David M. Anderson, Richard H. Scheller, Eric H. Davidson**

Individual members of four separate repetitive sequence families have been isolated, along with several thousand nucleotides of flanking DNA, from the sea urchin genome cloned in the bacteriophage λ. Three major projects using these clones are now in progress. (1) The DNA sequence organization around repetitive sequence elements is being analyzed with respect to repetitive and single copy sequences, and any cross-homologies in the DNA flanking different members of the same family. We

have observed both the typical interspersed pattern of sequence organization, and several regions of clustered repetitive DNA. Individual members of a repetitive family frequently occur within 15,000 nucleotides of each other. Preliminary data indicate that members of one repetitive family tend to occur near each other at several locations in the genome, although not always in the same orientation. (2) The repetitive DNA families that were used in the initial screening of the λ library were selected because they exhibited embryonic stage-specific changes in their representation in nuclear RNA populations. We are now studying the transcription of single copy DNA flanking several members of a repetitive family highly represented in gastrula nuclear RNA. One of these single copy sequences is represented in gastrula nuclear RNA at 100 times the average single copy DNA transcript concentration. (3) The relationships between individual members of a given repetitive sequence family are also being examined. We have identified subfamilies in two of our repetitive families by determining the thermal stability of hybrids formed between individual family members, and comparing the data to those obtained from hybrids formed with total genomic DNA. It now seems clear that for the repetitive families we have studied, the total number of family members in the genome has arisen through several probably independent multiplication events. Each of these multiplications appears to have generated a subfamily of closely-related elements. We are now isolating one such subfamily.

29. PRIMARY STRUCTURE OF REPETITIVE SEQUENCE ELEMENTS IN SEA URCHIN DNA

Investigators: **James W. Posakony, Eric H. Davidson**

In order to investigate the internal organization of repetitive sequence elements, we have determined the nucleotide sequence of seven such elements cloned from sea urchin DNA (Scheller et al., 1977). Each clone contains a representative of a different repetitive sequence family; these families show a wide variation in both the number and sequence divergence of their members (Klein et al., 1978).

A variety of computer techniques (R. F. Murphy, unpublished) were used to search the nucleotide sequences both for internal structural features and for sequence blocks shared among different repeats. These studies revealed the statistically significant occurrence of direct and inverted repetitions of varying lengths and of alternating runs of AT and GC base pairs. Furthermore, five of the seven repeats contain a sequence matching at least seven out of eight nucleotides with the sequence TTCAGGAT; the other two contain a six out of eight match. Aside from the unlikelihood of a chance occurrence of this homology, the sequence is of interest because of its close similarity to a consensus sequence representing the junctions between intervening and coding sequences in several eukaryotic genes (Breathnach et al., 1978). Thus, repetitive sequence elements in sea urchin DNA appear to have a highly nonrandom internal organization and, in addition, different repetitive families may be related by short sequence homologies not detectable by physical-chemical methods.

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30. REPETITIVE SEQUENCE TRANSCRIPTS IN SEA URCHIN EGGS AND EMBRYOS

Investigators: **Frank Costantini, William H. Klein, Eric H. Davidson**

We have previously reported that the sea urchin egg contains transcripts representing most of the repetitive sequence families in the genome, in widely varying prevalence, and that most repetitive sequence transcripts (RSTs) are 1000 to 2000 nucleotides or longer. Recently, we have found that many of the egg RSTs are polyadenylated. We have isolated egg poly(A)-RNA, labeled it in vitro with ^{125}I, and isolated those transcripts containing repetitive sequences by hybridization to mercurated total repetitive DNA and chromatography on sulfhydryl Sepharose. About 25% of the poly(A)-RNA molecules (average fragment length about 1400 NT) appear to contain a repetitive sequence. Hybridization kinetics with excess sea urchin DNA showed that these transcripts consist mostly of single copy sequence, and only about 20% of their length, on the average, is repetitive sequence. Thus most of the egg repetitive sequence transcripts are linked to single copy transcripts. Since most of the single copy sequences in egg RNA are maternal mRNAs, these results suggest that the egg RSTs may be linked to single copy maternal mRNAs.

By isolating gastrula polysomal mRNA that is only slightly degraded in length (about 1500 NT weight average length) we have observed a previously undetected fraction of polysomal mRNA consisting of covalently-linked single copy and repetitive sequences. These transcripts were detected by the same methods used for polyadenylated egg RNA, and also by hydroxyapatite chromatography. At least 20% of labeled polysomal RNA consists of such transcripts. These molecules are not contaminating nuclear transcripts, as shown by pulse-labeling experiments. The presence of repetitive sequences in gastrula polysomal RNA has also been detected by hybridization of cloned individual repetitive sequences with polysomal RNA.

31. DOUBLE-STRANDED hnRNA FROM SEA URCHIN GASTRULA

Investigators: **Laurence A. Lasky, Eric H. Davidson**

This project has revolved around the isolation and characterization of RNA:RNA duplex molecules from hnRNA of sea urchin gastrula stage embryos. Previous work from this laboratory has shown that interspersed repetitive sequence elements are symmetrically represented in hnRNA of gastrula. hnRNA from gastrula was labeled in vivo, isolated, and reassociated. Duplex RNA was isolated by cellulose-ethanol chromatography. The reaction showed second-order kinetics. Approximately 15% of the total radioactivity was bound to the column after a 10 min pulse-label and incubation to Rot 2000. The isolated duplex fraction was characterized with respect to size, thermal melting behavior, and structure in the electron microscope. These results suggested that hnRNA duplexes consisted of covalently-linked double- and single-stranded regions. The isolated hnRNA duplex fraction was also used to titrate a number of interspersed repeat element clones. These experiments demonstrated that the RNA transcripts for most of the elements tested were ~70-fold more concentrated in the duplex fraction. Further experiments with the hnRNA duplex fraction will include a characterization of the adjacent single-strand regions which are covalently linked to the duplexes.

32. CONSERVATION OF THE PATTERN OF REPEATED DNA TRANSCRIPTS IN SEA URCHIN OOCYTES

Investigators: **Gordon P. Moore, Roy J. Britten**

Previous studies with cloned repetitive DNA sequences of the sea urchin Strongylocentrotus purpuratus demonstrated that repetitive elements are transcribed in a specific fashion into RNA which is then stored in the mature oocyte (Costantini et al., 1978). That is, some repetitive DNA transcripts are highly abundant and others less so. Another series of measurements (Scheller et al., 1978) also demonstrated specific contrasting patterns of repeat transcripts in nuclear RNA of various embryonic stages of development and adult tissues.

We have initiated the study of the evolutionary stability of these specific patterns of transcripts by measuring the concentration of transcripts in a related species of sea urchin. The evolutionary lines leading to S. purpuratus and S. franciscanus diverged approximately fifteen million years ago. We have measured the concentration of RNA sequences in S. franciscanus oocytes complementary to the same set of cloned repetitive DNA elements of S. purpuratus using kinetic and titration techniques. Costantini et al. (1978) isolated a fraction of the total repetitive DNA sequences of S. purpuratus complementary to the abundant class of repetitive oocyte transcripts. We have reacted this tracer with S. franciscanus oocyte RNA. Results clearly show that the pattern of transcript accumulation is conserved. That is, repetitive transcripts abundant in S. purpuratus are also abundant in S. franciscanus. Evolutionary conservation of the pattern of repeat sequence transcript accumulation lends support to the view that the specific pattern of repeat transcription is physiologically important to the development and maintenance of the organism.

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Scheller, R. H., Costantini, F. D., Kozlowski, M. R., Britten, R. J. and Davidson, E. H. (1978) Cell **15:** 189-203.

33. INITIAL STUDIES OF THE EVOLUTION OF SEQUENCE ORGANIZATION IN SEA URCHINS

Investigators: **Gordon P. Moore, Roy J. Britten**

Repetitive DNA in the sea urchin genome falls into two categories. About 65% of repeats are short relative to a structural gene and generally interspersed with single copy DNA. The remaining 35% are greater than 2000 nucleotides in length. The two classes can be separated by intermediate Cot reassociation, treatment with S1 nuclease and chromatography on Sepharose 2B. The long repeats reassociate precisely to yield high thermal stability duplexes whereas the short repeats are divergent and form low melting hybrids (Britten et al., 1976; Eden et al., 1977).

We have examined the sequence homologies between the long and short classes of repeat DNA and individual cloned repetitive elements, each a representative of a family of repeats. Kinetics of hybridization indicate that each repeat family is predominately contained in one class or the other. Nevertheless, each cloned element reacts to some extent with the other class of repeat. Thus the two classes are overlapping in the sense that some sequences are shared between them.

Why are the long repeats precisely matched? One possibility is that selection acts to prevent sequence divergence during evolution. Alternatively, long repeats might be the products of recent multiplication. We approached this question by preparing a high thermal stability fraction of S. purpuratus repeats in 2.4 M tetraethyl ammonium chloride which suppresses the effect of base composition on thermal stability. We reacted this fractionated S. purpuratus repeat DNA with an excess of total DNA from the related urchin species S. franciscanus and melted the resulting duplexes. Results indicated that the high thermal stability repeats had diverged to the same extent as the average repeat. We conclude that precisely matched repeats are not more highly selected than the average repeat and that recent multiplication is a more plausible mechanism to explain their relative lack of sequence divergence.

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34. POLYMORPHISM OF SINGLE COPY DNA SEQUENCES

Investigators: **Terrence J. Hall, Roy J. Britten**

The 4% single copy DNA sequence polymorphism of Strongylocentrotus purpuratus reported last year has been confirmed and the measurements extended. New procedures, including the measurement of the length of duplexed DNA after S1 digestion, have increased the accuracy and clarity of interpretation of these measurements. Several additional pairs of individuals have been studied and the average sequence difference between individual genomes remains about 4° with an uncertainty of less than 1°. The length of the duplexed regions is very slightly shorter for reassociated interindividual DNA compared to that from the same individual. We do not know if this is due to rearrangement events or the digestion of short regions of high local sequence divergence.

Studies of the single copy DNA sequence polymorphism of a second species (S. drobachiensis) have been initiated. DNA of one individual collected at Woods Hole, Massachusetts, was labeled and reassociated with that of another individual from Woods Hole and with the mixed DNA from several individuals collected near Vancouver, British Columbia. In both cases the length-corrected melting temperatures were about 1° below that observed for reassociation with driver DNA from the same individual. The earlier measurements for S. purpuratus show the polymorphism to be widespread and possible homozygosity due to inbreeding in local populations was not observed. Thus heterozygosity was large and the average thermal stability for reassociated DNA from one individual was halfway between that of perfect duplex and that observed for interindividual reassociated DNA. If the same situation is true for S. drobachiensis it appears that the single copy DNA polymorphism of this species is half of that for S. purpuratus or about 2% average nucleotide sequence difference. It also appears that there is no greater sequence difference between East and West Coast individual genomes than between the two genomes of East Coast individuals.

35. CORRELATION BETWEEN INTRASPECIES AND INTERSPECIES DNA SEQUENCE DIVERGENCE

Investigators: **John W. Grula, Roy J. Britten**

Measurements of sequence divergence between the single copy DNAs of two species show a wide spectrum of degrees of sequence difference. Further, in studies of polymorphism a majority of the DNA appears to show little sequence difference between individual genomes while a smaller fraction exhibits a large sequence difference. Is this range of divergence due to chance or natural selection?

If the same set of single copy DNA which showed small polymorphism also displayed small interspecies divergence, it would support the natural selection alternative and indicate that these sequences are being conserved in evolution. Therefore we have isolated two fractions of labeled Strongylocentrotus purpuratus single copy DNA on the basis of thermal stability after reassociation with DNA of a different individual of the same species. To test the fractionation these preparations were reassociated with mixed S. purpuratus DNA and gave T_ms

of 58° and 54° (compared to 60° for perfect duplexes of their length in the 2.4 M tetraethyl ammonium chloride system). When they were reassociated with S. franciscanus DNA, T_ms of 52° and 47°, respectively, were observed. These measurements suggest a correlation between degree of polymorphism and interspecies divergence. Complementary measurements in which the fractionation is carried out on the basis of interspecies sequence differences also suggest a correlation, but it may be somewhat weaker.

Thus it appears that a large fraction of the single copy DNA sequences of these sea urchins is under selective pressure. It is worth noting that only a much smaller fraction of the single copy DNA (6%) has been shown to be made up of structural genes on the basis of its transcription into the complex class of maternal message in mature oocytes. The single copy sequences other than those coding for structural genes appear to be under sequence-dependent selective pressure.

36. EVOLUTIONARY SEQUENCE CONSERVATION OF TRANSCRIBED SINGLE COPY DNA

Investigators: **Terrence J. Hall, Roy J. Britten**

Comparisons have been made in sea urchins between the interspecies sequence divergence of total single copy DNA and the fraction of the single copy DNA which is transcribed to yield nuclear RNA or mature egg RNA. Labeled single copy DNA of Strongylocentrotus purpuratus was hybridized with gastrula nuclear RNA or total egg RNA and the duplexed fragments isolated on hydroxyapatite. The resulting specific tracers are termed nDNA and oDNA and consist of short fragments of single copy DNA (100-500 nucleotides) which contain regions which are transcribed. However their full length is not necessarily represented in the RNA. These tracers were reassociated with an excess of sheared DNA from S. purpuratus or S. franciscanus, the resulting duplexes were digested with S1 nuclease to remove unpaired regions, and the duplex length measured. Samples were heated to a series of temperatures, cooled, digested with S1 and the remaining duplexes assayed. Least squares methods were used to determine the T_m (temperature at which half the duplexes were melted) and the resulting values were corrected for the length by adding C = (500/duplex length). The interspecies duplex lengths were about half of intraspecies duplex lengths, presumably as a result of digestion of clusters of unpaired nucleotides, and the length reduction was somewhat less for the specifically transcribed tracers.

Compared to intraspecies duplexes (between different individuals) the interspecies duplexes with total single copy tracer were reduced in T_m by 9.7°. The same comparison for nDNA tracer yielded 6.5°. With the oDNA tracer, S. franciscanus duplexes showed a T_m only 4.4° below those with S. purpuratus. A clear conclusion can be drawn that these transcribed regions have been conserved in sequence relative to total single copy DNA during the more than 15 million years of independent evolution of S. franciscanus and S. purpuratus.

37. COMPLEXITY OF RNA SEQUENCES IN THE EGGS OF TWO SPECIES OF FLIES

Investigators: **Barbara R. Hough-Evans, Marcelo Jacobs-Lorena*, Michael R. Cummings**, Eric H. Davidson**

We have extracted RNA from the eggs of two species of flies, the housefly Musca domestica and the fruit fly Drosophila melanogaster, and measured its complexity (that is, the number of nucleotides of diverse sequence represented). Musca and Drosophila diverged in evolution at least 63 million years ago, and their genomes differ in several respects: (1) there is 5 times as much total DNA in Musca, and (2) 3-1/2 times as much single copy DNA; (3) the single copy and repetitive sequences of Musca DNA are arranged in a short-period interspersion pattern like those of Xenopus, sea urchin, and most other eukaryotic DNAs, while Drosophila DNA is arranged in a long-period interspersion pattern, with both nonrepetitive and repetitive sequences of 5 to 10 times the length of those observed in Musca.

The egg RNA complexities were determined by hybridization of excess RNA with radioactively-labeled nonrepetitive DNA. We found that the egg RNA of Musca contains twice the complexity of Drosophila egg RNA (2.4 vs. 1.2×10^7 nucleotides). Musca egg RNA is thus similar in complexity to the RNA of eggs of Xenopus and sea urchin, while Drosophila egg RNA is unusually low in complexity.

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38. THE EXPRESSION OF HISTONE GENES IN SEA URCHIN OOGENESIS

Investigators: **Barbara R. Hough-Evans, Alex Mauron*, Eric H. Davidson**

The histone genes of the sea urchin Strongylocentrotus purpuratus exist in the genome as a cluster of repeated genes, with the "spacer DNA" between them equally repeated. These genes and the spacer regions have been reproduced as recombinant DNA in the bacterial plasmid pBR322 in the Kedes laboratory at Stanford. Cloned fragments containing single genes or spacers are now being prepared. The adjacent individual genes coding for histones H2B and H3, and the spacer DNA between them, are to be strand-separated, labeled to high specific activity, and hybridized to mature sea urchin egg RNA and to cytoplasmic and nuclear RNAs extracted from vitellogenic oocytes and previtellogenic oocytes. The goal of the project is to determine the time of appearance, and the accumulated amounts, of stored histone mRNAs during oogenesis. We are also interested in finding out if spacer regions are transcribed, and whether the mRNAs of all five genes are produced and stored at the same rate.

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39. SIZE DISTRIBUTION OF POLY(A) TRACTS IN EARLY MOUSE EMBRYOS

Investigators: **Lajos Pikó*, Kerry B. Clegg***

Previously we have shown that the unfertilized mouse egg contains about 0.7 pg of poly(A) sequences in its cytoplasm. The amount of poly(A) remains unchanged in the fertilized 1-cell egg but drops sharply, to about 0.25 pg per embryo, at the 2-cell stage. The poly(A) content increases progressively from the 4- to 8-cell stage onward to about 1.5 pg in the early blastocyst (32 cells).

To explore the significance of the changes in poly(A) content, we have analyzed the size distribution of poly(A) tracts (defined as material resistant to RNase A and T_1) in total unlabeled RNA isolated from unfertilized and fertilized 1-cell eggs and 2-cell embryos. The poly(A) tracts were fractionated by polyacrylamide gel electrophoresis, eluted from the gel slices and assayed by hybridization with [^{3}H]poly(U). Essentially identical patterns were obtained for the three stages, with a sharp peak of 20 to 30 nucleotides and a broad peak of up to 250 nucleotides (the majority ranging between 120 to 200). The number average molecular weight of poly(A) tracts varied within narrow limits, from 63 to 75. Labeling experiments with [^{3}H]adenosine have failed to detect any significant turnover of the poly(A) sequences or synthesis of poly(A)-containing RNA in the 1-cell fertilized egg, although newly synthesized poly(A)-containing RNA is clearly detected at the 2-cell and later stages. These results indicate that poly(A)-containing RNA is stored in the mouse egg and that neither the amount nor the size of the poly(A) sequences changes significantly upon fertilization. Protein synthesis in the 1-cell embryo may utilize stored messenger RNAs exclusively. The significant drop in poly(A) content at the 2-cell stage cannot be accounted for by a reduction in poly(A) length and may represent a turnover of maternally inherited mRNA. Poly(A)-containing RNA is synthesized in progressively larger amounts from the 2-cell stage onward.

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40. EXPRESSION OF GENES CODING FOR RARE SEA URCHIN EMBRYO MESSAGES

Investigators: **Ze'ev Lev, Terry L. Thomas, Amy Shiu Lee, Robert C. Angerer*, Eric H. Davidson**

Measurements are presented of the number of transcripts in sea urchin embryos and in adult intestine cells complementary to two cloned genomic single copy sequences, Sp88 and Sp34. These sequences apparently code for maternal mRNAs stored in the egg. About 1000 and 1400 transcripts are stored per egg, close to the average value for maternal mRNAs. RNAs complementary to the cloned sequences are also found in the polysomal message of early embryos. The number of polysomal transcripts representing each cloned sequence is typical of the "complex," or "rare" class of message in sea urchin embryos. Expression of these putative complex class structural genes is regulated developmentally. Thus polysomal transcripts of both sequences essentially disappear by the pluteus stage. One sequence is again represented at a low level in adult intestine cytoplasmic RNA while the other is absent. However, in all the developmental stages and tissues examined the number of nuclear transcripts of the cloned sequences remains about the same as for any average single copy sequence transcript.

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41. TRANSCRIPT SIZE OF SEA URCHIN MESSAGES

Investigators: **Amy Shiu Lee, Eric H. Davidson**

Two sea urchin recombinant DNA clones, Sp88 and Sp34, contain sequences which code for maternal mRNA stored in the egg. RNAs complementary to the cloned sequences are also found in nuclear transcripts of all developmental stages examined, as well as polysomal message of 16 cell embryos (Biology 1978, Nos. 32 and 33). The size of these transcripts as they occur in different developmental stages is being examined by DMSO gradients, as well as by RNA blotting techniques. Preliminary results indicate that in the maternal RNA population of the egg, Sp88 hybridizes to two RNA transcript sizes, ~4000 NT and 1600 NT. These transcripts appear to be polyadenylated. In the case of Sp34, the major egg transcript size is 4000 NT. The 16-cell polysomal RNAs reacting with Sp88 are of typical message length; they are ~1800 NT long. However, at the gastrula stage when the Sp88 transcript is not represented in polysomal or cytoplasmic RNA populations, the transcripts which are detected in the nucleus are over 6000 NT long. Work is in progress to determine if Sp34 transcripts expressed during embryonic stages are similar in size to the Sp88 transcripts.

42. cDNA CLONES FROM EGGS AND GASTRULAE OF SEA URCHIN

Investigators: **Laurence A. Lasky, Jay W. Ellison, Ze'ev Lev, Terry L. Thomas, Eric H. Davidson**

cDNA clones have been constructed using polyadenylated RNA from eggs and gastrulae of Strongylocentrotus purpuratus. These clones have been constructed using the double-stranded DNA approach in the vector pBR322. Several clones have been isolated from both stages and characterized with respect to insert size and restriction enzyme sites. One such clone from gastrula has been found to be represented at about 20 copies per cell in gastrula polysomal RNA, but appears to be absent from blastula messenger RNA. In addition, a new library of cDNA clones is being constructed by cloning of RNA:cDNA hybrids. This library will consist of a large number of clones which can be screened with various probes. In this way, it should be possible to isolate several cDNA clones which correspond to mRNA sequences specific for given stages in early development.

43. SYNTHESIS AND DECAY RATES OF STRUCTURAL GENE TRANSCRIPTS

Investigators: **Jay W. Ellison, Eric H. Davidson**

A recent observation concerning transcription in sea urchins was that structural gene transcripts absent from polysomes of a given tissue are nonetheless present in the nuclear RNA of that tissue (Wold et al., 1978). This finding implied that post-transcriptional events play some role in the regulation of those structural genes. Specific predictions of the nature of this level of regulation have recently been proposed (Davidson and Britten, 1979).

The present study is designed to ask questions concerning the extent of transcriptional control of the expression of structural genes. Specifically, we wish to determine whether mRNAs which differ in prevalence in the cytoplasm also differ in their rates of synthesis. Embryos are labeled with exogenous [^{3}H]guanosine and the specific activity of the GTP pool is determined as a function of labeling time. Nuclear RNA is prepared at various times after labeling and is hybridized with an excess of a cloned DNA sequence complementary to a cytoplasmic poly(A)-containing RNA. From the GTP-specific activity measurements and the flow of radioactivity into the complementary transcript, we can derive synthesis and decay rate constants for the cDNA transcript. Comparison of these parameters for cDNAs representing mRNAs of varying abundance should shed some light on the degree of transcriptional regulation of structural gene expression.

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Davidson, E. H. and Britten, R. J. (1979) Science **204:** 1052-1059.

Wold, B. J., Klein, W. H., Hough-Evans, B. R., Britten, R. J. and Davidson, E. H. (1978) Cell **14:** 941-950.

44. DISTINCT SINGLE COPY SEQUENCE SETS IN SEA URCHIN NUCLEAR RNAs

Investigators: **Susan G. Ernst, Eric H. Davidson**

The purpose of this study was to determine whether nuclear RNAs (nRNA) of sea urchin embryos and adult tissues contain identical or partially distinct sets of single copy sequence transcripts. A DNA tracer was prepared consisting mainly of sequences absent from gastrula nRNA; 3.6% of this tracer reacted with adult intestine nRNA but not with gastrula nRNA. The existence of a differentially transcribed DNA fraction was verified by its partial purification and rehybridization to intestine and gastrula nRNAs. About one-third of the genomic single

copy sequence is represented in both nRNAs, or about 2 x 10^8 nucleotides. The differentially transcribed portion of the single copy genome identified in this work includes about 3.5 x 10^7 nucleotides.

45. GENE ORGANIZATION IN THE SEA URCHIN

Investigators: **Ze'ev Lev, Eric H. Davidson**

This project is concerned with the relation between hnRNA and mRNA in a differentiating animal cell system. The sequence complexity and transcript length of both mRNA and hnRNA have been measured in several embryonic stages and adult tissues of the sea urchin. It has been shown that the sequence complexity of nuclear RNA is 30 to 40% of the complexity of total single copy sequences existing in the sea urchin genome. The complexity of mRNA varies, according to the tissue tested, between 0.8 and 4.5% of total DNA single copy sequence complexity. It is reasonable to assume that about 10% of the whole single copy genome are mRNA sequences. Recent results suggest that most, or even all, mRNA sequences appear in hnRNA of every tissue (Wold et al., 1978; see Biology 1979, No. 40). It is concluded that 10%/40% = 25% of hnRNA sequences are sequences included at some time in mRNA (Wold et al., 1978). This is about the same as the ratio of sizes between the two RNA populations. The size of mRNA molecules is about 1.8 kb and the size of hnRNA molecules is about 8.0 kb.

There are many possible explanations for this relationship. Two of the most simple possibilities are: (1) Each hnRNA molecule contains one mRNA sequence which constitutes about 25% of its length; (2) all mRNA sequences are confined to a 25% class of hnRNAs and 75% have no mRNA sequences at all, but perform other functions, possibly regulative. In the first case, where each hnRNA molecule carries one mRNA sequence, it would be predicted that at the DNA level the gene organization is such that the genes are at least 8.0 kb apart. The second alternative predicts that the genes are clustered in groups of about four genes per group.

In order to distinguish between these two possibilities, we use cDNA clones as a probe to screen the cloned library of sea urchin genomic DNA. Selected genomic clones will be purified and the positions of the sequences coding for mRNA and hnRNA prepared from a given embryonic stage or adult tissue will be determined, using biochemical and electron microscopy techniques. If the results show clustering of genes, this will be considered as a confirmation of model (2). However, the experiment is inconclusive if only one gene is found to be expressed within a long DNA sequence. Thus, it is always possible that genes included in this sequence are expressed only at some time during the life cycle of the sea urchin other than that from which the diagnostic mRNA was obtained. We shall try to overcome this difficulty by testing mRNA preparations made from many different tissues.

Reference:

Wold, B. J., Klein, W. H., Hough-Evans, B. R., Britten, R. J. and Davidson, E. H. (1978) Cell **14**: 941-950.

46. ISOLATION OF CLONED DNA SEQUENCES CODING FOR INSULIN-LIKE mRNAs

Investigators: **Terry L. Thomas, Argiris Efstratiadis*, Eric H. Davidson**

The importance of insulin in mammalian physiology is well known. Interestingly, there are a number of examples of insulin-like activities in invertebrates, including the starfish, an echinoderm. We are currently attempting to isolate insulin-like structural genes from the sea urchin. This is being done by screening our Strongylocentrotus purpuratus λ libraries with a cloned rat insulin cDNA probe. This clone was constructed by Efstratiadis et al. of Harvard University Medical School. At present we have isolated three different λ recombinants sharing some homology with the rat insulin probe. We are determining the extent of this homology by hybridization methods and by direct DNA sequencing.

Our interests in the insulin clones are directed at the genome organization surrounding the insulin structural gene and in the expression of a structural gene with a known gene product in either embryonic or adult tissues. Model tests similar to those described for Sp88 and Sp34 are also of considerable interest. Efstratiadis will compare the DNA sequence of the rat insulin intervening sequences with any intervening sequences found in the sea urchin insulin gene.

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47. IMMUNOGLOBULIN (IgA) GENE TRANSCRIPTS IN NUCLEAR RNA AND mRNA

Investigators: **Terry L. Thomas, Kathryn L. Calame, Leroy E. Hood, Eric H. Davidson**

We are examining the expression of the IgA heavy chain constant region structural gene in the nuclear RNA and mRNA of several myelomas and mouse tissues. This

sequence has been cloned as a full length cDNA. We have prepared strand-separated radioactively-labeled tracers of the IgA constant region sequence and have reacted these tracers with nuclear RNA and mRNA from the above sources. All transcripts are completely asymmetric. In a myeloma producing large quantities of IgA, there are $>10^4$ mRNA transcripts loaded on polysomes; there are $\leq 2 \times 10^3$ transcripts per nucleus. In nuclear RNA from mouse liver and a myeloma that does not produce IgA, there are $\sim$0.6 and 0.2 copies per nucleus, respectively, of IgA constant region transcript. At this point we are not sure these latter observations are real because we cannot rule out contaminating lymphocytes as a source for these transcripts, although this seems unlikely for the nonproducing myeloma. We are now making more measurements on nuclear RNA and mRNA preparations from tissues (fetal brain and adult brain) less likely to be contaminated with circulating lymphocytes. These experiments will provide a quantitative measure of the level of expression in both nuclear RNA and mRNA of the Ig genes.

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Summary:

"For the real amazement, if you want to be amazed, is the process. You start out as a single cell derived from the coupling of a sperm and an egg, this divides into two, then four, then eight, and so on, and at a certain stage there emerges a single cell which will have as all its progeny the human brain. The mere existence of that cell should be one of the great astonishments of the earth...No one has the ghost of an idea how this works and nothing else in life can ever be so puzzling."

From "On Embryology" by Lewis Thomas (1979)

From the time over fifty years ago when the Division of Biology was founded and Thomas Hunt Morgan was its first chairman, numerous Caltech scientists have studied and speculated on the process of embryogenesis. It was probably clear from the start that the surfaces of

cells somehow differed from each other and that these differences play an important role in the elaborate cell interactions and migrations which even then were known to take place. Caltech Professors Albert Tyler (1946) and Roger Sperry (1951, 1963) have been among those who worked and speculated on the subject of cell-cell interactions. Yet, after more than fifty years of incredible advances since the era of Morgan, the whole subject remains a fascinating mystery.

In previous years we have, together with Lee Hood, used the immune system as a model for studying developmental processes (Dreyer et al., 1967; Hood et al., 1977; Huang and Dreyer, 1978). A most intriguing series of programmed gene cutting and splicing events occurs as the diverse cells of the immune system are generated. Each differentiated cell makes one particular antibody molecule that is the product of an arrangement of spliced genes which exist in that particular cell and which will be replicated in all of its progeny. The initial germline cells contain the same genes but they are not spliced together in the same way as in the differentiated cell (see Biology 1979, Nos. 58, 61, and 62). This somatogenetic process of determination and cellular differentiation appears to occur in the same systematic and reproducible fashion in the immune system of different embryos as they undergo development (Biology 1978, No. 51). The fact that these events are developmentally programmed in this interesting and unexpected manner immediately makes one think of ways in which similar events would help explain the development of other cell lineages. The developing immune system thus does provide a most unexpected and interesting "ghost of an idea" as to how cells become different during embryogenesis, and also how different cell-surface recognition molecules may be formed in different cells: antibody molecules do, after all, function first as cell-surface receptors and the lineage of cells which display these receptors is generated by an elaborate and systematic series of gene cutting and splicing events. Other cell lineages, such as those involved in brain development, also undergo systematic and reproducibly programmed steps of differentiation at precise stages of development. Processes like those of the immune system, including gene splicing, may well play a role in generating other cell lineages--even those which form the human brain.

We are now developing a new research program aimed at studying cell-surface receptors on cell lineages other than those of the immune system. In previous research, tumors of the immune system provided an important means for cloning single differentiated cells from within a large and heterogeneous population. The antigens (idiotypes) displayed on these plasmacytomas differed from each other due to differences in the cell-surface receptors, the antibodies. Tumors of skin cells also differ from each other in antigenic properties (individually specific tumor antigens, ISTA). We are studying antigen molecules displayed on such tumors with the aim of determining the molecular basis for this type of variation. Do the antigenic molecules differ on each skin tumor because they are cell recognition molecules unique to the particular line of differentiation of the original normal cell, as is true for tumors of the immune system? Or is it possible that some sort of abnormal mutational or viral event has made these cells genetically different as a part of the process of carcinogenesis? The program which we are developing is aimed at answering questions like these and, in general, learning more about molecules which are displayed on the surface of differentiated cells.

One important approach to our study of cell-surface antigens on cloned (tumor) cell lines utilizes classical methods such as fractionation and isolation of cellular membranes, fractionation of solubilized membrane glycoproteins by lectin affinity chromatography, and immunoprecipitation combined with two-dimensional gel electrophoresis. One extremely intriguing family of different but related proteins has been identified in the cell-surface glycoprotein fraction. These are glycoproteins with apparent molecular weights of 70 to 90 daltons (gp70-90) and which are related to a large family of molecules generally referred to as gp70 in C-type viruses. Various related molecules have been detected on a wide variety of different normal cell types; however, they are also found as the primary cell-surface recognition molecules exposed on the membranes of C-type viruses. It appears that: (1) many (all?) vertebrates carry several families of genes coding for gp70-90-type molecules (Chattopadhyay et al., 1978; Rowe, 1978); (2) specific different types of gp70-90 molecules appear on different tissues and this expression is under developmental control (Del Villano and Lerner, 1976; Lerner et al., 1976); (3) in mice, most of these genes are expressed systematically even in strains which lack genetic information for producing C-type viruses (McClintock et al., 1977); (4) the isolated molecules show

a high specificity of interaction with cell surfaces (Fowler et al., 1977). While their function in the viruses is known to involve specific cell-surface recognition, the role of these molecules in normal tissues has not been explored experimentally. It should also be noted that one class of gp70-90 molecules found on our tumor cell lines is a "tumor antigen" and may be important in the host's diverse immunological responses to tumor cells. As seen in the abstracts which follow, we are using a number of different experimental approaches in order to answer some of the many questions that can be asked concerning the gp70-90 molecules and the genes which code for them. Since the expression of these related families of molecules is under precise developmental control in the absence of infectious viruses it is possible that they, too, are among those classes of molecules which play a role in cell recognition processes during normal development.

While many laboratories have been interested in molecules of this type, very little is known about them since such molecules (and many other interesting cell-surface proteins) are extremely difficult to isolate in quantities sufficient for conventional types of analysis at the molecular level. It is for this reason that, together with Lee Hood, we continue our program of development of new, powerful, and highly-sensitive methods and an advanced protein chemistry laboratory. This has been a long-term effort which is now beginning to produce very exciting and important results.

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48. ANTIGENS ON THE CELL SURFACE OF UV LIGHT-INDUCED FIBROSARCOMAS

Investigators: **Carol Readhead, Jeanne P. Patalano**

Long exposure to ultraviolet light induces transformation of skin cells in mice and men. In mice, not only does UV light induce fibrosarcomas but, at low levels, it also has an immunosuppressive effect. Animals that would normally reject implanted UV skin tumors are unable to do so if they have been subjected to UV light (Kripke and Fisher, 1976; Daynes et al., 1977). Immunological experiments in vivo and in vitro (see Biology 1978, No. 47 and Kripke, 1974) have shown that UV light-induced fibrosarcomas have both individually specific and cross-reactive antigens. We have been trying to isolate these antigens from the cell surface.

One of the most striking results of these studies of cell-surface proteins from these tumors is the finding of a number of glycoproteins with a molecular weight between 70,000 and 90,000 daltons (gp70-90). Some of these molecules have antigenic determinants in common with the envelope or surface proteins of AKR and/or Rauscher leukemia viruses and they are recognized by antisera to these viruses.

It is not particularly surprising that viral antigens are on the surface of these C3H tumor cells, and recent collaborative studies with Dr. Robert Nowinski have shown that each of these tumor cells expresses an N-ecotropic virus; however, it is surprising to find a number of different families of molecules (at least three) in the gp70-90 range on these tumors.

Alloantisera raised to UV light-induced tumors have antibodies which recognize one subgroup of gp70-90 molecules on the cell surface. Antisera raised to different UV light-induced tumor lines all recognized the same subgroup of antigens. Accordingly, these molecules may be termed cross-reactive tumor antigens.

These results raise a number of interesting questions: Are the several different families of gp70-90 molecules which we have found on these tumors related to differentiation antigens which may be present on the normal stem cells from which these tumors are derived? Are they expressed as a part of the process of carcinogenesis and concordant genetic alteration of normal somatic cells? What is the significance of the N-ecotropic virus(es) that have been isolated from several different tumor lines? Are the gp70-90 molecules on these viruses always identical or do they vary? If such

molecules are expressed on normal skin cells as well as on tumors might this help explain the various phenomena of the immunology of this tumor system such as rejection of transplanted tumors by normal mice, and also the production of specific suppressor T cells (and tumor growth) in animals exposed to UV light?

This project is still in an early phase of development but we believe that the results obtained so far in this molecular level approach suggest that it may generate answers to some of the many provocative questions concerning both tumor immunology and the related topics of fetal and developmental antigens.

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49. SEROLOGICAL ANALYSIS OF TUMOR CELL SURFACE MOLECULES BY MONOCLONAL ANTIBODIES FROM HYBRIDOMAS

Investigators: **David E. Levy, Robert C. Nowinski*, Carol Readhead, Jeanne P. Patalano, Janet M. Roman**

We are investigating the cell surface molecules displayed on a series of UV-irradiation-induced murine fibrosarcomas. Some of these molecules share antigenic determinants with molecules expressed on the membranes of murine C-type retroviruses. We are exploiting the exquisite specificity of monoclonal antibodies to probe the structure of these molecules and to determine the similarities and differences which exist among the various tumors.

Using monoclonal antibodies specific for six different epitopes on the envelope gene products of murine leukemia viruses, we have shown variations in the reactivity patterns among four cultured tumor lines. We are presently analyzing the molecules responsible for this reactivity on the tumors and on the endogenous viral isolates of these tumors. Our aim is to isolate these proteins using specific antibodies and characterize the structural relationships within this family of molecules by peptide mapping and primary sequence analysis. In a related project, we are attempting to extend this analysis of the cell surfaces by producing additional monoclonal antibody reagents from syngeneic and allogeneic animals immunized with tumor cells, tumor fragments, and tumor extracts.

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50. METHODS FOR THE ISOLATION OF MONOCLONAL ANTIBODIES REACTIVE WITH TUMOR ANTIGENS

Investigators: **David E. Levy, Janet M. Roman, John Wen-Kiang Chu**

We are preparing hybridomas from spleen cells of syngeneic and allogeneic mice immunized against C3H fibrosarcomas. The hybridoma technique will allow the selection and amplification of monoclonal antibodies specific for tumor antigens. The unique specificity of each antibody will allow a fine-point analysis of cell-surface molecules to a precision not previously possible due to the limitations of syngeneic immune sera.

One assay system often used for the detection of monoclonal antibodies reactive with surface components involves the attachment of viruses, whole cells, or cell extracts to the wells of microtiter plates. To improve the ease and reproducibility of this assay, we use isolated cell membranes as the antigenic target, thus increasing the antigen concentration in each well and obviating the need for cultured cells. The membranes are prepared from disrupted C3H tumor cells by standard procedures of differential centrifugation. Isolated membranes are then sonicated to create small vesicles which are adsorbed to the test wells. Testing hybridoma culture fluids against a panel of different C3H tumor membranes will allow the identification of clones secreting antibodies that react with cross-reacting or individually specific antigenic determinants.

51. SEROLOGICAL ANALYSIS OF COMMON AND UNIQUE ANTIGENIC DETERMINANTS ON TUMOR CELL SURFACES

Investigators: **Janet M. Roman, David E. Levy, Gayle-Linda Westrate**

From an extensive immunization of C3H mice with syngeneic UV-irradiation-induced fibrosarcomas, we have collected a panel of antisera reactive with tumor cell surface molecules. These sera cross-react with common determinants on different tumor lines and react in an individually specific manner with determinant(s) on each tumor tested to date. We also have a variety of high titer antisera of other types which cross-react with some of the

same tumor antigens. Immunoprecipitations with these sera of detergent-solubilized proteins from [^{35}S]methionine-labeled tumors and cultured tumor lines are being analyzed by polyacrylamide gel electrophoresis to characterize the molecule or molecules responsible for the common and individually specific antigenicity. Previous studies have indicated the likelihood that these antigens are glycoproteins, allowing the judicious use of lectin affinity chromatography on a micro scale to concentrate the antigens, remove interfering proteins, and reduce the background of the tumor lysates. We are also attempting to increase the sera's titer and specificity by preadsorption on glutaraldehyde-fixed tumor cells followed by a "cell-phase" precipitation with this reversed immunoabsorbent. This approach complements our work utilizing monoclonal antibodies and, again, our ultimate goal is the isolation of sufficient quantities of these tumor antigens for peptide mapping and primary sequence analyses to investigate their structural interrelationships. These same experimental procedures will be used to explore the relationship between these tumor molecules and a number of different but related developmental antigens which are expressed in specific patterns on differentiated tissues.

52. INVESTIGATIONS OF INAPPROPRIATE ANTI-H-2 REACTIVITIES OF TUMOR CELLS

Investigators: **Janet M. Roman, Benjamin Bonavida***

We were interested in the numerous reports which indicate that tumor cells display antigenic specificities not detected on normal cells of the same inbred strain of mice. To attempt to explain this puzzling phenomenon we investigated the cell surface molecules on the tumors of the SJL/J strain of mice which reacted serologically and in cell-mediated cytotoxicity experiments with antisera and killer cells directed towards H-2 antigenic specificities normally detectable only on other strains of mice. Radiolabeled molecules from normal SJL/J lymphocytes, from SJL/J tumor cells, and from lymphocytes of the BALB/c strain of mice, were precipitated with antisera appropriate for either the SJL/J strain (H-2^s haplotype) or the BALB/c strain (H-2^d haplotype) and analyzed by two-dimensional gel electrophoresis. As predicted by the serological reactivity of SJL/J tumor cells, patterns of molecules isolated from these tumors very closely resembled the molecules from normal BALB/c lymphocytes. Furthermore, and most unexpectedly, based on serological reactivities with intact cells, H-2 molecules from normal SJL/J lymphocytes also closely resembled those from BALB/c lymphocytes. These results are preliminary, but they raise many questions concerning the polymorphism of H-2 molecules, their presentation in the cell membrane, and their role in tumor-rejection and cell-cell interactions.

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53. CONTINUED DEVELOPMENT OF IMMUNOMICROSPHERES, VERSATILE NEW IMMUNOLOGICAL REAGENTS

Investigators: **Alan Rembaum*, William J. Dreyer**

Labeled antibodies and other molecules with similar biospecific affinities have long been used in biological research to identify or isolate molecules and structures of particular interest. One of the deficiencies of this approach has been the limited amount of a chemical or physical tag that can be attached to each antibody molecule. This amount is often insufficient to produce the required definition. We have greatly increased this specific labeling by incorporating any of a variety of tags into specially synthesized microscopic packets to which antibody or similar molecules are then attached.

These immunomicrospheres have been synthesized to be (1) highly radioactive for ultrasensitive immunoassays; (2) intensely fluorescent, giving increased definition to fluorescence microscopy and better signal-to-noise resolution in cell-sorting instruments; (3) ferromagnetic for a novel approach to the physical separation of cells; (4) highly colored or electron-dense as specific tags in light or electron microscopy; and (5) carrying cytotoxic compounds as a drug delivery system targeted against specific undesirable cells.

These applications are presently being evaluated using various types of synthetic microspheres in conjunction with highly specific monoclonal antibodies derived from hybridomas.

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54. DEVELOPMENT OF AN EXPANDED TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS SYSTEM

Investigators: **William J. Dreyer, David E. Levy, Janet M. Roman, Barbara L. DeOgny**

The combination of isoelectric focusing followed by SDS gel electrophoresis results in an extremely high resolution fractionation of proteins. This system, as originally described by O'Farrell, is being used routinely in our laboratory for the analytical determination and preparative isolation of cell membrane proteins and their proteolytic fragments (Biology 1979, No. 56). Nevertheless, the original system is limited in its generality by a narrow pH gradient, only retaining proteins with isoelectric points within the range of approximately 6-8. The method is further constrained by the resolving power of the molecular weight fractionation dimension. We have found that an expansion of the original 10 x 13 cm gels to a 28 x 28 cm size greatly increases the fractionation range and thus the applicability of the method.

The elongation of the focusing dimension has extended the gradient to encompass a pH range of from less than four to greater than nine. The inclusion of a 10/16% step gradient in the enlarged second dimension has given us increased resolution over a larger fractionation range of approximately 3000 to 100,000 daltons. We have designed and constructed simplified apparatus for casting and electrophoresing ten gels simultaneously, resulting in high reproducibility as well as in a considerable saving of time.

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55. AMINO ACID SEQUENCE ANALYSIS USING GAS-PHASE SEQUENCE TECHNOLOGY

Investigators: **Rodney M. Hewick, Suzanna J. Horvath, David B. Helphrey, Michael W. Hunkapiller, Leroy E. Hood, William J. Dreyer**

A prototype miniaturized gas/solid phase protein sequencer has been built which employs gas-phase reagents at critical points in the Edman reaction. In this approach to automatic sequential degradation, the protein or peptide is immobilized by drying or precipitation onto a porous support held in a cartridge, and chemical bonding is not required. During degradation the material is exposed only to gas-phase reagents or to mild washing with nonpolar solvents so that it remains fixed on the support. The miniaturized reaction cartridge, with reduced surface area, offers the potential for reducing chemical noise in the analytical system used to identify the amino acid derivatives.

Using this system we have sequenced proteins for greater than 50 cycles at the subnanomole level and small peptides to completion at the nanomole level. This instrument takes less than half the time required by a commercial spinning cup sequencer to complete each cycle of degradation and has a greatly reduced rate of reagent consumption.

We are also in the process of building a new gas-phase sequencer which we anticipate will be capable of sequencing less than 100 picomoles of protein or peptide. The various modifications include miniaturized delivery valves to further reduce chemical noise, improved cartridge and solid support design, and a versatile new solid state programmer. The performance of the prototype instrument already surpasses that of commercially available sequenators and the instrument under construction should be far superior. The low chemical background noise and the very small sample requirements are of particular importance as the sensitivity of the related analytical instruments is improved (see Biology 1979, No. 57).

56. SEQUENCE ANALYSIS OF CELL SURFACE MOLECULES OF BIOLOGICAL INTEREST

Investigators: **Rodney M. Hewick, Suzanna J. Horvath, Barbara L. DeOgny, William J. Dreyer**

Our laboratory is particularly interested in the interrelationship between the primary structure of the envelope proteins gp70 and p15(E) of ecotropic, amphotropic, and xenotropic murine leukemia viruses, and in how these relate to the structure of similar molecules located on the cell surface of various mouse tissues.

To study these molecules in detail requires the ability to purify, peptide map, and sequence subnanomole amounts of protein. With this aim in mind, we have concentrated on using high resolution two-dimensional polyacrylamide gel electrophoresis (isoelectric focus-

ing/SDS PAGE) as the final step in the preparative isolation of proteins of interest. Electrophoretic or diffusional elution and precipitation is then used to transfer the proteins directly onto the solid phase support used in the gas/solid-phase sequencing instrument mentioned in the previous abstract, or the proteins may be placed directly in the spinning cup of one of the new instruments of that type which are in routine operation in the protein chemistry facility. Preliminary results with subnanomole quantities of proteins are very encouraging, indicating little loss in sequencing or peptide mapping efficiency after this mode of purification.

57. A NEW, EXTRAORDINARILY SENSITIVE ANALYTICAL SYSTEM FOR RESEARCH AND MEDICINE

Investigators: **Charles E. Giffin*, Heinz G. Boettger*, David D. Norris*, David B. Helphrey, Aron Kuppermann**, Michael W. Hunkapiller, Leroy E. Hood, William J. Dreyer**

In most areas of biochemistry and clinical chemistry, the specific method of instrumental analysis available usually determines the speed, sensitivity, accuracy, manpower requirements, and even the types of data which it is possible to obtain. Mass spectrometers have found wide application in these fields because of their ability (1) to separate virtually any vaporizable molecule or molecular fragment from all those of different molecular weights, (2) to electronically monitor the results in a form compatible with data systems, and (3) to present the results of the analysis in an overall time which rarely exceeds a few minutes and can be much faster. We have not yet used this method in our new protein chemistry facility because commercially available mass spectrometers are neither as sensitive nor as suitable for routine, automated analysis of large numbers of samples as the liquid chromatographic systems now in use.

As reported in several previous years, we have joined with scientists at Caltech's Jet Propulsion Laboratory to conceive, design and construct a new type of mass spectrometer which is at least 1000 times more sensitive than existing instruments. The key innovation is an electro-optical array of ion detectors which acts much like an eye with its array of extraordinarily sensitive photon detectors. Obviously, developments of this type require a number of years before real rewards are evident. Accordingly, those of us who have worked on it are very pleased this year because two different JPL mass spectrometers of this type have been constructed; tests have proven that they surpass design specifications. In the particular application of analysis of amino acid derivatives this means that rather than being restricted to the picomole range (n x 10^{-12} moles) we can now work in the femtomole range (n x 10^{-15} moles). It is already apparent that in other areas of biochemistry and, in particular, in clinical analysis systems, this added factor of a 1000-fold increase in sensitivity will make possible a number of very exciting applications. We expect to make increasingly routine use of this new type of instrument as this long-range program continues.

*Jet Propulsion Laboratory (numerous other members of the JPL staff have contributed substantially to this program).
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Summary: Our laboratory employs protein chemical, recombinant DNA, and immunological techniques to study several complex multigenic families related to the vertebrate immune system.

Antibodies are encoded by three unlinked gene families, two encoding light chains, lambda (λ) and kappa (κ), and the third encoding heavy chains (H). Antibody polypeptides are encoded by three (or more) gene segments--V (variable), J (joining), and C (constant). The V and J gene segments encode the V regions of light and heavy chains which in turn form the antigen-recognition domain. The C gene segments encode those regions of the antibody molecule which trigger the elimination or destruction of the antigen and these are termed the effector domains. There are 8-10 C_H genes that code for the various classes and subclasses of immunoglobulins (e.g., IgM, IgG, and IgA, etc.). These classes have distinct effector functions. We will refer to the protein segments encoded by the V and J gene segments as the V and J segments, respectively, to differentiate them from the V region which is composed of the V plus J segments. These gene segments are rearranged at the DNA level during the differentiation of the antibody-producing or B cell. Accordingly, the antibody gene families provide a unique opportunity to study eukaryotic differentiation because they couple DNA rearrangements directly to differentiation and gene expression.

During the differentiation of the B cell several phenotypic changes occur that can be correlated with DNA rearrangements. First, IgM molecules are expressed initially in B-cell differentiation. Only one V_L and one V_H region (V domain) is expressed in each B cell. If an animal is heterozygous for kappa alleles, in individual B cells only the maternal or the paternal allele is expressed. This phenomenon, termed allelic exclusion, is unique to antibody genes. Second, a B cell or its progeny may shift from the expression of IgM molecules to the synthesis of other classes of immunoglobulins. In a given B-cell lineage, the same V domain is expressed throughout this process of C_H switching. We have used recombinant DNA techniques to analyze heavy chain genes in terms of V-J rearrangements, C_H switching, and allelic exclusion.

The vertebrate organism is capable of synthesizing 10^6 or more different antibody molecules. We also are interested in characterizing the genetic mechanisms responsible for antibody diversity. To this end, we are characterizing three sets of closely-related V regions--those from antibodies binding two simple antigens (haptens), α-1,3 dextran, and phosphorylcholine, and those belonging to a particular group of kappa chains denoted $V_{\kappa 21}$. We are studying these V regions at the protein and DNA levels to assess the relative contributions of multiple germline V gene segments, combinatorial joining of V and J gene segments and somatic mutation to antibody diversity.

Antibody molecules exist in two very different environments--as integral membrane receptors and as hydrophilic serum antibodies. We are characterizing the differences between the membrane-bound and soluble IgM molecules in order to understand the chemical basis for these two different proclivities.

The cell-surface proteins encoded by the major histocompatibility complex (MHC) fall into two distinct categories--transplantation antigens and Ia antigens. Both play fundamental roles in various aspects of the vertebrate immune response. Both are encoded by small multigenic families that are closely linked to one another. Both are extremely polymorphic. We are analyzing the protein diversity patterns of the transplantation antigens and Ia antigens in the mouse in order to gain insights into the gene organization and evolution of these important molecules. We are now in the process of using recombinant DNA techniques to further characterize the MHC or H-2 complex of the mouse.

Our laboratory has developed new microsequence techniques that have permitted us to analyze polypeptides at the 100 picomole level without using radiolabel. Recently we have applied these microsequencing techniques to a variety of medically interesting molecules available in very small quantities including several interferons and neurohormones.

In summary, our conviction is that the mechanisms for differentiation and DNA rearrangement found in the immune system will be shared by other complex eukaryotic systems. We feel that it is unlikely that nature would have evolved sophisticated mechanisms of DNA rearrangements and then use them only in a single system. Thus the antibody gene families pose intriguing questions for other eukaryotic systems such as the major histocompatibility complex.

58. GENERATION OF DIVERSITY IN LIGHT CHAINS

Investigators: **James W. Schilling, Tim Hunkapiller**

The variable region of both light and heavy chains of immunoglobulin molecules is constructed from two independently-assorting portions: the V (variable) segment and J (joining) segment. DNA sequences coding for the V and J segments of light chains are known to exist separately within the genome of undifferentiated (germline) cells. An as yet undetermined mechanism of DNA rearrangement must bring together one J gene segment with one V gene segment to form the continuous, transcriptionally-active variable region unit found in a mature, antibody-producing B cell. This type of combinatorial joining between various J and V gene segments appears to be random with respect to which J and V segments are involved.

Extensive protein sequence analyses carried out in this laboratory on a group ($V_{\kappa 21}$) of closely-related myeloma kappa (κ) chains from NZB mice (Biology 1978, No. 58) has helped to define the role this V-J joining plays in the generation of the diversity exhibited by murine antibodies. Out of 28 partially or completely sequenced $V_{\kappa 21}$ regions, there are at least ten different J segments in association with at least six V subgroups. If the sequence diversity generated by this combinatorial joining of V and J segments is to be considered biologically significant, it should lead to distinct antigen-binding sites. The most variable residue in ten different J segments is at the site of V-J joining, a site that lies within the putative third hypervariable (antigen-binding) region. In addition, recent work indicates that single residue deletions and insertions can also be expressed at the joining point.

The diversity of J segments within this kappa group can be explained by postulating ten different germline J gene segments. Alternatively, we have proposed that there are only four to five germline J gene segments and that the mechanism responsible for V-J joining allows or even promotes intra- as well as intercodon splicing between the immediate, 3' flanking codon of the V gene segment and the 5' codon of the J gene segment as long as the reading frame is conserved. This putative intracodon splicing generates a junction diversity consistent with the observed protein diversity.

Other single residue differences found between the J_{κ} segments and the proposed germline-coded sequences can all be explained as somatic point mutations, none of which reside in the hypervariable region. Their biological significance is clouded by their myeloma origin and the possibility that some variation arose during the multiple passages of these tumors.

Protein sequencing has proven to be invaluable in studying the novel genetic mechanisms associated with antibody production. It has allowed us to place important constraints on proposed models of antibody diversity and to pose realistic questions that can now be approached at the DNA level.

This work has been done in collaboration with Dr. Martin Weigert at the Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania.

Reference:

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59. SEQUENCE STUDIES OF α-1,3 DEXTRAN-BINDING ANTIBODIES

Investigator: **James W. Schilling**

We have continued our study of α-1,3 dextran-binding antibodies. We had previously determined the complete variable region sequence of a dextran-binding myeloma protein, M104E (Biology 1978, No. 53). We now have determined the complete variable region sequence of a second dextran-binding myeloma, J558 and ten homogeneous dextran-binding antibodies derived from hybrid cell lines (hybridomas) of spleen and myeloma cells (Schilling et al., 1979).

A detailed comparison of these twelve sequences leads to several important conclusions: (1) The variable

regions can be divided into three regions comprising residues 1-99, 100-101, and 102-117. We and others have previously shown that light chain variable regions are encoded by two gene segments, termed V and J (Biology 1978, No. 52). Residues 1-99 of the heavy chain appear to constitute the V segment and residues 102-117 the J segment of the heavy chain. Thus far we have identified four V and three J segments in dextran antibodies. (2) Residues 100 and 101 are extremely variable. They do not appear to be encoded directly as part of either the V or J segment. Thus it is possible that the diversity at positions 100 and 101 may arise from somatic variation occurring during V-J joining. Alternatively, these residues may constitute a third gene segment. These questions will be resolved at the DNA level (see Biology 1979, No. 64).

As previously reported (Biology 1978, No. 53), there are two types of idiotypic determinants which have been described in dextran antibodies. Both M104E and J558 have unique determinants, termed IdIM104 and IdIJ558, which correspond to specific pairs of amino acids at positions 100 and 101. A second determinant, termed IdX, is shared by M104E and J558. This determinant appears to involve a carbohydrate attached to residue 55 and possibly adjacent amino acid residues. These observations indicate that caution should be employed with the use of idiotypes for genetic mapping studies because the idiotypes may represent different gene segments (V and J) or even carbohydrate moieties.

These studies have been carried out in collaboration with Drs. Brian Clevinger and Joseph Davie of the School of Medicine, Washington University, St. Louis, Missouri.

Reference:

Schilling, J., Clevinger, B., Davie, J. and Hood, L. (1979) Nature. Submitted for publication.

60. PHOSPHORYLCHOLINE-BINDING ANTIBODIES

Investigator: **Nelson D. Johnson**

We have determined the complete V_H region amino acid sequences of seven heavy chains derived from myeloma proteins binding phosphorylcholine. We have screened nine hybridoma antibodies to phosphorylcholine by partial N-terminal amino acid sequence analysis. Three additional hybridoma antibodies have been extensively sequenced in their V_H regions. The myeloma and hybridoma immunoglobulins generally are quite similar to one another in the sequences of their V regions. We plan to determine the complete amino acid sequences of ten V_H regions from hybridoma antibodies to phosphorylcholine. The diversity patterns of these V regions should provide insights into the phenotypic patterns of variability in these antibodies. These analyses will be useful in posing specific questions for our analysis of this system at the DNA level (see Biology 1979, No. 63).

These studies were carried out in collaboration with Dr. Patricia Gearhart of the Carnegie Institution of Washington, Baltimore, Maryland.

61. IMMUNOGLOBULIN HEAVY CHAIN GENE REARRANGEMENTS. I. V-J JOINING AND THE CONSTANT REGION SWITCH

Investigators: **Mark M. Davis, Kathryn L. Calame**

There are five known classes of antibodies produced by mammalian lymphocytes, IgM, IgG, IgA, IgD, and IgE. The heavy chain constant region determines the class of an antibody and the constant region gene segments are referred to as mu, gamma, alpha, delta, or epsilon, respectively. These heavy chain constant regions mediate immunoglobulin effector functions such as complement fixation which lead to the elimination or destruction of antigens. During the course of differentiation, antibody-producing or B cells are able to switch the class of immunoglobulin they produce without changing their antigen-binding ability or variable region domain. In this report we discuss evidence that this heavy chain switch occurs at the DNA level.

We have analyzed an alpha heavy chain gene, $\alpha 6$, isolated from a partial Eco RI genomic library of DNA from the IgA-producing myeloma tumor, M603. Heteroduplex and restriction analyses demonstrate that the $\alpha 6$ clone consists of 11 kb of 5' flanking sequence, the joined V_H and J_H gene segments, 6.8 kb of intervening DNA sequence, the C_α gene segment, and $\sim$3.5 kb of 3' flanking sequence (Early et al., 1979). We have isolated from undifferentiated or germline DNA (sperm) clones corresponding to the V_H, J_H, and C_α gene segments, none of which are contiguous. The germline V_H clone has $\sim$11 kb of homology with the $\alpha 6$ clone, including the 5' flanking sequence and the V_H but not the J_H gene segment. In the $\alpha 6$ clone the J segment is joined to about 5 kb of the intervening DNA sequence which in the germline is adjacent to the C_μ gene segment. Thus the DNA joining of V_H and J_H gene segments creates a functional μ gene and could lead to expression of antibody of the IgM class. The germline alpha constant region clone accounts for the remaining 2 kb or so of the intervening DNA sequence of

the α6 clone, the C_α gene segment, and the 3' flanking sequence to C_α. Therefore, the α6 clone is derived from three separate regions of the chromosome--the V_H, J_H-C_μ, and C_α gene segments--requiring at least two separate DNA rearrangement events to produce the joined alpha gene. These DNA rearrangements are V-J joining (to specify the V region) and a C_H switch ($C_\mu \rightarrow C_\alpha$) to allow different effector functions to share a given specificity (Davis et al., 1979).

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Davis, M. M., Calame, K., Early, P. W., Livant, D. L., Joho, R. H., Weissman, I. R. and Hood, L. (1979) Nature. Submitted for publication.

Early, P. W., Davis, M. M., Kaback, D. B., Davidson, N. and Hood, L. (1979) Proc. Nat. Acad. Sci. USA **76**: 857.

62. IMMUNOGLOBULIN HEAVY CHAIN GENE REARRANGEMENTS. II. ALLELIC EXCLUSION

Investigators: **Kathryn L. Calame, Mark M. Davis**

We are studying DNA rearrangements which occur during the differentiation of antibody-producing cells and presumably commit a given cell to the expression of a unique antibody molecule. These DNA rearrangements result in the creation of coding regions for variable (V) domains (V-J joining), constant (C) region switching (see preceding abstract), and in allelic exclusion, the phenomenon whereby an antibody-producing cell expresses either the maternal or paternal alleles for a particular immunoglobulin family, but not both alleles. This phenomenon is unique to immunoglobulin genes.

We have compared the forms of the C_α gene segment in germline (sperm) and differentiated (M603, an IgA-producing tumor) DNA by Southern blot analysis. M603 DNA has three forms of the C_α gene segment, none of which are identical to the germline form. These same three forms also occur in the DNA of H8, a tumor line whose V_H region is closely related to that of M603. These three M603 α gene segments have been isolated as Charon 4A clones. The α6 clone has a $V_H + C_\alpha$ gene segments, whereas the α9 and α30 clones appear to have no associated V_H gene segments. The absence of a germline C_α form in the M603 DNA suggests that both maternal and paternal chromosomes coding for heavy chain genes have been rearranged. Thus, allelic exclusion may require DNA rearrangements even for the unexpressed allele.

Restriction enzyme analysis and heteroduplex comparisons of the α6, α9, and α30 clones demonstrate that, although the three forms share 2.7 or more kilobases of homology just 5' to the C_α gene, each clone is distinct in the more 5' regions. We are currently investigating whether there is a universal switch point in the 5' flanking sequence to the C_α gene which may mediate C_α rearrangements leading to α chain expression and allelic exclusion.

Reference:

Davis, M., Early, P., Calame, K., Livant, D. and Hood, L. (1979) In: Eukaryotic Gene Regulation, R. Axel, T. Maniatis and C. F. Fox (Eds.), Vol. VIII. ICN-UCLA Symposia on Molecular and Cellular Biology, Academic Press. In press.

63. STRUCTURAL REARRANGEMENT AND DIVERSITY IN MOUSE IMMUNOGLOBULIN HEAVY CHAIN GENES

Investigators: **Philip W. Early, Henry V. Huang**

The differentiation of antibody-producing cells requires expression of a vast repertoire of immunoglobulin molecules, differing both in antigen specificity and effector functions. We are using the techniques of recombinant DNA to study immunoglobulin genes both in the germline of the mouse and in immunoglobulin-producing cells (Early et al., 1979). One aspect of this work is the study of diversity in the heavy chains of immunoglobulins specifically binding phosphorylcholine. We have shown that the mouse germline contains about ten gene segments homologous to a cloned cDNA probe encoding the antigen-binding portion (variable region) of a phosphorylcholine-binding (PC) heavy chain. By sequence analyses of recombinant clones containing homologous germline and differentiated variable region genes of the PC type, we have determined that a portion of the variable region, important for antigen-binding specificity, is encoded by at least one other independent genetic element in addition to the variable gene segment. In the differentiated cell, a DNA rearrangement has occurred joining the germline V gene segment to the other element (J gene segment), forming the complete heavy chain variable region. This process, called V-J joining, has previously been shown to occur in light chains (Max et al., 1979; Sakano et al., 1979). It now appears to play an even more significant role in the generation of variable region diversity in heavy chains because the J segment and junction region encompass virtually the entire third hypervariable (antigen-binding) region. Interestingly, a short palindromic sequence found adjacent to light chain V and J gene segments in the germline is also found next to the germline heavy chain V gene segment that we have

sequenced. Whatever role this sequence plays in the V-J joining event, it seems to be common to both light and heavy chain gene families. We are currently studying heavy chain J gene segments in the germline to further delineate their significance for variable region diversity and the differentiation of antibody-producing cells.

References:

Early, P. W., Davis, M. M., Kaback, D. B., Davidson, N. and Hood, L. (1979) Proc. Nat. Acad. Sci. USA **76:** 857-861.

Max, E. E., Seidman, J. G. and Leder, P. (1979) Proc. Nat. Acad. Sci. USA. In press.

Sakano, H., Hüppi, K., Heinrich, G. and Tonegawa, S. (1979) Nature. In press.

64. ANTIBODY V REGION DIVERSITY AND V-J JOINING: THE α-1,3 DEXTRAN SYSTEM

Investigator: **Donna L. Livant**

How does an organism generate the enormous V region diversity necessary for an immune response to each of the thousands of unique antigens encountered during its lifetime? Three current hypotheses are that diversity is encoded in the germline genes of the zygote, that diversity arises through somatic mutation of only a few germline genes during the lifetime of the organism, or that diversity arises partly through the encoding of families of V and J gene segments separately in the germline DNA. In the third instance, the combinatorial joining at the DNA level of V gene segments with J gene segments creates unique V genes. Experimental support for the third hypothesis comes both from protein data from this laboratory (Weigert et al., 1978), and from studies of light chain genes in other laboratories (Brach et al., 1978; Sakano et al., 1979).

In order to investigate the third hypothesis in detail, we are currently dissecting at the DNA level the murine response to α-1,3 dextran, a bacterial polysaccharide. Several questions arise. First, recent protein sequence analyses from this laboratory (Schilling et al., 1979) have demonstrated that there are two or more dextran V_H segments as well as three distinct J segments. What is the molecular basis for the V-J joining event? Are there identical palindromes 3' to the V_H sequences and 5' to the J sequences as has been shown for light chains (Sakano et al., 1979; Seidman and Leder, 1979)? These palindromes may form a stem-like structure juxtaposing the V and J gene segments so that site-specific recombination across the base of the stem can join the V and J gene segments, thereby deleting the intervening DNA separating these coding regions in the germline (Sakano et al., 1979). Second, protein sequence analyses also have shown that the region at the V-J junction in dextran antibodies is extremely diverse (Schilling et al., 1979). Does this diversity arise from the V-J joining event itself, or does this region actually derive from yet a third separate family of DNA segments (i.e., D or diversity gene segments)? Finally, what is the overall organization of the dextran V_H region family (V_{HDex}) in germline DNA? How does the germline organization compare with the organization in differentiated DNA? How does V_{HDex} gene organization compare with that of other V region families in the mouse?

In order to begin to answer some of these questions, we have made a cDNA probe to a dextran V region from a cloned M104E cDNA. We are currently screening a mouse library made from sperm (germline) DNA in order to isolate germline V_{HDex} clones. Using DNA from dextran hybridomas made in collaboration with Dr. J. Davie as examples of differentiated DNA, we will construct libraries in Charon 4A, and screen them for V_{HDex} genes. We will sequence the germline clones at the 3'-end of their V regions in order to answer questions concerning V-J joining and V-J junctional diversity. We will use clones from the germline library, as well as clones from the hybridoma libraries to investigate the organization of the V_{HDex} family before and after the V-J joining event.

References:

Brach, C., Hirama, M., Lenhard-Schuller, R. and Tonegawa, S. (1978) Cell **15:** 1.

Sakano, H., Hüppi, K., Heinrich, G. and Tonegawa, S. (1979) Nature. In press.

Schilling, J., Clevinger, B., Davie, J. M. and Hood, L. (1979) Nature. Submitted for publication.

Seidman, J. G. and Leder, P. (1979) Nature. In press.

Weigert, M., Gatmaitan, L., Loh, E., Schilling, J. and Hood, L. (1978) Nature **276:** 785.

65. DO T CELLS EXPRESS IMMUNOGLOBULIN GENES?

Investigator: **Mitchell Kronenberg**

Lymphocytes are divided into two general classes: B cells and T cells. B and T cells differ with respect to expression of cell-surface molecules, migration patterns, and functions in the immune response. It is well established that B cells use immunoglobulin to specifically recognize antigens. While T cells also demonstrate exquisite specificity when presented with antigens, the molecules involved in T-cell antigen recognition are completely uncharacterized. After ten years of con-

troversy, serological and biochemical approaches to T-cell antigen recognition molecules have not yielded definitive results. Interest in this question has been heightened in recent years by the finding that T cells generally do not recognize antigens alone, but must recognize antigen in a complex manner in conjunction with major histocompatibility complex gene products. Moreover, it appears that V_H gene segments are utilized in coding for T-cell receptors.

Our approach to the T-cell receptor problem has been to utilize cloned cDNA probes to test for immunoglobulin gene rearrangement and expression in T cells. DNA from normal T cells and from the T lymphoma Wehi 22 was digested with Eco RI, run on agarose gels, and transferred to nitrocellulose filters. When radioactive probes containing the constant regions of κ light chain and μ and α heavy chain are hybridized to the filters, the fragment sizes containing sequences complementary to these constant regions are identical to those in germline (liver or embryo) DNA. Thus, no evidence for the DNA rearrangement of the C_κ, C_μ, or C_α gene segments has been obtained.

Poly(A)+ total cell RNA from the thymus and from the Wehi 22 tumor has been prepared and will be analyzed for the presence of immunoglobulin sequences using the cDNA probes. We hope to extend these studies by using probes complementary to the V or J gene segments and by analyzing cloned lines of T-effector cells which have recently become available.

66. COMPARISON OF THE STRUCTURE OF SOLUBLE AND MEMBRANE-BOUND MOUSE IgM

Investigators: **Marilyn R. Kehry, Sandra J. Ewald**

The IgM molecule is secreted into the serum as a 19S pentamer and also exists as a membrane-bound 7S monomer on the surface of immature lymphocytes. The membrane IgM is an antigen receptor that triggers small lymphocytes to divide and differentiate into antibody-secreting plasma cells. This class of molecules can therefore exist in soluble form and apparently as an integral membrane protein. We are examining how these dual functions are carried out by comparing the detailed structure of secreted IgM heavy (μ) chains with 7S membrane-bound μ chains derived from the same cell line.

We have purified radiolabeled membrane-bound μ chains from the nonsecreting mouse B lymphocyte cell line Wehi 279. The cyanogen bromide fragments of these membrane-associated μ chains have been isolated and their NH_2-terminal sequences determined by microsequencing methods. Secreted 19S Wehi 279 IgM was isolated from a cell line created by fusion of the mouse myeloma MPC 11 (secreting IgG-2b) and the Wehi 279 lymphoma (hybrid cells a gift of W. Raschke, Salk Institute). Cyanogen bromide fragments from these secreted μ chains have also been purified and are being examined by microsequence analysis at their NH_2-termini. These sequencing studies have allowed us to identify the variable and constant region peptides by direct comparison with the sequence of the mouse myeloma μ chain MOPC 104E (Kehry et al., 1979). The membrane-associated μ chains appear to be lacking a COOH-terminal cyanogen bromide cleavage site that is present in the μ chains secreted by the Wehi 279 hybrid cell line. Since this COOH-terminal region is where we expect the μ chain to interact with the cell membrane, we are determining a partial amino acid sequence of the COOH-terminal cyanogen bromide fragment from membrane-bound μ chains in order to compare it with its counterpart from the secreted μ chains. This structural characterization of the antigen receptor on B lymphocytes may indicate how a membrane-bound receptor interacts with the cell surface to trigger proliferation and differentiation of the immature lymphocyte.

Reference:

Kehry, M., Sibley, C., Fuhrman, J., Schilling, J. and Hood, L. (1979) Proc. Nat. Acad. Sci. USA **76**: 2932-2936.

67. SYNTHESIS OF A MEMBRANE-BOUND IgM

Investigators: **Sandra J. Ewald, Marilyn R. Kehry**

The mouse lymphoma B-cell line, Wehi 279, synthesizes a membrane-bound 7S IgM molecule. Two forms of the heavy (μ) chain of this immunoglobulin are found in Wehi 279 cells when they are grown in radiolabeled amino acids for up to 18 hr. The two forms of μ chain differ in molecular weight and charge. By labeling only the cell-surface proteins or the complex carbohydrate structures of cell-surface molecules, we have determined that the higher molecular weight and more acidic form of μ chain is present on the cell surface and the other form of μ chain is present inside the cell. Pulse-chase studies indicate that the intracellular μ chain (μ_i) may be a precursor to the membrane-bound μ chain (μ_m).

The cyanogen bromide peptides of μ_i and μ_m have

been isolated and their amino terminal sequences determined by microsequencing methods (see Biology 1976, No. 134). We find that the polypeptide structures of μ_m and μ_i are identical. The differences in molecular weight (and probably charge) seem to be due entirely to glycosylation differences in the complex carbohydrate structures. These studies are important not only from the standpoint of the cellular physiology of these molecules, but also because such preliminary characterization is necessary for the detailed biochemical analysis of membrane IgM (see Biology 1979, No. 66).

These studies are being carried out in collaboration with Dr. C. Sibley at the Department of Genetics, University of Washington, Seattle.

Reference:

Kehry, M., Sibley, C., Fuhrman, J., Schilling, J. and Hood, L. Proc. Nat. Acad. Sci. USA **76:** 2932-2936.

68. ISOLATION OF THE mRNA OF MEMBRANE-BOUND MU CHAINS

Investigator: **Martha W. Bond**

Immunoglobulin M (IgM) functions in two ways. It is a serum antibody molecule, being both the first immunoglobulin class expressed in the organism's development and the predominant class in the primary immune response to antigenic stimulation. It also is present as a membrane-bound protein on the surface of unstimulated B lymphocytes and is believed to be the antigen-specific receptor that triggers B-cell proliferation and differentiation necessary for antibody synthesis (Kettman et al., 1979). Structural studies of both the membrane (m) and the secreted (s) forms indicate that these proteins differ in the carboxyl terminus of the heavy chain constant region (C_μ), i.e., at the locus of attachment to the cell surface for m-IgM (Kehry et al., 1979; M. Kehry, unpublished results).

An important question is how does the cell synthesize these two proteins which are identical over much of their structure but are expressed at different stages of B-cell development and have radically different functions? Do μ_m and μ_s chains arise from the post-translational modification of the same precursor protein? The available sequence data are somewhat ambiguous on this question. Are they encoded by two separate C_μ genes which differ at the 3'-ends under the influence of different regulatory elements? The data thus far available on the germline arrangement of the heavy chain C genes provide no evidence for more than one C_μ gene (Davis et al., 1979). Do they arise from the same initial RNA transcript which is then processed differentially? Or are they products of DNA rearrangements of the same C_μ gene element with two DNA sequences separately encoding the carboxyl termini of the secreted and membrane-bound chains (Bernard et al., 1978)?

In order to answer these questions we are currently isolating the mRNA for mC_μ from a B-cell lymphoma line (Wehi 279) which has μ on its surface but does not secrete IgM. The poly(A)+ RNA fraction from these cells has been prepared. The cDNA copies are being synthesized, cloned, and screened with a restriction fragment complementary to the 3'-end of a sC_μ derived from a myeloma tumor (MOPC 104E) (D. L. Livant, unpublished results). The nucleic acid sequence of the 3' portion of the mC_μ mRNA will be determined and compared to that of cDNA copies of sC_μ mRNA. The mC_μ cDNA also can be used as a probe of genomic libraries of both germline (sperm) and differentiated (lymphoma) DNA to ascertain whether the amino acid sequence differences are due to post-translational modifications, RNA processing, or DNA rearrangement.

References:

Bernard, O., Hozumi, N. and Tonegawa, S. (1978) Cell **15:** 1133.

Davis, M., Calame, K., Early, P., Livant, D., Joho, R., Weissman, I. and Hood, L. (1979) Nature. Submitted for publication.

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Kettman, J. R., Cambier, J. C., Uhr, J. W., Ligler, F. and Vitetta, E. S. (1979) Immunol. Rev. **43:** 69.

69. CHEMICAL CHARACTERIZATION OF Ia ANTIGENS OF THE MOUSE MAJOR HISTOCOMPATIBILITY COMPLEX

Investigator: **Minnie McMillan**

The Ia antigens are encoded in the I region of the major histocompatibility complex (H-2) of the mouse. The I region controls a series of phenotypic traits which are intimately involved in the immune response. By recombinational analysis the I region has been divided into five subregions, I-A, I-B, I-J, I-E, and I-C.

The only gene products from the I region which have been directly identified are the Ia antigens. These molecules are expressed predominantly on B lymphocytes and are integral membrane proteins which appear to be highly polymorphic by serological analysis. They are composed of at least two subunits of approximate molec-

ular weights 35,000 (α) and 28,000 (β). Distinct Ia molecules are encoded by the I-A and I-E subregions.

I have developed a method for analyzing radiolabeled tryptic peptides of integral membrane proteins, such as Ia antigens, using high-pressure liquid chromatography (McMillan et al., 1979). This technique has several advantages over conventional chromatography. It is 10-20 times faster, has excellent resolution, and gives good yields. It has high reproducibility so that tryptic peptide maps can be compared on polypeptides which have not been prepared and digested at the same time.

I have used the technique to compare the tryptic peptides of the α and β polypeptides of Ia molecules from two differing haplotypes which are encoded by the I-A and I-E subregions. I have also determined the partial N-terminal amino acid sequences of these Ia polypeptides using a microsequence technique developed in this laboratory.

Several preliminary conclusions about the evolution and organization of Ia genes can be drawn from these tryptic peptide maps and partial N-terminal sequence analyses of B-cell Ia antigens. Certain constraints also can be placed on the functional role of Ia antigens.

(1) The E_α polypeptides show striking homology with the human α chains. Likewise, the E_β polypeptides also demonstrate some homology to their human counterpart. These homology relationships suggest that the corresponding genes in the I region (or D locus in the case of man) have descended from a common ancestral gene.

(2) The A_α, A_β, and E_β polypeptides show multiple haplotype-associated peptide and amino acid differences. This observation implies that the genes (structural or regulatory) controlling the synthesis of these polypeptides are located within the H-2 complex, since the molecules are isolated from congenic mice, which have distinct H-2 complexes superimposed on genetically identical B10 backgrounds.

(3) The peptide map data suggest that the diversity of the Ia polypeptides is limited. This observation is inconsistent with the supposition that the Ia antigens constitute a diverse receptor system.

I am currently extending these studies to determine the primary structure of the A_α and A_β polypeptides of Ia molecules of the $H\text{-}2^k$ haplotype.

These experiments are being carried out in collaboration with Dr. H. O. McDevitt of the Stanford Medical School, Stanford, California and Dr. D. B. Murphy of the Yale School of Medicine, New Haven, Connecticut.

Reference:

McMillan, M., Cecka, J. M., Hood, L., Murphy, D. B. and McDevitt, H. O. (1979) Nature **277**: 663-665.

70. PRIMARY STRUCTURE ANALYSIS OF HUMAN AND MOUSE INTERFERONS

Investigator: **Michael W. Hunkapiller**

Interferons are small proteins secreted by mammalian cells infected by virus that render nearby cells resistant to the viral infection. They are active against a wide spectrum of viruses, and the lack of significant side effects with interferon therapy makes it a promising treatment for such diseases as hepatitis B, herpes zoster, and rabies, as well as for prophylaxis or treatment of viral infections in patients experiencing immunosuppression associated with cancer therapy or organ transplants. Since interferons, in addition to possessing this antiviral activity, also inhibit cell proliferation, they have aroused considerable interest as a potential treatment for certain cancers. Clinical trials in Sweden have shown a doubling of the survival rate with post-operative interferon treatment, compared to standard chemotherapy, for victims of osteogenic sarcoma. Similar results have been obtained in preliminary trials with multiple myeloma, melanoma, and non-Hodgkin's lymphomas.

Exploration of interferon's potential as a therapeutic agent has been hindered by the small amounts of the interferons available. Human leukocytes obtained from blood donors have so far been the chief source of interferon used in the clinical studies, although production facilities using either human fibroblasts or a human lymphoblastoid cell line have been started. Production of sufficient interferon for use in routine medical treatment, however, awaits detailed structural analysis that will allow its synthesis by chemical or recombinant DNA techniques.

The scarcity of the interferons and the difficulties inherent in their purification have thwarted their chemical characterization by classical methods of protein sequence analysis. Therefore, we have begun, using the microsequencing techniques developed in this laboratory (see Biology 1978, No. 67, and Hunkapiller and Hood, 1978), analysis of the primary structure of interferons from three sources: human fibroblast (Ernest Knight, E. I.

Du Pont de Nemours), human lymphoblastoid (Christian Anfinsen, NIH), and mouse Ehrlich cell (Peter Lengyel, Yale). N-terminal sequence runs have been performed using highly purified fibroblast (1.6 μg), lymphoblastoid (6 μg), and mouse (10 μg) interferon preparations.

Reference:

Hunkapiller, M. W. and Hood, L. E. (1978) Biochemistry **17:** 2124-2133.

71. A SIMPLE CHEMICAL CLEAVAGE FOR TRYPTOPHANYL PEPTIDE BONDS

Investigator: **Henry V. Huang**

Tryptophan is a relatively rare amino acid in proteins. Where it occurs, it is generally more conserved than any other amino acid (Dayhoff, 1976). Thus cleavage of a protein at tryptophan residues will usually generate a few large peptides, which may be useful for sequence studies using newly-developed sequenators with potential for extended runs (Hunkapiller and Hood, 1978). Further, a series of homologous proteins cleaved at tryptophan residues will produce similar sets of peptides when the peptides are separated by size. This will facilitate the comparative studies of homologous proteins.

We have determined the optimal conditions for the specific cleavage of proteins at tryptophanyl residues using a novel chemical method. This method employs the oxidation of tryptophan residues using dimethyl sulfoxide-hydrochloric acid and subsequent cleavage using cyanogen bromide. Cleavage efficiencies of 60 to 67% were obtained using human serum albumin as a model protein. The number and sizes of the peptides obtained from a panel of proteins cleaved with this method were exactly those predicted from their known amino acid sequences. No cleavage at methionines was detectable under these conditions, and there does not seem to be any neighbor-residue inhibition of cleavage at tryptophan using this method.

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Summary: The abstract by Hobby et al. that follows is probably the last that will appear in the Biology Annual Report on our attempts to simulate the results obtained on the surface of Mars by the Carbon Assimilation experiment. The Viking findings, which implied a small synthesis of organic matter from atmospheric carbon, were very surprising in view of the highly oxidizing nature of the Martian surface. The reaction was too thermostable to be considered biological; other Viking data also made it extremely unlikely that Mars is a habitat of life. Laboratory experiments summarized below suggest that reactions on the surface of iron-containing minerals are responsible for the Martian results. Gaps still remain in our reconstruction of the Martian events, but it is unlikely that they will be filled without further information on the chemistry and mineralogy of the surface of the planet.

Our studies of iron transport in Neurospora crassa are an outgrowth of the Mars Project, as explained in last year's summary. These studies have taken on a life of their own now. We report here on the isolation of mutants that are defective in the ability to transport the cyclic peptides (siderophores) that are produced by Neurospora and that are responsible for chelating and solubilizing ferric acid in the medium.

72. LABORATORY SIMULATIONS OF VIKING RESULTS

Investigators: **George L. Hobby*, Norman H. Horowitz, Jerry S. Hubbard****

The evidence is strong that iron oxides compose a significant fraction of the Martian surface material at the two Viking landing sites, and it is likely that clay minerals

or their degradation products are also present (Toulmin et al., 1977). Maghemite, or γ-hematite, is the most plausible magnetic component of the Martian surface fines (Hargraves et al., 1977). Adsorbed ammonia is also a possible component, since it was a major exhaust product of the lander descent engines (Husted et al., 1977).

The Carbon Assimilation, or Pyrolytic Release, experiment detected a weak, but significant, fixation of atmospheric CO/CO_2 by Martian surface material (Horowitz et al., 1977). Laboratory simulations show that carbon fixation comparable with that found on Mars is obtained with magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3) plus water vapor or ammonia, hematite (α-Fe_2O_3) plus water vapor, and montmorillonite enriched with ferric and ferrous iron (Hubbard, in press).

The absorptive capacity of the surface fines for CO + CO_2 was another Martian parameter measured by the Carbon Assimilation experiment. The Martian levels are mimicked by maghemite, hematite, and iron-enriched montmorillonite. Adsorption by magnetite is higher by an order of magnitude, however. The addition of water vapor to Martian samples enhanced the absorptive capacity, but this was not the case for any of the minerals. Addition of water vapor to the Martian samples furthermore had either no or an apparently inhibiting effect on carbon fixation, but it produced significant increases in fixation by the mineral species.

The results suggest that iron-rich minerals may be responsible for the activity observed in the Carbon Assimilation experiment on Mars, but the conditions of the laboratory simulations are incomplete. Obvious gaps in our knowledge include the actual Martian mineralogy and the identity and properties of the oxidant detected on Mars by other Viking experiments.

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73. ISOLATION OF NEUROSPORA MUTANTS DEFICIENT IN SIDEROPHORE TRANSPORT

Investigators: **Gisela Charlang, Norma P. Williams**

One approach to understanding the iron-transport system of Neurospora crassa is through the study of mutants defective for siderophore transport. We have used the filtration-enrichment method for isolating such mutants. Since wild-type conidia contain large stores of the siderophores ferricrocin and ferrichrome C (Horowitz et al., 1976), only a fraction of which can be removed without severe reduction in conidial viability, it was necessary to use a mutant blocked in siderophore synthesis for this purpose. We used the ornithine-deficient triple mutant constructed by Professor R. Davis (see Biology 1978, No. 70). Since the siderophores of Neurospora contain ornithine as an essential component, this mutant can be made siderophore-deficient by growing it on an ornithine-free medium with ascorbate present to maintain iron in the ferrous state. (Ferrous iron can be taken up without the intervention of siderophores.) Conidia from such cultures were mutagenized with UV and inoculated into glycerol liquid medium supplemented with ferricrocin. Conidia that germinated were removed by filtration. Ungerminated conidia were plated on solid medium containing ornithine, and 1000 presumptive mutants were isolated. These were subsequently tested for their ability to grow in liquid glycerol medium with ferricrocin. Eleven were found to be unable to utilize ferricrocin.

Reference:

Horowitz, N. H., Charlang, G., Horn, G. and Williams, N. P. (1976) J. Bacteriol. **127:** 135-140.

74. STUDIES OF SIDEROPHORE TRANSPORT IN NEUROSPORA

Investigator: **Gisela Charlang**

Conidia of Neurospora crassa develop a siderophore transport system before germination has been completed. In a medium containing glycerol as carbon source less than 10% of the conidia germinate in 3 hr at 30°C, but the siderophore uptake system is fully developed. Cycloheximide prevents appearance of the system, indicating that protein synthesis is required.

Kinetic studies in progress using ^{3}H-labeled siderophores show that the ornithineless triple mutant of N. crassa has K_m values for the uptake of ferricrocin and coprogen similar to those of wild type. Values of V_{max},

however, are greater in wild type than in the mutant. Eleven mutants deficient in siderophore utilization (see preceding summary) were tested for uptake of [^{3}H]ferricrocin and [^{14}C]phenylalanine. Five of the mutants were found to be deficient in ferricrocin transport and normal in phenylalanine transport. The other six were deficient in both uptakes and are therefore of no immediate interest in the present study. Measurements to determine K_m and V_{max} of ferricrocin and coprogen transport of the mutants are in progress.

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Research Fellows: Eugene T. Butler III, Edward F. Fritsch, Ross C. Hardison, Richard M. Lawn, C.-K. James Shen

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Research Staff: Catherine D. O'Connell, Diana H. Quon**

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Summary: During the past year, we have continued our studies on the structure and organization of mammalian globin genes using molecular cloning procedures. In the rabbit system, we have characterized four different, closely-linked β-like globin genes. Two of the genes hybridize to a cloned adult β-globin cDNA probe, while the other two genes are expressed during embryonic development. The structure of each gene, including the location and size of their intervening sequences was determined. In addition, the linkage arrangement and transcriptional orientation of the four genes were established. We have recently begun to study the transcription pattern of these genes using sensitive RNA-DNA hybridization procedures. A 15S nuclear RNA precursor of the adult rabbit β-globin mRNA has been detected using these procedures.

We have also examined the arrangement of repeated DNA sequences in the rabbit β-like globin gene cluster using two-dimensional blotting-hybridization procedures and heteroduplex analysis. These experiments have revealed an unexpectedly complex pattern of repeats consisting of over 20 different pairs of cross-hybridizing sequences within the 44 kb gene cluster. Both inverted and tandem repeat sequences were detected by electron microscopy. Although the functional significance of these sequences is not understood, it is interesting that one set of these repeat sequences hybridizes to a cDNA clone corresponding to an expressed repeat sequence. The analysis of repeat sequences in this gene cluster has also revealed that regions within the large intervening sequences of the adult β-globin gene hybridize to regions which map to the 5' side of the gene.

Our studies of the human globin gene family have also concentrated on the characterization of linked gene clusters. Last year we reported the isolation of bacteriophage λ clones containing both the δ- and β-globin genes. During the past year we have isolated and characterized a set of clones containing the fetal β-like globin genes, $^G\gamma$ and $^A\gamma$, and have established the linkage arrangements between these genes and the adult β-like globin genes. Recently, we have isolated at least one embryonic β-like gene, ε. Analysis of overlapping clones indicates that this gene is located on the 5' side of the $^G\gamma$-globin gene. Thus, we have mapped over 70 kb of chromosomal DNA containing five different β-like globin genes.

We have recently begun experiments to localize both naturally-occurring and in vitro-generated mutations which affect expression of β-globin genes. To establish a basis for comparison we are determining the complete nucleotide sequence of the normal β-globin gene. This

work is being carried out in collaboration with Argiris Efstratiadis and members of his laboratory at the Harvard Medical School. We have also begun to study the nature of naturally-occurring deletions in human β-like globin gene cluster as a means of identifying sequences which are necessary for the "switch" from fetal to adult globin gene expression. We have mapped the endpoints of a number of such deletions using the Southern blotting procedure and well-characterized subclones of genomic DNA as hybridization probes. This study revealed interesting differences between deletions which differentially affect expression of fetal globin genes in adults.

Analysis of human α-globin genes was also initiated this year. Two bacteriophage λ clones were isolated which contained both of the adult α-globin genes as well as genes which may correspond to the embryonic ζ chains. Thus, our efforts to isolate and characterize the entire human globin gene family are nearing completion. These clones should provide valuable tools for analyzing the expression of globin genes in normal and thalassemic individuals.

Another approach to the study of globin gene expression is to mutagenize sequences within and surrounding globin genes in vitro and then to assay the affect of this change by transcription in vivo or in vitro. To this end, we have been collaborating with Richard Axel's lab at Columbia University to introduce cloned globin genes into mammalian cells in culture. Initial experiments look promising since it was possible to transfer stably a cloned rabbit β-globin gene into a TK^- mouse L cell and to show that this gene is transcribed and processed.

Genetic transformation experiments are also being attempted using the alcohol dehydrogenase (Adh) system of Drosophila melanogaster. The Adh gene is developmentally regulated and its expression is tissue specific. The gene was isolated on the basis of differential hybridization to RNA from normal and mutant flies and is being structurally characterized. Adh was chosen because of the wealth of genetic information available and the fact that well-defined genetic selection procedures have been established. The primary objective of this project is to alter the cloned Adh sequences in vitro and to determine the effect of this mutagenesis on the developmental expression of these genes in vivo.

75. CHARACTERIZATION OF ADULT RABBIT β-GLOBIN GENES

Investigators: **Elizabeth H. Lacy, Ross C. Hardison, Diana H. Quon**

We have obtained nine clones containing sequences homologous to adult rabbit globin mRNA from a screen of the rabbit library. Four of these clones (designated RβG1, RβG2, RβG3, and RβG5) hybridize strongly to a plasmid carrying a cDNA copy of adult β-globin mRNA (Maniatis et al., 1976). Restriction mapping and hybridization experiments on the DNA from these four clones have shown that they do, in fact, contain the adult β-globin gene, which we have designated as gene β1. Furthermore, we found that clone RβG2 contains a second β-globin gene (β2) which lies approximately 8.5 kb 5' to gene β1 and that clone RβG5 contains a third β-globin gene which lies approximately 4.5 kb 5' to gene β2 (Lacy et al., 1979). The other five clones hybridize more strongly to a human γ-globin cDNA plasmid (Wilson et al., 1978) than to the rabbit β-globin cDNA plasmid. Restriction mapping and hybridization experiments have shown that these latter clones overlap with the four clones containing the adult β-globin gene (see Biology 1979, No. 76). In fact, each one of these five clones contains part or all of the third β-globin gene in RβG5.

Preliminary data suggest that gene β2 may code for a minor adult β-globin polypeptide. RNA blotting (Alwine et al., 1977) experiments have shown that gene β2 is more homologous to adult β-globin mRNA than to embryonic β-globin mRNA. Transcripts of β2 have been detected in adult rabbit reticulocyte and bone marrow RNA in a modified Berk-Sharp analysis (Berk and Sharp, 1977). Using hybridization probes containing either the 5' or 3' coding regions of the rabbit adult β-globin gene, we have determined that genes β1 and β2 are transcribed from the same strand of DNA. However, initial characterization of β2 suggests that its structure may differ slightly from that of gene β1. Nucleotide sequence analysis of the adult β-globin gene (Weissman et al., 1979; Efstratiadis, Lacy and Maniatis, in preparation) has shown that the large intervening sequence (Jeffreys and Flavell, 1977) located between amino acid codons 104 and 105 is 573 bp in length. Also, the large intervening sequence is flanked by a 5' Bam HI site and a 3' Eco RI site. Gene 2, on the other hand, contains a large intervening sequence that is approximately 730 bp in length and that is flanked by a 5' Bam site and a 3' Hind III site. Unlike gene β1 and genes

β3 and β4 (see Biology 1979, No. 76), gene 2 does not contain a RI site on the 3' side of the large intervening sequence. The Bam-RI fragment containing the β1 intervening sequence does not hybridize to the intervening sequence in gene 2 in Southern blotting hybridization experiments. Therefore, the two intervening sequences share little homology. A second intervening sequence (126 bp) has been located in β1 between amino acid codons 30 and 31 (van den Berg et al., 1978; Efstratiadis, Lacy and Maniatis, in preparation). No attempt has yet been made to locate a second intervening sequence in gene 2.

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76. ISOLATION, LINKAGE ARRANGEMENT, AND STRUCTURE OF RABBIT EMBRYONIC β-LIKE GLOBIN GENES

Investigators: **Ross C. Hardison, Eugene T. Butler III, Elizabeth H. Lacy, Diana H. Quon**

The molecular basis for the regulated ontogenetic expression of globin genes is being investigated using isolated, cloned fragments of the rabbit genome containing most of the β-like globin gene cluster. A set of nine overlapping clones containing a total of four β-like globin genes was isolated from a large collection of recombinant phage with randomly-generated inserts of rabbit genomic DNA (Maniatis et al., 1978). Four of the clones contain the major adult β-globin gene with another adult β-like globin gene; they are discussed by Lacy et al. (see Biology 1979, No. 75). The other five genomic clones also contain two β-related globin genes and were shown to overlap the adult β-globin clones by restriction mapping data. These two β-like genes encode embryonic globin polypeptides, as shown by their specific hybridization to embryonic globin mRNA, and are designated genes β3 and β4. Hybridization of the embryonic globin gene regions of cloned DNA with probes specific for the 5'- or 3'-ends of human γ-globin mRNA (Wilson et al., 1978), a related β-like globin, demonstrated that both globin genes are transcribed from the same strand of DNA. The four β-related globin genes are contained in a 40 kb region of the chromosome, each gene separated by 6 to 8 kb. All four genes are transcribed from the same strand of DNA and are arranged in the order 5' β4-β3-β2-β1 3'.

A large 800 bp noncoding sequence interrupting the coding region of both embryonic globin genes was found by examining hybrids of the cloned genes with embryonic globin mRNA (R-loops) in the electron microscope. A second, small intervening sequence of about 100 bp in gene β4 was revealed by a comparison of the restriction maps of the cloned gene and a cDNA plasmid constructed from β4 mRNA (see Biology 1979, No. 77). Although data are not yet available on a small intervening sequence in β3, it appears that the general structure of the embryonic globin genes closely resembles that elucidated for mouse, rabbit, human, and chicken adult β-globin genes. That is, the genes are encoded in three discontinuous segments separated by a small and large intervening sequence. Analysis of heteroduplexes formed between subclones containing all but the 3'-ends of the embryonic globin genes shows that the region of homology is limited to the 5' coding region. Thus, the 5' flanking region and intervening sequences are not homologous, although short regions of homology could be juxtaposed to the 5' coding region.

Preliminary blot-hybridization data suggests that at least one or possibly two more embryonic globin genes are present in rabbit chromosomal DNA. We are attempting to identify and isolate these genes and determine their linkage relationship to the β1 → β4 globin gene cluster. Further work on this system will focus on identifying transcribed regions of the gene cluster and elucidating processing pathways for the transcripts. We also will measure extents of homology in the coding and noncoding regions of the rabbit, human, and mouse β-related genes.

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77. CLONING AND CHARACTERIZATION OF DNA SEQUENCES COMPLEMENTARY TO EMBRYONIC RABBIT GLOBIN mRNA

Investigator: **Eugene T. Butler III**

The construction of embryonic rabbit globin cDNA clones was undertaken to provide specific hybridization probes for a study of the transcription of embryonic and adult globin gene clusters during development. Such clones, being representative of the end products of RNA splicing processes, would also facilitate the detection of small intervening sequences within the chromosomal copies of the embryonic globin genes.

The starting material for the preparation of embryonic globin cDNA clones was mRNA isolated from the blood islands of rabbit embryos between the eleventh and twelfth days of gestation. Rabbit blood island mRNA/cDNA hybrids were prepared using avian myeloblastosis virus RNA-dependent DNA polymerase (Myers and Spiegelman, 1978) and the mRNA/cDNA hybrids were inserted into a bacterial plasmid via the annealing of complementary homopolymer tracts added to the 3'-ends of the mRNA/cDNA hybrids and the plasmid vehicle using calf thymus terminal deoxynucleotidyl transferase (Wood and Lee, 1976; Zain et al., 1979). Synthesis of the second DNA strand of the inserts and their covalent ligation to the plasmid vector were expected to occur in vivo. Following the transformation of bacterial host cells and a drug selection for transformants, bacterial colonies were sought which carry plasmids that hybridized preferentially to regions of the β-globin gene cluster which were homologous to embryonic globin mRNA. Thus, cDNA clones complementary to each of the putative embryonic globin regions (see Biology 1979, No. 76) have been isolated. In addition, two other β-like cDNA clones have been identified which may correspond to more embryonic globin genes which have yet to be isolated. The screening of plasmids likely to bear embryonic rabbit α-like globin cDNA is in progress.

A cDNA clone, pRβ'G4, corresponding to gene β4 has been the most thoroughly characterized. Comparison of restriction maps of pRβ'G4 and a subclone of the β4-globin region confirmed the presence of a large intervening sequence in the gene (see Biology 1979, No. 76) and also revealed the existence of a small intervening sequence near the 5'-end of the gene. Determination of the nucleotide sequence of the cDNA clones and the genes from which their corresponding mRNAs were transcribed is being carried out to identify intron/coding sequence junctions and to verify that the embryonic globin cDNA clones correspond to the globin mRNAs which are translated in the embryonic rabbit reticulocytes.

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78. CLONING AND STUDY OF A REPETITIVE SEQUENCE REPRESENTED IN THE RNA OF EMBRYONIC RABBIT BLOOD ISLAND

Investigator: **Eugene T. Butler III**

In the course of screening recombinant cDNA plasmids for embryonic rabbit globin-specific sequences, a clone, designated h29, was detected which hybridizes to non-globin-coding regions of the rabbit β-globin cluster. Results of Southern transfer and hybridization experiments indicate that the cloned sequence in h29 bears homology with repeat family D2 of the rabbit β-globin region defined by Shen (Biology 1979, No. 79). Results of preliminary RNA gel transfer and hybridization experiments (Alwine et al., 1977) verify that the sequence represented in h29 is indeed transcribed and suggest that the sequence may be more abundant in the RNA extracted from embryonic rabbit blood islands than in that from bone marrow of adult rabbits. Liquid hybridization experiments are being undertaken to determine the subcellular distribution of h29 transcripts and the reiteration frequency of the h29 sequence in the rabbit genome. Questions pertaining to the symmetry of transcription of the h29 sequence and its possible linkage to single copy cytoplasmic transcripts are being addressed through construction of single-stranded hybridization probes by the subcloning of h29 on the single-stranded bacteriophage M13. We intend to determine if patterns of transcription of h29 may be correlated with states of cell differentiation in general and/or with globin gene expression in particular.

Reference:

Alwine, J., Kemp, D. and Stark, G. (1977) Proc. Nat. Acad. Sci. USA **74:** 5350-5354.

79. CHARACTERIZATION OF REPEAT SEQUENCES IN THE RABBIT β-LIKE GLOBIN GENE CLUSTER

Investigator: **C.-K. James Shen**

Regions of sequence homology within a 44 kb segment of rabbit chromosomal DNA containing four developmentally regulated β-like globin genes (β1, β2, β3, and β4) were mapped. This DNA segment was isolated in the form of overlapping bacteriophage recombinants from a library of rabbit liver DNA (see Biology 1979, Nos. 75 and 76). At least 20 sequences which are found more than once within the gene cluster were identified by using a two-dimensional electrophoresis-blotting-hybridization technique (2D-EBH) (Sato et al., 1977) as well as by the conventional Southern hybridization procedure (Southern, 1975). The Eco RI fragments of the gene cluster were classified into five groups (A, B, C, D, and E) according to the repeat elements they contain. Under the conditions used, the members of each group form stable hybrids with each other but not with the members of other groups.

Electron microscopic visualization of double-stranded regions formed by intrastrand base pairing on single strands of cloned DNA and of heteroduplexes between clones containing different regions of the gene cluster was used to determine the size, location, and relative orientation of the repeat sequences. Both direct and inverted repeats which range in size from 140 bp to 1.4 kb were detected. There are inverted repeats flanking one, two, three, or all four of the linked globin genes. Group B contains at least three repeated sequences, probably arranged in the same orientation (direct repeats) located in the region 4 to 7 kb to the 3'-end of β1, the gene which encodes the major β-globin polypeptide. Based on the size of the repeated sequences, groups C and D have been further divided into subgroups C_1, C_2, and D_1, D_2, D_3. The common repeat element of each of the subgroups is 280 bp, 190 bp, 1.4 kb, 420 bp, and 140 bp, respectively. Both groups C and D contain repeated sequences in both orientations. Group C encompasses the region of genes β2, β3, and β4, while the 1.4 kb inverted repeats of the subgroup D_1 are separated by 33 kb chromosomal DNA that includes all four β-globin genes.

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80. LARGE β-GLOBIN GENE INTRONS HYBRIDIZE TO SEQUENCES OUTSIDE THE GENES

Investigator: **C.-K. James Shen**

The characterization of cloned genomic DNA containing β-like globin genes ($^{G}\gamma$, $^{A}\gamma$, δ and β in human DNA and ε2, ε3, β2, and β1 in rabbit DNA) has revealed that all of these genes contain a large intron within the globin coding regions. All of the genes thus far studied also contain a smaller intron nearer to the 5'-end of the mRNA coding sequence. The large intron is approximately 1 kb in length in the human β-globin genes and 570 bp in the adult β-globin gene. In both species, Bam HI and Eco RI restriction sites flank the large introns. Cleavage of each of the genes with both Bam HI and Eco RI generates a fragment which contains the entire intron plus 70 bp of globin coding sequence. The Bam HI-Eco RI fragments from the rabbit β1, human δ and β genes were subcloned in the plasmid pBR322 and used as hybridization probes for Southern blots of restriction digests of the cloned DNAs containing each gene. We find that in addition to the expected hybridization to the Bam HI-Eco RI fragments containing the large intron (homoduplex formation), each probe also hybridizes to restriction fragments which are located immediately adjacent to the gene (heteroduplex formation). This suggests that there are sequences within the large introns that are repeated in the gene flanking regions. We are currently measuring the melting temperature (T_m) of the heteroduplexes to determine the extent of sequence homology. Preliminary data from melting experiments carried out on nitrocellulose filters indicate that the heteroduplexes have nearly the same T_m as the homoduplexes, suggesting that the repeated sequence contains few, if any, mismatched bases. We are attempting to map the location of these sequence homologies by electron microscopy. The analysis of intron sequences flanking each gene may provide insight into the functional significance of intervening sequences.

81. DNA-MEDIATED GLOBIN GENE TRANSFER

Investigators: **Elizabeth H. Lacy, Vann P. Parker**

The rabbit adult β major globin gene was introduced into mouse fibroblasts by DNA-mediated gene transfer (Wigler et al., 1977) in order to study the ability of mouse cells to transcribe and process a heterologous globin gene. Mouse L cells were co-transformed with a mixture of

intact DNA from the rabbit β-globin clone RβG1 and herpes simplex viral DNA containing the thymidine kinase (TK) gene (Wigler et al., 1979). Six TK^+ transformants were isolated which contained rabbit β-globin sequences. Hybridization experiments indicate that, in at least one of these transformants, rabbit β-globin sequences are expressed in the cytoplasm as a discrete polyadenylated 9S RNA at a concentration of five copies per cell (Wold et al., 1979). A Berk-Sharp analysis (Berk and Sharp, 1977) of the polyadenylated RNA of this transformant has shown that the mouse L cells spliced out the small and large intervening sequences in the rabbit β-globin gene correctly. However, either the initiation of transcription or 5' processing of the message is not occurring properly.

We plan to extend this approach to wild-type human globin genes as well as naturally occurring and in vitro-constructed mutant human globin genes to provide an assay for the functional significance of sequence organization.

This work was done in collaboration with Michael Wigler, Barbara Wold, Richard Axel, and Saul Silverstein at Columbia University.

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82. PHYSICAL LINKAGE OF THE HUMAN β-LIKE GLOBIN GENE FAMILY

Investigators: **Edward F. Fritsch, Richard M. Lawn**

There are at least five known β-like globin genes in man. An embryonic ε gene is expressed early in embryonic development, the $^{A}\gamma$ and $^{G}\gamma$ genes later in fetal development, and the δ- and β-globin genes in adult life. The genes represent a closely-related, differentially-expressed gene family, and it is hoped that the study of the globin genes and polypeptides at a molecular level will help elucidate the mechanisms of eukaryotic gene control. As one part of this study, we have determined the linkage arrangement of the β-like globin genes. Previous genetic evidence (Weatherall and Clegg, 1972; Bunn et al., 1977) and structural analysis of fusion proteins (Baglioni, 1962; Huisman et al., 1972) suggested that the fetal and adult β-like genes are linked. Physical linkage of the δ- and β-globin genes (Flavell et al., 1978; Lawn et al., 1978) and of the $^{G}\gamma$- and $^{A}\gamma$-globin genes (Little et al., 1979) was demonstrated using blot hybridization (Southern, 1975) and gene cloning (Maniatis et al., 1978) procedures. Recently, we have established physical linkage between the δ-β and $^{G}\gamma$-$^{A}\gamma$ loci using the blot hybridization procedure (Fritsch et al., 1979).

We have continued to screen for and isolate β-like globin gene clones both from the library of normal human DNA (Lawn et al., 1978) and from a recently constructed library of DNA from an individual with β^{o}-thalassemia (see Biology 1979, No. 83). To date, almost all of the 70 kb of DNA beginning approximately 25 kb 5' to the $^{G}\gamma$ gene and ending 10 kb 3' to the β gene have been isolated on clones from these libraries. Cloning of a Hpa I fragment was carried out to obtain one small region not isolated from either library. Preliminary evidence indicates that an embryonic ε-globin gene is located about 15 kb 5' to the $^{G}\gamma$ gene. Another β-like globin gene, possibly a second ε gene, has also been isolated although linkage to the other globin genes has not been determined. We hope eventually to obtain clones containing all the genes and their surrounding sequences which should prove invaluable in the studies of globin gene expression and of hereditary diseases in human globins.

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83. CLONING OF DNA FROM INDIVIDUALS WITH GLOBIN DISORDERS

Investigators: **Edward F. Fritsch, Richard M. Lawn, Kurt Runge***

The correlation of hematological data with studies at the levels of DNA and RNA of patients with various thalassemias and related globin disorders may contribute to an understanding of gene expression in higher organisms. We have prepared a cloned library in a phage λ system from the DNA of an individual with β°-thalassemia, a genetic disease marked by absence of the major adult hemoglobin. A clone containing the linked δ- and β-globin genes has been isolated from this library and characterization of the DNA has begun. We have detected no large alterations within or near to the β-globin gene in the β° clone and are in the process of carrying out a fine structure analysis.

We are also in the process of cloning the DNA from a person synthesizing hemoglobin Lepore, which is a fused protein containing portions of both the δ- and β-globin polypeptides. Previous blot hybridization experiments indicated that the Lepore gene was the result of a crossover between the δ- and β-globin genes (Flavell et al., 1978), probably within the 5'-end of the intervening sequence (our unpublished results). We hope to carry out direct DNA sequence analysis of the Lepore gene to determine the exact site of the crossover.

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84. CHARACTERIZATION OF DELETIONS WHICH AFFECT THE EXPRESSION OF FETAL GLOBIN GENES IN MAN

Investigators: **Edward F. Fritsch, Richard M. Lawn**

Hereditary persistence of fetal hemoglobin (HPFH) and δβ-thalassemia are two genetic disorders in humans marked by the lack of the adult δ- and β-globin proteins. The diseases differ, however, in the levels of fetal globin gene expression. An individual with HPFH is clinically normal due to continued production of the fetal γ-globin proteins whereas an individual with δβ-thalassemia is anemic, due to only partial fetal globin production. Solution hybridization experiments indicated that part or all of the δ- and β-globin genes were deleted from the DNA of individuals with either disease. Using recently acquired knowledge of the organization of the human β-like globin gene cluster (Flavell et al., 1975; Lawn et al., 1978), and radioactive probes synthesized from cDNA plasmids (Wilson et al., 1976) or DNA fragments isolated from clones containing the δ-, β-, and γ-globin genes, we have determined the extent of deletions present in the DNA of four individuals homozygous for either of these disorders (Fritsch et al., 1979). In both examples of HPFH we studied, a deletion begins approximately 4000 bp 5' to the δ gene and extends through the β- and δ-globin genes. In one case of δβ-thalassemia, the deletion begins within the δ gene and extends through the β gene. This result is consistent with the suggestion (Huisman et al., 1974) that a region 5' to the δ gene is involved in the normal turning down of γ-globin production at birth. In the second case of δβ-thalassemia an extremely large deletion removed δ, β, one of the two γ-globin genes, and most of the region between the two γ-globin genes. At least 4.5 kb of DNA 5' to the δ gene was removed. Although apparently inconsistent with the suggestion described above, the interpretation of the deletion in the second case of δβ-thalassemia was complicated by the extensive deletion within the γ-globin region which might considerably affect the level of fetal globin compensation. By studying other examples of HPFH and δβ-thalassemia with these techniques, we hope to learn more about the genetic elements involved in the switch from fetal to adult globin gene production in man.

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85. PHYSICAL LINKAGE OF THE HUMAN α-LIKE GLOBIN GENE FAMILY

Investigator: **Joyce E. Lauer**

Genetic evidence indicates that human adult α-globin genes, located on chromosome 16, are duplicated and that both genes are expressed (Bunn et al., 1977). Genomic blotting experiments have demonstrated physical

linkage of these genes (Orkin, 1978). There exists also an α-like embryonic globin chain designated ζ, whose chromosomal location and linkage relationship to the adult α genes have not been previously established.

We have isolated the linked human α-globin genes from a cloned library of human DNA. By hybridization with an adult α-globin cDNA probe, two regions have been identified which correspond to the duplicated adult genes. These genes are 3.8 kb apart, in agreement with the published genomic blotting data. Two additional regions, approximately 4 and 8.5 kb 5' to the 5' adult gene, hybridize faintly to the human adult cDNA probe but relatively strongly to embryonic rabbit globin cDNA, and we have therefore tentatively called these ζ genes. To determine whether this assignment is correct we will sequence the hybridizing regions and attempt to align the nucleotide sequences with codons for the known amino acid sequence of ζ-globin.

The similarity of restriction enzyme sites surrounding the adult genes, and the tendency of this region to delete, suggest that there may be interesting homologies between the regions flanking the two genes. We also plan to use these cloned segments of DNA to study the RNA transcripts of α-globin genes of normal and thalassemic individuals, in order to determine the molecular basis of defects in α-globin expression.

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86. CLONING OF THE DROSOPHILA ALCOHOL DEHYDROGENASE GENE

Investigator: **David A. Goldberg**

Genomic clones containing the alcohol dehydrogenase (Adh) gene have been isolated from a library of Drosophila melanogaster DNA carried in bacteriophage λ vectors. The gene has been identified by differential hybridization to RNA from a wild-type and homozygous-deficiency strain, by in situ hybridization, and by in vitro translation of RNA which hybridizes to this clone. A detailed restriction map has been determined.

At present, a characterization of transcribed regions within the close vicinity of the Adh gene is under way, and clones from genetically homogeneous, characterized strains are being isolated. These clones will be used to attempt genetic transformation of germ line tissue of mutants of Adh by microinjection of cloned DNA into early embryos. In this way, we hope to study the DNA sequences which are involved in the stage- and tissue-specific expression of Adh.

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Professor: Herschel K. Mitchell
Research Associate: Peter H. Lowy
Gosney Senior Research Fellow: Nancy S. Petersen
Graduate Students: Anne Chomyn, Galina Moller, Loveriza A. Sarmiento
Research Staff: Carol L. Shotwell

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Summary: The center of attention in our research group continues to be concerned with the molecular biology and biochemistry of development in Drosophila. We have, in the past year, made use of protein polymorphisms in heat shock proteins to show by standard genetic means that one of the heat shock chromosome puffs is the source of message for three different proteins at least. We have elaborated on the heat shock problem by showing that protection against death from a high temperature shock can be obtained by a lower temperature pretreatment. Analysis of this phenomenon on a molecular basis is in process. We have made good progress in an analysis of salivary gland functions in the pupation process of Drosophila. We hope to relate this eventually to the complex behavior pattern which is characteristic during pupation. In another area of investigation the program continues with the objective of relating the functions of specific genes such as hk and jw through transcription and translation to the development of elaborate structures of the scutellar bristles. This program is carried on at all levels from electron microscopy of external and internal structures through analysis of development-related protein and RNA synthesis. Work has also continued on the chemical nature of the cross-linking substance produced in sclerotization of Drosophila cuticle.

87. PROTECTION AGAINST THE STRESS OF HEAT SHOCK

Investigators: **Herschel K. Mitchell, Galina Moller, Nancy S. Petersen, Loveriza A. Sarmiento**

In earlier work (Mitchell and Lipps, 1978) we demonstrated that a heat shock of Drosophila at a high temperature (41.5°C) turns off both transcription and translation. As the animals recover at a normal temperature, translation resumes before transcription. With this implication of a mechanism of storage of mRNA dependent on production of heat shock proteins, we examined the protection phenomenon originally described by Milkman (1962). We have observed that a pretreatment of larvae, pupae or adults, as well as a cell line at temperatures between 30° and 38°C prevents death that would result from a subsequent treatment at a higher temperature. Similarly a phenocopy that would result from a heat shock at a specific stage of development can be prevented by a pretreatment during the sensitive period. Studies on protein and RNA synthesis under these conditions support a model which involves heat shock proteins in transport and protection of mRNAs. Protection occurs only under circumstances where normal mRNAs and heat shock proteins are produced at the same time.

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88. SELECTION OF MUTANTS WITH ALTERED HEAT SHOCK RECOVERY GENES

Investigators: **Nancy S. Petersen, Herschel K. Mitchell**

The effect of preheating at 35°C on survival at higher temperatures (see preceding abstract) provides the basis for a screen to select mutants which affect the expression of heat shock genes required for survival at high temperatures. The mutant screen is being done in two ways. The first is designed to look for dominant mutants which cause constitutive production of the protecting molecules. Oregon-R males were mutagenized

with EMS (Lewis and Bacher, 1968) and mated to virgin Oregon-R females in a population cage. Progeny were collected as pupae and heated 80-82 hr after puparium formation to 41.5°C for 45 min (normally lethal to 100% of pupae at this stage). Fifteen survivors were collected and mated to a CyO/TM6 balancer stock. Progeny of this cross will be retested for temperature resistance and then for alterations in the level of heat shock protein synthesis.

The second mutant screen is designed to look for recessive mutations which either increase or decrease the level of heat shock gene expression. In this case we are testing stocks which are homozygous for mutagenized (EMS) third chromosomes. Flies from one stock are divided into two vials, the first vial is pretreated at 34°C and then both vials are heated for 20 min at 40.5°C. Flies which survive the 40.5°C treatment without pretreatment and flies which die at 40.5°C in spite of the pretreatment have been picked for retesting. We will use extra long SDS acrylamide gels to look at the levels of synthesis of 25 of the heat shock proteins. Mutants which show altered levels of heat shock protein synthesis will be mapped using conventional genetic methods and they will be tested in combination with different electrophoretic variants of the heat shock protein(s) affected to see whether the regulatory effects are cis or trans acting.

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89. EFFECTS OF HEAT SHOCK ON GENE EXPRESSION AND SURVIVAL IN EARLY EMBRYOS OF DROSOPHILA MELANOGASTER

Investigators: **Nancy S. Petersen, Herschel K. Mitchell**

Preblastoderm embryos are much more temperature-sensitive than older embryos, pupae, or adults. Twenty minutes at 36°C kills 90% of preblastoderm embryos. This is the temperature which induces heat shock gene expression in older animals and protects against death at higher temperatures. Treatment of preblastoderm embryos at 34°C or 35°C does not improve survival at temperatures above 36°C. During late blastoderm and early gastrula stages, there is a rapid increase in the ability of the embryos to survive heat treatment. The survival of older embryos at high temperature can also be improved by a lower temperature pretreatment. We suggest that the difference in temperature sensitivity between preblastoderm embryos and later stages in the animals' life cycle may be due to the lack of ability of preblastoderm embryos to synthesize heat shock messenger RNA. We are testing this hypothesis by isolating RNA from heat shocked preblastoderm and post-gastrula embryos. The messages present will be translated in vitro to determine whether heat shock messages are present in older embryos, but not in preblastoderm embryos.

90. PHENOCOPIES FROM HEAT SHOCK DURING METAMORPHOSIS IN DROSOPHILA

Investigators: **Herschel K. Mitchell, Carol L. Shotwell**

Drosophila is the most resistant to heat shock during the period in the life cycle from pupation time (+12 hr after preparation) to eclosion (96 hr). There are highly significant differences between stocks but the present Ore-R strain survives 10 min at 41.5°C to the extent of at least 50% at all of these stages. At the other extreme, 50% survive this temperature for 40 min at 48 hr but all are killed before 40 hr and after 53 hr. There are especially sensitive periods around 29, 55, 64, and 82-96 hr and especially resistant periods around 24, 40, 49, and 66 hr. In general heat shock phenocopies are most easily obtained during periods of greatest heat resistance. We have noted about 25 that are produced with high penetrance in the 12 to 96 hr range by shocks at 41.5°C. These include abnormalities in the structures of bristles, hairs, and wings.

91. GENES CODING FOR THREE HEAT SHOCK PROTEINS ARE LOCATED IN THE 67B PUFF AND ARE EXPRESSED COORDINATELY

Investigators: **Nancy S. Petersen, Galina Moller, Herschel K. Mitchell**

We have used electrophoretic variants of the 23K, 27K, and 28K proteins to map the position of the genes coding for these proteins by linkage to recessive visible markers. All three map to a region of the chromosome which includes only one heat shock puff, the 67B puff. A DNA clone from the 67B puff has been isolated which codes for the 23K protein, the 26K protein, and one more unidentified protein (Betty Craig, personal communication). This implies that the coding regions for at least four proteins are located in the 67B heat shock puff. These four genes are not simply slightly altered copies of the same gene. Tryptic digest fingerprints of the smaller heat shock proteins show that the 23K protein, at least, is very different from the 26K, 27K, and 28K (Mirault et al.,

1978). We have found that the kinetics of synthesis of these proteins during recovery from heat shock is coordinate and distinctly different from the kinetics of synthesis of the 84K, 70K, 68K, and 22K heat shock proteins. This suggests that the gene organization may have an effect on the time of gene expression.

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92. THE AROMATIC CROSS-LINK OF INSECT CUTICLE

Investigators: **Peter H. Lowy, Herschel K. Mitchell**

The cuticle which functions as the skin and external skeleton of insects consists of protein and chitin. During pupation the pliable larval cuticle changes to the hardened (sclerotized) pupal cuticle as aromatic cross-links, derived from tyrosine via N-acetyldopamine, are formed. To learn the nature of this linkage one needs to split cuticle into low molecular weight fractions containing the aromatic cross-link attached to portions of protein and/or chitin. Enzymatic digestions of cuticle have yielded glycopeptides indicating linkages of protein to chitin, but only after pretreatment with N-bromosuccinimide which destroys aromatic components. Heating with dilute acids has yielded several ketocatechols, suggesting that the beta-carbon atom of N-acetyldopamine is oxidized to allow linkage to amino and hydroxyl groups in protein or chitin. Because of artifact interactions between protein and carbohydrate during acid treatment one would have to achieve enzymatic digestion to obtain the desired piece with the aromatic link in intact form.

There is recent evidence that a protease present in molting fluid from tobacco hornworm renders cuticle chitin susceptible to digestion by the chitinase which is also present. This suggests that a combination of chitinase and other enzymes in molting fluid might digest even sclerotized cuticle.

93. DROSOPHILA CHITINASE

Investigators: **Peter H. Lowy, Herschel K. Mitchell, Lynn M. Hildemann***

Chitinase is present throughout the life span of Drosophila larvae even during periods of heavy chitin synthesis, and the levels of its activity were measured earlier by Winicur using a viscometric assay which depends on strict control of pH and salt concentrations. We have adapted the chemical determination of N-acetylglucosamine released by the enzyme for use with Drosophila fractions. By precipitation at 33 to 66% ammonium sulfate saturation, followed by dialysis and freeze-drying, most of the activity can be obtained in stable form.

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94. PUPATION PROTEINS IN DROSOPHILA MELANOGASTER

Investigators: **Loveriza A. Sarmiento, Herschel K. Mitchell**

One aspect of developmental regulation that we are studying concerns the pupation process. The organism undergoes a series of rapid changes shortly before pupation which leads us to ask what regulatory elements are possibly involved in the process.

A series of pulse-label experiments using [^{35}S]-methionine on salivary glands of Drosophila melanogaster from the late larval to the pupal stage show a pronounced decrease both in total protein content and in newly synthesized proteins. Injection of larvae with labeled methionine and subsequent examination of protein turnover at regular intervals in salivary glands, brains, pupation fluid, and hemolymph suggest a transfer of specific proteins from the salivary glands to the pupation fluid. The other tissues do not show such a change in their protein patterns. The pupation fluid is present within a few hours after puparium formation and might be essential for the organism to undergo the various contractions involved in pupation.

We hope eventually to select one of the protein components and follow its course through the pupation process. A comparison is also being made on proteins being synthesized during regular time periods from late larval to pupal stage. Concurrent studies of the puffing patterns are being done in the hope of being able to correlate a specific puff site for the message coding for any one of these regulatory proteins. A more accurate visualization of the puffs is done by autoradiography of glands labeled with [^{3}H]uridine at time periods similar to those in pulse label and turnover experiments. More label is expected to accumulate in sites where puffs are present.

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Associate Professor: James H. Strauss Jr.
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Summary: We are continuing our studies on the structural proteins of Sindbis virus. The glycoproteins of the virus, E1 and E2, are integral membrane proteins which are made on the rough endoplasmic reticulum and migrate to the surface of the infected cell. They are acquired by the virus particle when the nucleocapsid buds through the plasma membrane in the act of maturation. The virus thus furnishes a model system for the study of the integral membrane proteins of the plasmalemma. In addition it is closely related to a number of important animal pathogens. We are interested in knowing more about the biosynthesis and transport of the virion glycoproteins, their conformation in the plasma membrane and in the virion, and the interactions which occur among the RNA, proteins, and lipids of the virus. We intend to determine the primary sequence of these proteins as an aid in understanding the virion structures. Many of the following reports detail various approaches used in these studies. We are also engaged in a long-term study of virus genetics as an aid in understanding the molecular biology of virus replication.

95. STUDIES ON THE CONFORMATION OF THE SINDBIS GLYCOPROTEINS

Investigator: **John R. Bell**

Any mechanism which is proposed to describe the synthesis and insertion into the membrane of the glycoproteins E2 (as the precursor PE2) and E1 must account for their final conformation. The conformation of E1 and E2 in the virus has been studied by protease digestion of the virus particle and the results show that, although most of the mass of these proteins is external to the membrane, a small region of each protein near its C-terminal end is protected from digestion by the lipid bilayer. I am studying the effect in mechanically disrupted cells of protease digestion of PE2 and E1 shortly after their synthesis. Under these conditions, the contents of the lumen of the disrupted endoplasmic reticulum, topologically equivalent to the external surface of the virus particle, are protected from the protease, while the membrane surface corresponding to the inner face of the viral membrane is accessible to the protease. It has recently been shown that PE2 is slightly shortened by protease treatment under these conditions.

One hypothesis I am examining results from the analysis of the cyanogen bromide cleavage products of E2, which suggests that the C-terminal end of this protein is glycosylated and is thus external to the viral membrane. If this is true, then both ends of the molecule are outside the membrane and the protein must loop into and through the lipid bilayer and back to the outer surface. This possibility is being examined by attempting to confirm that the presumed C-terminal cyanogen bromide-produced peptide is in fact C-terminal, and by attempting to

determine the number of fragments generated from PE2 by proteolytic digestion from what corresponds to the inner face of the viral membrane in mechanically disrupted cells.

96. ACETATE LABELING OF SINDBIS PROTEINS

Investigator: **John R. Bell**

An attempt to sequence the capsid protein of Sindbis virus by Edman degradation has shown that the N-terminus of this protein is blocked, and preliminary results suggest that the same is true of the nonstructural protein PE2. One of the two common types of modified N-termini in eukaryotic proteins is an acetylated amino group, and it has been shown that in some cases this group can be specifically labeled through the incorporation of radioacetate. I am attempting to incorporate tritiated acetate specifically into acetylated N-termini of proteins in the cell types used for growing Sindbis virus, monitoring the specificity of incorporation by examining the extent of labeling of actin, which is known to be acetylated, relative to the labeling of total protein. If successful, this technique should reduce the chances of purifying a protein for N-terminal sequencing only to find it blocked. In addition, radioactive acetate would make a convenient "tag" for following the N-termini of acetylated proteins during the proteolytic processing which occurs during the maturation of the Sindbis viral proteins. Growth of cells in the presence of tritiated acetate, followed by hydrolysis of the proteins and amino acid analysis, has shown that a significant conversion of acetate to proline and glutamic acid occurs. Efforts are under way to reduce this metabolic conversion in order to make the labeling specific enough to be useful.

97. SEQUENCING OF PRECURSORS OF THE SINDBIS PROTEIN

Investigators: **John R. Bell, Jeffrey T. Mayne, Charles M. Rice III**

All three structural proteins of Sindbis are formed by proteolytic cleavage of a single precursor molecule. Most of these cleavages occur while the protein is still nascent, but uncleaved products can be found intracellularly during virus infection of certain cell types or during infection by mutants of the virus. We have previously determined the sequences of the N-terminal 50 amino acids of the structural proteins E1 and E2. We now wish to determine the N-terminal sequences of several of the uncleaved forms of the precursor to these proteins in order to compare these sequences with the ends of the final products. We also hope to sequence some of the regions of the precursor which are not represented in the structural proteins of the virus, in particular the hypothetical "signal sequence" responsible for the insertion of the envelope glycoproteins into the membrane.

Two approaches are being used, which differ in the manner of detection of the amino acids released sequentially from the proteins by the automated sequences. In one case the proteins are produced (in vivo) in the presence of radioactive amino acids, and the amino acids detected after sequenation by assaying for radioactivity. Not all amino acids can be labeled in this manner and the method is tedious. In the second approach, sufficient protein is used to detect the released amino acids chemically. Toward this end, we are attempting to increase the size of our cell cultures and we are developing schemes to purify the proteins of interest from the vast excess of cellular protein by using antibodies directed against the viral structural proteins.

98. STRUCTURAL STUDIES OF SINDBIS VIRUS GLYCOPROTEINS

Investigator: **Charles M. Rice III**

Digestion of intact Sindbis virions with α-chymotrypsin gives rise to a particle of lighter density containing two protease-resistant fragments of the glycoproteins embedded in the lipid bilayer as well as the intact nucleocapsid. The apparent molecular weights of these fragments (called "roots") are 10,000 and 5,000 daltons. Two-dimensional thin layer as well as high pressure liquid chromatographic techniques for separation of tryptic and/or chymotryptic digestion products have shown that the 10K dalton root is derived from E2 [RE2], the 5K dalton root from E1 [RE1]. In a two-phase aqueous-organic system at neutral pH, RE1 is much more soluble (~4-fold) in the organic phase than is RE2. Other workers have shown that when Sindbis is grown in cells prelabeled with [^{3}H]palmitate, both E1 and E2 are labeled. Digestion of such labeled virus with α-chymotrypsin gives rise to palmitate-labeled material which co-migrates with RE1 and RE2. The percentage of [^{3}H]palmitate recovered in the roots accounts for most, if not all, of the glycoprotein-associated palmitate label.

Further studies will include the amino acid compositions and sequence analysis of these hydrophobic peptides,

as well as attempts to determine their orientation in the lipid bilayer and with respect to the viral nucleocapsid.

99. CROSS-LINKING STUDIES ON SINDBIS VIRUS GLYCOPROTEINS

Investigator: **Charles M. Rice III**

We have studied the subunit structure of Sindbis virions. Using monospecific rabbit antisera against each viral envelope glycoprotein and both reversible and irreversible cross-linkers, we found that the glycoproteins in intact virions, infected cells, and Triton X-100-solubilized virions, are assocated as a heterodimer of E1 and E2. We have been unable to show an equivalent association of PE2 (the precursor to E2 in infected cells) and E1 by either cross-linking or immunoprecipitation of Triton X-100-solubilized, pulse-labeled, infected monolayers.

100. PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST SINDBIS PROTEINS

Investigators: **Jeffrey T. Mayne, Charles M. Rice III**

Using a technique for the fusion and selection of hybrid mouse cells producing antiviral antibodies we have isolated several cell lines producing anti-E1 antibodies. One IgG producing clone reacts equally well with Triton X-100 or SDS solubilized E1. We plan to further characterize this and other clones with respect to their specificity to particular regions of the protein, and their possible effects on viral infectivity, virus-induced hemagglutination, and binding to infected cells. We are also attempting to isolate hybrid lines producing antibody against E3 isolated from the culture supernatant of infected cells. One intent is to obtain a probe specific for PE2 which would be useful for studies on virus maturation in chicken and mosquito cells by fluorescence and electron microscopy.

101. PURIFICATION AND CHARACTERIZATION OF E3 IN THE SINDBIS VIRUS INFECTION

Investigators: **Jeffrey T. Mayne, Charles M. Rice III, Ellen G. Strauss**

The two best studied alphaviruses are Sindbis virus and Semliki Forest virus (SFV). One of the major differences between the two viruses is the presence of E3 (a small, heavily glycosylated protein) in the membrane of the SF virion and its lack in the Sindbis virion. In SFV, E3 is produced at a late stage of virus maturation when PE2 is cleaved to produce E2 and E3. But in the Sindbis infection PE2 is cleaved to produce E2 only, and the fragment equivalent to E3 is lost.

E3 has been isolated from the culture fluid of Sindbis-infected cells by our lab and other investigators (Welch and Sefton, 1979). Pulse-chase and tryptic digestion experiments indicate that it is cleaved from PE2 and released into the culture fluid as PE2 is processed to E2.

E3 runs as multiple (3-5) closely-spaced species on SDS acrylamide gels. Initial evidence suggests that this is due to differential degrees of glycosylation.

A purification scheme has been devised which purifies E3 20-40,000-fold. This scheme uses differential precipitation by ethanol, gel filtration, ion exchange chromatography, and a lectin affinity column.

The purified E3 is being used to do biochemical studies, including amino acid compositions and sequencing. By comparison of sequences of the carboxy terminus of E3, the amino terminus of E2, and the appropriate CNBr peptide of PE2, we hope to determine some of the fine structure of the cleavage of PE2 → E2 + E3.

We are also investigating the kinetics of synthesis and the location of E3 during the infection cycle in mosquito cells. In chick cells the virus buds through the plasma membrane and is released into the medium. In mosquito cells, in contrast, the virus seems to be assembled in cytoplasmic "factories," although there is some controversy over this point. The location of E3, whether extracellular or intracellular, will help resolve this question.

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102. CLONING OF SINDBIS VIRION RNA

Investigators: **Jing-hsiung James Ou, Charles M. Rice III**

We plan to clone the Sindbis virion 49S RNA using reverse transcriptase to synthesize a complementary ssDNA copy followed by insertion of the RNA-DNA hybrid into an appropriate vector. Using the known amino terminal sequences of the glycoproteins and carboxy terminal sequence data for each structural protein, alignment with the corresponding DNA sequence from the cloned 49S should be possible. In this way we hope to delineate clearly the sites of proteolytic processing and compare their peptide sequences. Short-term projects include the sequencing of the 3'- and 5'-ends of the cloned 49S RNA.

103. A BIOCHEMICAL CORRELATE WITH PLAQUE MORPHOLOGY IN SINDBIS VIRUS

Investigator: **Ellen G. Strauss**

It has been well known for several years that many strains of Sindbis virus and other alphaviruses exhibit heterogeneous plaque morphology. Large plaque (LP) and small plaque (SP) variants can be isolated from these stocks by single plaque isolation, producing homogeneous populations which are genetically stable for this trait. SP variants of Sindbis appear to have a selective advantage in mammalian cells; SP grows to higher titer in these cells and produces plaques with a higher efficiency than LP strains. Successive passages of an uncloned Sindbis stock in BHK cells, for example, will result in predominantly SP virus. On the other hand, LP variants are favored in avian cells and successive passages in chick embryo fibroblasts results in primarily LP virus. Many attempts in both our laboratory and others to establish a biochemical or physiological basis for plaque morphology have been only marginally successful and have implicated both the kinetics of virus production in a particular host as well as the charge on the particle and its interaction with the solid support during the plaque assay as important parameters.

Recently we have found one biochemical distinction between LP and SP Sindbis strains. When the proteins synthesized in infected monolayers are examined by polyacrylamide gel electrophoresis, PE2 (a precursor to one of the envelope glycoproteins of the mature virion) from SP-infected cells always migrates more rapidly than PE2 from LP-infected cells. This difference between the PE2s is retained in ts mutants derived from SP and LP strains respectively. During normal Sindbis maturation, PE2, which is an integral membrane protein of the host cell plasmalemma, is cleaved to E2 at the moment when the particle is budding from the cell. The product which is removed, E3, is released into the culture fluid. Although the differences between PE2 from LP and SP are reproducible and easily discernible, we have not been able to detect strain differences in either of the cleavage products (E2 or E3). These differences may be masked, however, by the heterogeneity of the glycosylation of these two species.

104. CHARACTERISTICS OF THE $RNA^{\pm}$ MUTANTS OF SINDBIS VIRUS

Investigator: **Ellen G. Strauss**

In previous reports we have discussed the isolation and preliminary characterization of about 100 temperature-sensitive mutants of Sindbis virus. These have been grouped into three broad classifications on the basis of their degree of incorporation of uridine at the nonpermissive temperature. RNA^{+} mutants incorporate 60 to 100% as much as the parental strain, RNA^{-} mutants incorporate 0 to 10% as much, and the remainder, $RNA^{\pm}$, make 10 to 60% as much RNA at the nonpermissive temperature. The RNA^{+} mutants can be assigned to three complementation groups corresponding to defects in the three structural proteins of the virion while the RNA^{-} mutants fall into four groups. Recently we have begun to determine the physiological and genetic characteristics of the $RNA^{\pm}$ mutants. In general, these mutants appear to belong to the RNA^{-} class for they produce thermostable particles at the permissive temperature (indicating that the defect is not in the structural proteins) and give a pattern of protein synthesis at the nonpermissive temperature which is very similar to a mock infection. This means that virus-specific proteins are not produced in measurable quantities and that host protein synthesis is not shut off. Temperature shift experiments reveal the presence of polyprotein precursors to the nonstructural proteins similar to those seen with RNA^{-} mutants. The particular pattern of these higher molecular weight precursors cannot in any case be unambiguously correlated with complementation group at the current time.

Finally, most of the $RNA^{\pm}$ mutants complement all groups of RNA^{+} mutants. Some of them can be provisionally assigned to particular RNA^{-} groups, but several of them complement poorly with more than one RNA^{-} group. In summary, the $RNA^{\pm}$ mutants resemble RNA^{-} mutants except for their incorporation of uridine into RNA at the nonpermissive temperature. These isolates may contain one or more physiologically leaky defects in the nonstructural genes.

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CELLULAR BIOLOGY AND BIOPHYSICS

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Summary: Evidence has accumulated which indicates that the bending movements of cilia and flagella are generated by a sliding microtubule process, similar to the sliding filament process responsible for muscle contraction. Control mechanisms are needed to cause oscillatory bending and to maintain the phase differences between bending in different regions which are required for propagated bending waves. Two quite independent mechanisms for flagellar oscillation have been proposed; both mechanisms also lead to bending wave propagation under at least some conditions.

Our work makes particular use of ATP-reactivated movements of demembranated sperm flagella as a source of experimental data, and computer programs which simulate the movement of model flagella to relate theoretical mechanisms to experimental data. We hope to identify the types of control mechanisms which actually exist in flagella and cilia, to understand how parameters of movement such as frequency, wavelength, and bend angle are controlled, and to use this understanding to enable detailed study of the active process which generates sliding in flagella and muscle.

105. CALCIUM-INDUCED ASYMMETRICAL BEATING OF DEMEMBRANATED SEA URCHIN SPERM FLAGELLA

Investigator: **Charles J. Brokaw**

Asymmetrical bending waves can be obtained by reactivating demembranated sea urchin spermatozoa at high Ca^{2+} concentrations. Moving-film flash photography shows that asymmetrical flagellar bending waves are associated with premature termination of the growth of the bends in one direction (the reverse bends) while the bends in the opposite direction (the principal bends) grow for one full beat cycle, and with unequal rates of growth of principal and reverse bends. The relative proportions of these two components of asymmetry are highly variable. The increased angle in the principal bend is compensated by a decreased angle in the reverse bend, so that there is no change in mean bend angle; the wavelength and beat frequency are also independent of the degree of asymmetry. This new information is still insufficient to identify a particular mechanism for Ca^{2+}-induced asymmetry.

When a developing bend stops growing before initiation of growth of a new bend in the same direction, a modification of the sliding between tubules in the distal portion of the flagellum is required. This modification can be described as a superposition of synchronous sliding on the metachronous sliding associated with propagating bending waves. Synchronous sliding is particularly evident in highly asymmetrical flagella, but is probably not the cause of asymmetry. The control of metachronous sliding appears to be unaffected by the superposition of synchronous sliding.

Photographs of asymmetrical flagella taken during exposure of demembranated flagella to CO_2 show that the gradual inhibition of the amplitude of bending by CO_2 occurs without any change in the degree of symmetry measured by the difference between principal and reverse bend angles. This provides additional evidence for the independence of asymmetry and other wave parameters.

In order to obtain symmetrical bending waves, it is not only necessary to reactivate the sperm flagella at a low Ca^{2+} concentration, but they must also be first exposed to a high Ca^{2+} concentration. We now find that the amount of Ca^{2+} required for this high Ca^{2+} exposure is much less if Mg^{2+} is not present. This has allowed us to study the kinetics of these processes. The effect of high Ca^{2+} exposure is gradual. Initially it is reversible by MgEGTA, but only if the flagella concentration is high. These results suggest a model in which a high Ca/Mg ratio causes the release from the flagellum of some component which is required for the generation of asymmetrical bending waves. It is interesting that the ability to produce asymmetrical bending waves in response to Ca^{2+} is also very sensitive to trypsin digestion.

106. DIGESTION OF SPERM FLAGELLA WITH ELASTASE

Investigator: **Charles J. Brokaw**

Sea urchin sperm flagella were demembranated with Triton X-100 and reactivated in solutions containing 1.6 $\mu gm\ ml^{-1}$ elastase and soybean trypsin inhibitor. The spermatozoa were motile for 3 to 4 min, and then stopped and disintegrated by tubule sliding as in earlier observations with trypsin (Summers and Gibbons, 1971; Brokaw

and Simonick, 1977). During the period of digestion, there is a gradual increase in the amplitude of the flagellar bending waves, as measured by bend angle, accompanied by a decrease in frequency, so that the product of frequency and bend angle, proportional to the rate of sliding between flagellar tubules, is approximately constant. These changes are opposite to changes reported during digestion by trypsin. When spermatozoa are demembranated at very low calcium concentrations, their subsequent movements are asymmetrical, and remain asymmetrical throughout the period of digestion by elastase, while trypsin digestion causes a decrease in asymmetry under these conditions.

The stability of the axonemal structure appears to be maintained in part by interdoublet linkages composed of a protein which has been named nexin (Stephens, 1970). The linkages must be highly extensible in order to accommodate the sliding which occurs during normal flagellar oscillation. Nexin is therefore likely to be an elastic protein with amino acid composition similar to elastin, resilin, abductin, etc. and particularly sensitive to digestion by elastase. The changes in movement seen during elastase digestion are consistent with the hypothesis that the elastic resistance of the interdoublet linkages is a factor in regulating the amplitude of flagellar bending, and that these linkages are preferentially digested by elastase. Because of its apparently more specific effects on the interdoublet linkages, elastase may be more suitable than trypsin for experiments involving observation or measurement of sliding disintegration of flagellar and ciliary axonemes.

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107. ANTI-TUBULINS AND FLAGELLAR MOTILITY

Investigator: **David J. Asai**

Antibodies binding to sea urchin flagellar outer doublet tubulin have been purified from rabbit sera by affinity chromatography--"induced" anti-tubulins from rabbits immunized with SDS denatured preparations of tubulin and "spontaneous" anti-tubulins from unimmunized rabbits. Spontaneous anti-tubulin is found in normal serum at approximately one-third the concentration of induced anti-tubulin. Both preparations are composed exclusively of IgG class antibodies. Both are specific for tubulin when presented with a crude mixture of flagellar proteins. Only induced anti-tubulin precipitates tubulin in an immunodiffusion assay. Both anti-tubulins and their monovalent Fab fragments bind to sea urchin axonemes in a simple radiobinding assay. Induced anti-tubulins from four different immunizations were all found to reduce the bend angle and the symmetry of the movement of demembranated spermatozoa without affecting the beat frequency. At identical concentrations, spontaneous anti-tubulins (five samples) and both of the Fab preparations had no effect on bend angle, symmetry, or frequency. This is the first report of an anti-tubulin having an inhibitory activity on microtubule-associated movement.

At a concentration of induced anti-tubulin capable of completely paralyzing reactivated flagella, the ATP-induced sliding disintegration of elastase-digested axonemes was not inhibited. However, incubation with induced anti-tubulin did raise by approximately 3-fold the threshold concentration of ATP necessary to induce disintegration by sliding. These results are in contrast to the total inhibition of tubule sliding obtained with the ATPase-inhibiting anti-dynein fragment A antibody. The lack of inhibition of sliding by induced anti-tubulin under paralyzing conditions indicates that this antibody does not interfere with the active sliding process and suggests that the anti-tubulin affects mechanisms controlling bending.

108. INHIBITION AND RELAXATION OF FLAGELLA BY VANADATE

Investigator: **Makoto Okuno**

Direct measurements of the stiffness (elastic bending resistance) of demembranated sea urchin sperm flagella were made in the presence of $MgATP^{2-}$ and vanadate (to prevent spontaneous bending). Under these conditions, the flagellum is in a relaxed state, with a stiffness of about 0.5×10^{-21} N m^2, which is about 1/20 the stiffness obtained in the rigor state in the absence of $MgATP^{2-}$. $MgADP^-$ binds to flagella as a competitive inhibitor of beat frequency but does not substitute for $MgATP^{2-}$ in producing the relaxed state. Vanadate enhances competitive inhibition of beat frequency by $MgADP^-$ and causes the flagellum to relax more quickly in the presence of low concentrations of $MgATP^{2-}$.

These observations indicate that vanadate inhibits and causes relaxation by binding preferentially to a detached cross-bridge state which is accessible by

$MgATP^{2-}$ binding but not by $MgADP^{-}$ binding. However, since vanadate can also bind in the presence of $MgADP^{-}$, relaxation by vanadate does not result from a specific affinity for detached cross-bridges. Vanadate does not bind in the absence of $MgATP^{2-}$ or $MgADP^{-}$.

109. COMPUTER SIMULATION OF FLAGELLAR MOVEMENT

Investigators: **Charles J. Brokaw, Edward F. Pate**

Our basic program for computer simulation of flagellar movement has been augmented by the inclusion of thermodynamically realistic models for the action of dynein-tubulin cross-bridges. Programs involving both two-state and four-state cross-bridge models have been developed and tested. These models can generate realistic-looking flagellar movement, but there are important discrepancies between the behaviors of the model and of real flagella. Our efforts to perfect the models are currently concentrating on the behavior of cross-bridge systems in "rigor."

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Summary: Our long-standing puzzle, the short-term growth and tropic effects of barriers, winds, and confinements, has continued to occupy our attention. We have now improved the mathematical model calculations and have reached the conclusion that the simple assumption of an avoidance gas (Cohen et al., 1975) is inadequate and must be replaced by assuming that the avoidance gas is converted on impact with air or barrier from a precursor form to the active form. Experiments to test these new notions are now under way.

Phycomyces belongs to the zygomycetes, a group of fungi of uncertain position on the tree of life. We have been attempting to locate its position by sequencing of cytochrome c. The cytochrome c sequence shows an amazingly distant relationship to the alleged closest of kins of the zygomycetes, the ascomycetes, and a possibly less distant relation to higher plants (Jayaram and Hunkapiller).

For the same purpose, and also because of its intrinsic interest, we have also looked at the DNA sequence organization in Phycomyces. The DNA organization shows "long-range interspersion" of the middle repetitive DNA which is abundant in Phycomyces, with the single copy DNA (Harshey et al.). This is similar to the organization of the DNA in a few other organisms with small genomes (the fly Drosophila and oomycete Achlya), and in strong contrast to all other organisms studied.

Our efforts in direct pursuit of the blue-light photoreceptor have been limited during the past year to its chromophore riboflavin. Two new riboflavin auxotrophs were isolated (Wischhusen) and a considerable number of riboflavin analogs were studied for their competition effects on growth and uptake. Riboflavin

kinase, the enzyme which converts riboflavin after uptake into flavin mononucleotide (FMN) has been isolated and characterized (Otto and Kochevar).

A principal problem in the isolation of the photoreceptor, as well as in other studies involving subcellular elements (mitochondria, nuclei, plasmids), has been the difficulty of breaking open the hyphae and getting hold of the plasma membrane. Effective digestion of the cell wall is needed. In the case of Phycomyces and its close relatives this digestion is known to require two enzymes: chitinase and chitosanase. The former is available commercially; the latter has to be isolated and purified from a suitable microorganism. We have initiated such efforts with the chitosanase obtainable from a myxobacterium (Mechaber).

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110. AVOIDANCE, WIND RESPONSES, HOUSE EFFECT

Investigators: **Paul Meyer*, Max Delbrück**

In 1975 (Cohen et al.) we proposed to explain the then known effects by postulating a substance produced, emitted, and sensed by the growing zone of the sporangiophore of Phycomyces. The distribution of this substance in the vicinity of the specimen was supposed to be modified by purely aerodynamic effects.

New experimental results, reported last year (Biology 1978, Nos. 109 and 110), as well as closer mathematical analysis undertaken this year, made this scheme untenable.

We now propose instead a conversion by the obstacle of the substance emitted primarily, the precursor substance, Y, to the active form, the effector gas, X. The diffusion constant of Y must be considerably lower than that of O_2. Conversion by the emitting sporangiophore is inefficient, but is responsible for the wind effects in absence of a barrier. Quieting of random winds is responsible for the house effects, as before.

A series of experiments to test these new ideas is now under way.

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111. SEQUENCE OF CYTOCHROME C FROM PHYCOMYCES

Investigators: **Makkuni Jayaram, Michael W. Hunkapiller**

Phycomyces belongs to a class of fungi called zygomycetes. The zygomycetes are characterized by gametangiogamy, by a haploid vegetative phase, nonmotile spores, and the presence of chitin in the cell wall. The phylogenetic position of this class in relation to other fungi is not understood. Sequencing of cytochrome c (cyt c) from Phycomyces was undertaken with a view to shedding light on this problem since the evolutionary divergence of this protein has been most extensively studied. The evolutionary history of Phycomyces has become particularly interesting following the finding that Phycomyces has nearly 35% medium repetitive DNA arranged in a rather unexpected pattern of sequence organization (see Biology 1979, No. 112). The cyt c could not be directly sequenated in an Edman-type automated sequenator as the protein is blocked at the N-terminus, presumably by acetylation. All the fungal cyt c molecules so far sequenced have a free amino terminus whereas in higher plants and animals the amino terminus is blocked. The Phycomyces cyt c has one methionine and one tryptophan per molecule and from evolutionary conservation one can assign them positions 59 and 80, respectively. The molecule was cleaved chemically at these residues and the sequences of peptides (from residue 60 to 80 and 81 to the C-terminus) were determined. The succinylated cyt c was cleaved at arginine residues by trypsin. One of the peptides obtained by this method (starting with residue 39) has been sequenced.

The available sequence of Phycomyces cyt c shows weak relations to other fungal, to certain plant sequences, and to those of the flagellates Crithidia and Euglena.

112. SEQUENCE ORGANIZATION IN THE PHYCOMYCES GENOME

Investigators: **Rasika M. Harshey, Makkuni Jayaram, Margaret E. Chamberlin**

The sequence organization of fungal genomes is poorly understood. Most fungi have small genome sizes and contain relatively small amounts of repetitive DNA. Recently it has been shown that the water mold Achlya has 16% repeated DNA, arranged in a "long-period" interspersion pattern with the single copy and repetitive sequences being much longer than in Drosophila (Hudspeth et al., 1977). Since Phycomyces is known to contain high

amounts of repetitive DNA (Dusenbery, 1975), we were curious to know (1) whether the repetitive DNA of Phycomyces is interspersed with single copy sequences, and (2) what the lengths of the single copy and repeat sequences are.

Reassociation analysis of 300 NT long DNA fragments using hydroxyapatite (HAP) chromatography shows that approximately 35% of the genome consists of middle repetitive sequences. The genome size may be estimated from the reassociation rate to be 6.6×10^7 NTP. Increasing the length of the reassociating fragments up to 4100 NT does not cause an appreciable increase in the middle repetitive fraction as assessed by HAP chromatography. Therefore, if the genome is interspersed, the length of the single copy sequences must be greater than 4100 NTP. It could also be shown that the length of the repetitive sequences is greater than 4100 NTP. We have performed a more sensitive test for linkage between single copy and repetitive sequences by enriching a medium repetitive fraction of ^{3}H-labeled DNA (fragment length = 1650 NTP). This fraction was sheared to 400 NT long fragments and reassociated in the presence of unlabeled total Phycomyces DNA fragments (also 400 NT long). The reassociation data indicate that 19% of the enriched DNA consists of single copy sequences. We can then calculate that the single copy and repetitive sequences are interspersed approximately 1440 times in the genome. Since single copy and medium repetitive sequences constitute approximately 55 and 35% of the genome, the average length of the single copy sequences is 2.5×10^4 NTP and that of the repetitive sequences is 1.6×10^4 NTP. This pattern of organization is similar to what has been found in Achlya.

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Dusenbery, R. L. (1975) Biochim. Biophys. Acta **378:** 363-377.

Hudspeth, M. E. S., Timberlake, W. E. and Goldberg, R. B. (1977) Proc. Nat. Acad. Sci. USA **74:** 4332-4336.

113. RIBOFLAVIN AUXOTROPHS OF PHYCOMYCES

Investigator: **Nancy K. Wischhusen**

To help elucidate the pathway of riboflavin synthesis in Phycomyces I am attempting to isolate riboflavin auxotrophic mutants from the wild-type NRRL 1555(-) corresponding to the various steps of the pathway. I have developed a protocol of enriching for riboflavin auxotrophs by first recycling UV-mutagenized spores on rich medium, growing the next generation of spores in riboflavin-deficient medium, then using heat and filtration to kill and remove riboflavin prototrophs. I have thus far obtained three auxotrophs belonging to at least two complementation groups. Further work should generate more auxotrophs and, I hope, show that this method is applicable for many types of Phycomyces auxotrophs.

114. METABOLISM OF RIBOFLAVIN AND ANALOGS IN PHYCOMYCES

Investigators: **Manfred K. Otto, Laura E. Kochevar**

The photoreceptor of Phycomyces contains a riboflavin derivative as photopigment. Our approach to study the photoreaction in the photoreceptor is to replace the flavin pigment by analogs that are structurally related but differ in their light absorbance properties from riboflavin.

In all flavoenzymes, riboflavin-5'-phosphate (FMN) and/or flavin adenine dinucleotide (FAD) rather than free riboflavin serve as coenzymes. We therefore believe that 5'-phosphorylation of riboflavin (or analogs) is the first enzymatic step in the biosynthesis of the photoreceptor pigment. The respective enzyme is riboflavin kinase. In order to compete at the level of kinase (with riboflavin) the analog has to be taken up by the organism. The riboflavin present in the mycelium is either produced internally by endogenous biosynthesis or taken up across the membrane. Our studies therefore include (1) regulation of riboflavin biosynthesis in Phycomyces, (2) transport of riboflavin and analogs across the cytoplasmic membrane, (3) riboflavin kinase of Phycomyces and its interaction with analogs.

(1) Regulation of riboflavin biosynthesis. So far no positive evidence for regulation of riboflavin biosynthesis in Phycomyces has been obtained.

(2) Flavin transport. Phycomyces can utilize externally offered riboflavin. [2-^{14}C]riboflavin in the growth medium is taken up into the sporangiophores. Studies with riboflavin analogs indicate that a flavin-specific transport system is involved.

We studied the incorporation into Phycomyces mycelium of [2-^{14}C]riboflavin over a short period of time. During the first 3 min a constant uptake rate was observed. With riboflavin concentrations from 10^{-8} M up to 5×10^{-7} M increase in uptake was found. Higher concentrations showed no further increase in uptake rate. From a Lineweaver-Burke diagram an apparent K_m of 6×10^{-8} M was calculated. From these data it follows that

Phycomyces has a highly specific uptake system for riboflavin.

In some cases transport systems feed their substrate into closely coupled metabolic pathways whereby accumulation of free substrate and efflux is kept minimal. After several minutes of riboflavin uptake Phycomyces contains more than 60% as free riboflavin. Therefore riboflavin transport and subsequent enzymatic conversion are not closely linked.

The interaction of roseoflavin (8-dimethylamino-8-demethyl-riboflavin, the most promising candidate for incorporation into the photoreceptor) with the transport system was studied. [2-^{14}C]roseoflavin is taken up less efficiently than riboflavin. The apparent K_m is ca. 7 x 10^{-7} M. Roseoflavin inhibits uptake of riboflavin with an inhibition constant of the same order of magnitude (K_i $\sim 10^{-6}$ M); therefore the same transport system is reponsible for transport of both riboflavin and roseoflavin.

(3) Riboflavin kinase of Phycomyces. Riboflavin kinase catalyzes the transfer of a phosphate group from ATP to the ribityl side chain of riboflavin. Abundant phosphatases mask this activity in Phycomyces crude extracts. The enzyme can be obtained free of phosphatase activity by ammonium sulfate fractionation, ion exchange, and molecular sieve chromatography.

The Phycomyces enzyme differs from other known riboflavin kinases: it does not require metal ions (zinc and magnesium stimulate the enzyme only slightly), it is specific for riboflavin (K_m = 10^{-6} M), and uses ATP exclusively as phosphate donor.

115. LIGHT-INDUCED CAROTENE SYNTHESIS IN PHOTOTROPIC MUTANTS OF PHYCOMYCES

Investigators: **Leslie S. Leutwiler, Makkuni Jayaram**

Blue light stimulates the synthesis of β-carotene in Phycomyces in a biphasic manner. The low fluence response saturates at $\sim$20 J/m^2 and the high fluence response at $\sim$1000 J/m^2. It was of interest to determine if the low fluence and/or high fluence light-induced carotene synthesis was affected in mutants for the blue-light sensory pathway. Of the three mutants (madA, madB, madD) investigated, all showed lower induction than wild type in response to both low and high fluence irradiation. These data suggest a common photoreceptor for the low and high fluence responses.

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Assistant Professor: Elias Lazarides
Research Fellow: Clare M. O'Connor
Graduate Students: Joseph J. Falke*, David L. Gard, Jeff D. Gelles*, Bruce L. Granger, Bruce D. Hubbard
Research Staff: David R. Balzer Jr., Ilga Lielausis

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Summary: Our laboratory is interested in understanding the regulation of the maintenance of cell structure during muscle differentiation. Our emphasis is mainly on a newly identified filamentous system known as "intermediate filaments." We have found that this filamentous system and its major subunit in muscle cells, desmin, may be responsible for mechanically integrating laterally all myofibrils in a muscle fiber and thus bringing about the striated phenotype so characteristic of muscle morphology. In order to understand the molecular details of this process we utilize cell biology, biochemistry, and immunological techniques to study the synthesis, assembly, chemical modification, localization, and the association with membranous organelles of desmin filaments during myogenesis in tissue culture and in adult muscle.

116. PHOSPHORYLATION OF SUBUNIT PROTEINS OF INTERMEDIATE FILAMENTS FROM CHICKEN MUSCLE AND NONMUSCLE CELLS

Investigator: **Clare M. O'Connor**

The phosphorylation of the subunit proteins of intermediate (10 nm) filaments has been investigated in chicken muscle and nonmuscle cells by using a two-dimensional gel electrophoresis system. Desmin, the 50,000 dalton subunit protein of the intermediate filaments of muscle, had previously been shown to exist as two major isoelectric variants--α and β--in smooth, skeletal, and cardiac chicken muscle. Incubation of skeletal and smooth muscle tissue with $^{32}PO_4^{3-}$ reveals that the acidic variant, α-desmin, and three other desmin variants are phosphorylated in vivo and in vitro. Under the same conditions, minor components of α- and β-tropomyosin from skeletal muscle, but not smooth muscle, are also phosphorylated. Both the phosphorylated desmin variants and the nonphosphorylated β-desmin variant remain insoluble under conditions that solubilize actin and myosin filaments, but leave Z-discs and intermediate filaments insoluble. Primary cultures of embryonic chicken muscle labeled with $^{32}PO_4^{3-}$ possess, in addition to the desmin variants described above, a major nonphosphorylated and multiple phosphorylated variants of the 52,000 dalton, fibroblast-type intermediate filament protein (vimentin). Filamentous cytoskeletons, prepared from primary myogenic cultures by Triton X-100 extraction, contain actin and all of the phosphorylated and nonphosphorylated variants of both desmin and vimentin. Similarly, these proteins are the major components of the caps of aggregated 10 nm filaments isolated from the same cell cultures previously exposed to Colcemid. These results demonstrate that a nonphosphorylated and several phosphorylated variants of desmin and vimentin are present in assembled structures in muscle and nonmuscle cells.

117. DESMIN PHOSPHORYLATION BY cAMP-DEPENDENT KINASES

Investigator: **Clare M. O'Connor**

Our previous work has shown that the subunit proteins of intermediate filaments, desmin and vimentin, are phosphorylated in muscle and fibroblastic cells (O'Connor et al., 1979). When muscle cells are labeled with ^{32}P-inorganic phosphate, radioactive phosphate is incorporated into several isoelectric variants of desmin. Using desmin purified from smooth muscle as substrate, we have been characterizing the desmin kinase activities in muscle cells. The desmin kinase activity in chicken smooth and skeletal muscle is present in the low speed supernatants of cell extracts. When chromatographed on DEAE cellulose, desmin kinase activity co-elutes with the two cAMP-dependent kinases in chicken skeletal muscle. In addition, both desmin and vimentin are phosphorylated in detergent-extracted cytoskeletons by the catalytic subunits of the two cAMP-dependent kinase isozymes. The phosphorylation reaction is inhibited by a heat- and acid-stable protein similar to the inhibitor protein of cAMP-dependent kinases characterized in other tissues (Walsh et al., 1971). On one-dimensional peptide maps, the peptides phosphorylated by the cAMP-dependent kinases in vitro are similar to those phosphorylated in vivo. Together the results suggest that cAMP may be involved in the regulation of cytoskeletal structures by affecting the phosphorylation of intermediate filament subunits.

References:

O'Connor, C. M., Balzer, D. R. Jr. and Lazarides, E. (1979) Proc. Nat. Acad. Sci. USA **76:** 819-823.
Walsh, D. A. et al. (1971) J. Biol. Chem. **246:** 1977-1985.

118. COEXISTENCE OF DESMIN AND THE FIBROBLASTIC INTERMEDIATE FILAMENT SUBUNIT (VIMENTIN) IN MUSCLE AND NONMUSCLE CELLS

Investigators: **David L. Gard, Paul B. Bell Jr.**

Chicken embryo fibroblasts (CEF) and baby hamster kidney (BHK) cells were extracted with 1% Triton X-100 and 0.6 M KCl, and the resulting cytoskeletal residues were analyzed by two-dimensional isoelectric focusing-sodium dodecyl sulfate polacrylamide gel electrophoresis. Cytoskeletons of CEF cells are composed primarily of a 52,000 dalton protein, identified as the fibroblastic intermediate filament subunit (vimentin), and a small amount of β- and γ-actin. A minor component with a molecular weight of 50,000 has been identified as α-desmin, one of the two major isoelectric variants of desmin, the intermediate filament subunit of avian smooth muscle. BHK cytoskeletons exhibit a composition similar to CEF cytoskeletons, containing vimentin, β-, γ-actin, and the mammalian variant of desmin.

Cytoskeletons prepared from fibroblast-free cultures of chicken embryonic muscle contain vimentin, α-, β-, γ-actin, and desmin. These data suggest that two distinct 10 nm filament subunit species, namely the

fibroblastic intermediate filament protein (vimentin), and the smooth muscle intermediate filament protein (desmin) may coexist in cells of a single type of both muscle and nonmuscle origin.

One-dimensional peptide analysis of these cytoskeletal proteins reveals that there is considerable homology between both avian and mammalian vimentins, between mammalian and avian desmins, and between vimentin and desmin from the same species. Peptide analysis of ^{32}P-labeled desmin and vimentin from cultured skeletal muscle suggests that there is considerable similarity in the phosphorylation sites of these proteins. These results indicate that vimentin and desmin might be related evolutionarily.

119. THE ASSOCIATION OF DESMIN AND VIMENTIN WITH MYOFIBRIL Z-DISCS

Investigators: **Bruce L. Granger, David L. Gard**

Desmin, the major subunit of intermediate filaments from smooth muscle, is also found in striated muscle. Here, it surrounds each Z-disc of a myofibril, and may be responsible for linking together and promoting the registration of adjacent myofibrils. Two-dimensional polyacrylamide gel electrophoresis shows that vimentin, the major subunit of intermediate filaments from most cells of mesenchymal origin, is also a constitutent of isolated myofibrils. Indirect immunofluorescence of myofibrils and Z-disc sheets (Granger and Lazarides, 1978) reveals that vimentin has the same distribution as desmin: it forms a collar around each Z-disc, and exhibits a staining pattern complementary to that of α-actinin within a given Z-plane. Both subunits are also found in the interiors of Z-discs of those myofibrils that are presumably undergoing longitudinal subdivision.

In vitro labeling of chick myogenic cultures with [^{35}S]methionine shows that vimentin is synthesized throughout the period from plating of mononucleate myoblasts to 20 days post-fusion in culture; desmin synthesis is not detectable immediately after plating but commences within one day of plating and continues through 20 days. Initially, desmin and vimentin exist in a filamentous form in the cytoplasm and are readily aggregated by Colcemid. Within a week of fusion, they begin to associate with the Z-lines and become resistant to this induced rearrangement.

The transition of desmin and vimentin from cytoplasmic filamentous forms to Z-disc-associated forms thus exemplifies the development of a mechanically integrative structural system during the maturation of skeletal muscle fibers. It suggests that during myogenesis and myofibril assembly there is some sort of molecular transformation or stimulus that serves to bring together desmin, vimentin, and the Z-disc.

Reference:

Granger, B. L. and Lazarides, E. (1978) Cell **15:** 1253-1268.

120. AN ACTIN-DESMIN COMPLEX FROM CHICKEN SMOOTH MUSCLE

Investigators: **John J. Ngai*, Bruce D. Hubbard**

Repeated extraction of chicken gizzard with 10 mM EGTA solubilizes a small number of proteins, among them actin, desmin, α-actinin, tropomyosin, filamin, and other as yet uncharacterized proteins of 118K, 129K, and 137K molecular weights. Several lines of evidence indicate that actin and desmin coexist in a complex in smooth muscle which is at least partially solubilized with EGTA. When the EGTA extract is applied to a DNase I affinity chromatography column (in which actin is specifically bound [Lazarides and Lindberg, 1974]), actin, desmin, 129K and 137K proteins are co-retained and co-elute under conditions which release specifically-bound actin. Further evidence for an actin-desmin complex is obtained with indirect immune precipitation with anti-actin or anti-desmin antibodies, wherein actin and desmin are the major precipitating components with either antibody; tropomyosin and α-actinin are not precipitated. The size, stability, and stoichiometry of the actin-desmin complex(es) have been characterized by velocity sedimentation of the EGTA extract. Actin-desmin complexes sediment over a broad 6S to 60S range. Upon resedimentation, the 50-60S species resediments to its original position on the gradient, indicating the complex's stability. This species has an approximate stoichiometry of five actin molecules per desmin molecule.

The evidence obtained thus far implies the existence of actin-desmin complexes in calcium-stabilized structures in smooth muscle, possibly dense bodies; chelation of calcium would induce these structures to slowly dissociate, thus causing the concomitant appearance of soluble actin-desmin complexes. Presently, we are attempting to reconstitute actin-desmin complexes in vitro from isolated actin and desmin. Our assay methods include sedimentation analysis and affinity chromatography (e.g.,

DNase I, anti-actin, and anti-desmin).

Reference:
Lazarides, E. and Lindberg, U. (1974) Proc. Nat. Acad. Sci. USA **71:** 4742-4746.

*Summer student.

121. USE OF FLUORESCENT DYES AS PROBES OF MEMBRANE STRUCTURE

Investigator: **Joseph J. Falke**

We are investigating the suitability of fluorescent dyes as probes of membrane structure in living cells; in particular it may be possible to group cell types into two categories on the basis of the staining properties they show with such dyes. Easton et al. (1978) claim that merocyanine 540 (MC 540), a fluorescent dye with a charged group as well as a large hydrophobic region that can presumably intercalate into a bilayer, can distinguish electrically-excitable cells from nonexcitable cells. These workers used fluorescence microscopy to visualize the fluorescence intensity of live cell plasma membranes incubated with MC 540, and evidently the intensity associated with the membranes of excitable cells is much greater than that seen in nonexcitable cells.

We are investigating these results for artifacts and plan to develop a different probe to look independently for corresponding cell type differences in plasma membrane solubilities. An assay has been devised to search for an appropriate probe, and if the cell type differences indicated in the prior work are real, the physical basis of the differences will be investigated. The binding affinities of the membranes for this probe will be quantitated, and with fluorescence energy transfer the location of the probe-binding sites in the membrane will be determined. The relationship between membrane composition and probe solubility will be examined. Ultimately, when the physical basis for the difference in probe solubility is better understood we will be able to investigate the biological reasons for maintaining this difference.

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Easton, T. G., Valinsky, J. E. and Reich, E. (1978) Cell **13:** 475-486.
Thomas, D. D., Carlsen, W. F. and Stryer, L. (1978) Proc. Nat. Acad. Sci. USA **75:** 5746-5750.

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Professor: Jean-Paul Revel
Visiting Associate: Paul B. Bell Jr.
Research Fellows: Malcolm E. Finbow, Eva B. Griepp, David J. Meyer, Nancy L. Shinowara, S. Barbara Yancey
Graduate Students: Richard H. Gomer, Bruce J. Nicholson, Chung Wang
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Summary: We have made good progress in our understanding of the gap junction in the past year. We had long suspected that the major gap junction polypeptide had a molecular weight of approximately 26K (Biology 1975, No. 29). This polypeptide can be isolated by new methods which minimize proteolysis. It seems to be the precursor of the 10K connexin of the literature and we are presently undertaking a detailed analysis of both the degraded 10K "core" and the "native" 26K components.

Also rewarding has been our continuing investigation of gap junctions in hepatectomized animals. Of particular interest have been the electrophysiological studies which we have initiated comparing cell-cell coupling at times when junctions cannot be detected morphologically with those times when they are found. Even at times when gap junctions are essentially absent from the surface of hepatocytes electrotonic coupling is unimpaired. However, the transfer of dyes such as carboxyfluorescein is greatly decreased.

As I conclude this overview I want to indicate my appreciation for the help Pat Koen, Dave Easter, and Jean Edens have given to the research described in the following abstracts.

122. BIOCHEMISTRY OF LOSS AND REAPPEARANCE OF GAP JUNCTIONS IN REGENERATING LIVER

Investigators: **S. Barbara Yancey, Malcolm E. Finbow, David W. Easter, Jean-Paul Revel**

Experiments in our laboratory have shown that during liver regeneration in weanling rats, the gap junctions are virtually absent during the span of 28 to 36 hr after hepatectomy. They reappear and reach near normal levels by the end of the second day after hepatectomy (Biology 1977, No. 123). Enzyme-treated junction fractions isolated by the two-phase and other methods (Biology 1977, No. 129) from 30 hr regenerating liver showed a loss of the major band at 10,000 daltons which is present in fractions isolated from normal rat liver (Biology 1979, No. 123). However, by three days of regeneration, this band was again present. When no proteolytic enzymes were used and junctions isolated by the urea method (Biology 1978, No. 119), a 26,000 dalton band was present in fractions from control and three day regenerating liver but absent in the fraction from 30 hr regenerating liver. Therefore, both the 10,000 and the 26,000 dalton bands are undetectable in fractions from 30 hr regenerating liver when it is known that the gap junctions have disappeared but they are present when gap junctions become morphologically recognizable. Since other experiments show that the 10,000 dalton band can be obtained by proteolysis of the 26,000 dalton component, the data support the conclusion that 26,000 dalton band represents a major gap junction component.

123. EFFECT OF TRYPSIN ON GAP JUNCTION FRACTIONS ISOLATED WITHOUT ENZYMES

Investigators: **Malcolm E. Finbow, David W. Easter, Jean-Paul Revel**

By the use of a two-phase polymer system, plasma membrane fractions suitable for the isolation of gap junctions can be obtained from rat, rabbit, and mouse liver (Biology 1977, No. 129). Extensive treatment of these fractions with collagenase and trypsin yields very clean fractions of gap junctions. Analysis of these fractions by SDS-PAGE shows one major band at about 10,000 daltons which very likely represents a proteolytic breakdown product of junctional protein. From two-dimensional gel electrophoresis of fractions from rat liver, this band seems to represent only one polypeptide species. In order to identify the native gap junctional protein, we have devised a method for the isolation of gap junction fractions without a proteolytic step by using a 6 M urea step to remove contaminants (Biology 1978, No. 119). On SDS-PAGE, these fractions show a prominent band at about 26,000 daltons as well as bands at about 35,000, 38,000, and 50,000 daltons. After trypsinization the major change is the rapid disappearance of the 26,000 dalton band and a concomitant appearance of a 10,000 dalton band which reaches maximum density at 5 min. These results strongly suggest that the 10,000 dalton band

represents a proteolytic fragment from the 26,000 dalton peptide and that the 26,000 dalton peptide represents a major protein component of the gap junction.

124. BIOCHEMISTRY OF THE GAP JUNCTIONAL PROTEIN

Investigators: **Bruce J. Nicholson, Jean-Paul Revel**

Methods for the purification of gap junctional protein (or proteins?), and of their tryptic fragments, have been developed in both this and other laboratories. Studies are in progress to characterize the 10,000 dalton peptide so that one may compare it to other protein components (such as the 26,000 dalton component) believed to be part of gap junctions. SDS polyacrylamide urea gels have been used to examine the peptides produced from cyanogen bromide digests of the 10,000 dalton peptide. Preliminary evidence suggests three sites of cleavage. A two-dimensional assay system, using isoelectric focusing and SDS polyacrylamide urea gels, will provide a more detailed fingerprint of the cyanogen bromide peptides and will be useful in comparative studies.

In addition to this mapping, sequencing studies have been started on the 10,000 dalton peptide. Preliminary evidence shows that the N-terminus of the 10,000 dalton peptide is not blocked and a preliminary sequence of 20 amino acids has been established.

It is to be hoped that a combination of tryptic and cyanogen bromide maps and sequencing of the various proteins found in preparations of gap junctions will allow us to establish the nature of the native gap junctional protein and its relationship to the "junctional" peptides reported in the literature.

Reference:

Finbow, M., Yancey, S. B., Johnson, R. and Revel, J.-P. (1979) Proc. Nat. Acad. Sci. USA. Submitted for publication.

125. RAPID ISOLATION OF GAP JUNCTIONS FROM LIVER

Investigator: **Richard H. Gomer**

Standard isolation procedures for gap junctions (Biology 1977, No. 129) require several days of work and give relatively low yields. We have developed a procedure which can be carried out in a few hours and gives both higher yields and purity as compared to previous techniques. Although this was developed for the isolation of gap junctions from chicken liver, we have shown that it also works with rat liver. The method takes advantage of the fact that most of the contaminants in gap junction preparations are solubilized by a solution containing potassium iodide, urea, and Sarcosyl NL-97.

126. CYTOLOGICAL CHANGES IN GAP JUNCTIONS DURING LIVER REGENERATION

Investigators: **S. Barbara Yancey, David W. Easter, Jean Edens, Jean-Paul Revel**

We have used freeze-fracture replicas and thin sections to examine the ultrastructural changes in gap junctions in the regenerating liver of weanling rats at the times of gap junction disappearance (24 to 28 hr postoperative) and reappearance (36 to 44 hr postoperative). At 24 hr most hepatocytes have a normal complement of gap junctions but by 26 hr only about 25% have normal junctions while 65% have none, and 9 to 10% display only small "remnants." It has previously been proposed that gap junctions are removed from the cell surface by internalization with resultant formation of cytoplasmic "annular" gap junctions; however, we found no evidence of internalization and no annular gap junctions during the 24 to 28 hr period when gap junctions were rapidly disappearing from the regenerating liver. We conclude that the disappearance does not involve any gross internalization of gap junctional membrane. However, we did find configurations of gap junctions that could be interpreted as intermediate stages in their disappearance, views of particle dispersion at the periphery of gap junctions, and small "remnants" of gap junctions occurring singly or in small groups within particle-poor areas of membrane. No evidence could be found to support the idea that membrane structures lost from junctions by a dispersal mechanism persisted elsewhere in the membranes since the density of nonjunctional intramembrane particles did not change as it would have been expected to do. By 36 hr after hepatectomy, numerous small aggregates of gap junctional particles reappear, often associated with tight junctional strands. "Formation plaques" described in other cell systems also can be found in regenerating liver, but they are rare.

127. ELECTROTONIC COUPLING AND DYE SPREAD AFTER DISAPPEARANCE OF GAP JUNCTIONS

Investigators: **David J. Meyer, S. Barbara Yancey, Jean-Paul Revel**

Previous work with weanling rats has shown that between 28 and 36 hr after partial hepatectomy, gap

junctions virtually disappear from the surfaces of the remaining hepatocytes (Yee and Revel, 1978). Using morphometric techniques to study freeze-fracture replicas, Yancey et al. (1979) found that at 28 hr after partial hepatectomy gap junctions occupied less than 2% of the membrane area occupied by gap junctions in control livers.

Electrotonic coupling was investigated in control animals, animals hepatectomized for 30 hr (no gap junctions), and animals allowed to regenerate for three days (gap junctions have reappeared). Surprisingly, the hepatocytes in regenerating liver in which gap junctions were essentially absent were as well coupled as in controls or in livers where gap junctions had reformed. The same result was found when either the frequency of coupling, the efficacy of coupling, or the spatial extent of coupling were compared. In contrast, the spread of the fluorescent dyes, carboxyfluorescein and lucifer yellow, was much reduced (but not absent) in regenerating as compared with control livers.

As we have been able to exclude coupling by extracellular current flow, our results admit three interpretations: (1) The number of junctional channels in regenerating liver is reduced but that does not affect the extent of electrotonic coupling we can measure. (2) The number of channels remains the same, but they are no longer detectable (however, the density of intramembrane particles on nonjunctional membranes does not fit with the idea of dispersion of junctional particles and examination of thin sections makes it implausible that cell contacts are still present but not detectable by freeze-cleaving). This hypothesis also requires that the permeability of the individual channels change to explain the reduction in dye spread. (3) The tight junctions, which persist after hepatectomy, are permeable to ions but not to dye molecules. At present we favor the first of these explanations since we have obtained evidence for an increase in the input resistance of the electrical syncytium. Our results emphasize the need for caution in drawing inferences about intercellular communication from morphological data alone.

References:

Yancey, S. B., Easter, D. and Revel, J.-P. (1979) J. Ultrastruct. Res. In press.
Yee, A. G. and Revel, J.-P. (1978) J. Cell Biol. **78**: 544.

128. OUABAIN DECREASES INTERCELLULAR COUPLING IN NORMAL AND REGENERATING RAT LIVER

Investigators: **David J. Meyer, S. Barbara Yancey, Jean-Paul Revel**

De Mello (1977) has reported that the cardiac glycoside ouabain decreases the efficacy of electrotonic coupling between cells in canine Purkinje fibers and in guinea pig liver. He speculated that the effect of this drug is due to decreasing the conductance of junctional membrane by raising intracellular ionized calcium.

We have examined the effect of this drug on normal and regenerating rat liver because it is a potentially useful tool for understanding the changes in regenerating liver reported by Meyer, Yancey and Revel (Biology 1979, No. 127). We find that exposure of both normal and regenerating liver to low concentrations of ouabain (2.5×10^{-6} M) leads to an approximately 75% reduction in the size of the electrotonic potential recorded in response to a 50 nA test pulse within 5 min. A preliminary study of the spatial extent of coupling revealed that it, too, had decreased. Measurements of input resistance with a two electrode technique showed that it was the same or lower in both normal and regenerating livers. These results indicate that the primary effect of ouabain may be to decrease the resistance of nonjunctional membranes.

Reference:

De Mello, W. C. (Ed.) (1977) In: Intercellular Communication, pp. 87-120, Plenum Press, New York.

129. THE EFFECTS OF ANOXIA OR ISCHEMIA ON RAT LIVER GAP JUNCTIONS

Investigators: **S. Barbara Yancey, David W. Easter, Jean-Paul Revel**

There is evidence suggesting that anoxia or interruption of the blood supply to some tissues prior to fixation may induce interiorization of gap junctions (Fawcett, 1978). We have used freeze-fracture and thin-sectioning techniques to examine the effects of anoxia or ischemia on rat liver gap junctions. In liver made anoxic or ischemic, curvature of gap junctional membranes suggestive of incipient internalization is very common and after 1 hr of ischemia, annular gap junctions may readily be found in the cytoplasm. This is contrary to what we have found to be the case during the disappearance of gap junctions in regenerating liver (see Biology 1979, No. 126) and these results offer support for our conclusion that the disappearance of gap junctions during regeneration does

not require gross internalization of gap junctional membrane.

Reference:
Fawcett, D. W. (1978) In: Electron Microscopy, Vol. 111, pp. 643-650, Microscopical Society of Canada, Toronto, Ontario.

130. MORPHOLOGY AND ELECTROPHYSIOLOGICAL PROPERTIES OF CELL JUNCTIONS IN DICYEMID MESOZOA

Investigators: **Chung Wang, Jean-Paul Revel**

In previous reports (Biology 1977, No. 133 and Biology 1978, No. 128) we have shown the existence of gap junctions between cells of dicyemids, very primitive organisms living as parasites in molluscs. We have now perfected a fixation method which allows us to define the architecture of the organism more clearly: we still cannot find any septate junctions, tight junctions, or desmosomes in thin sections or freeze-cleaved samples. It seems likely, therefore, that gap junctions are the only kind of cell junctions between cells of this primitive organism.

We have successfully cultivated the organism by using the method of Lapan and Morowitz (1975) with the following modifications: (1) we use filtration instead of centrifugation for isolation, and (2) the organism is kept at low density and stored under refrigeration. By using conventional electrophysiology techniques, we found the adjacent cells are electrically coupled and allow dye transfer. This result suggests that the gap junctions of dicyemids may be functionally identical to those of higher organisms.

Reference:
Lapan, E. A. and Morowitz, H. J. (1975) J. Exptl. Zool. **193:** 147-160.

131. EPITHELIAL MESENCHYMAL INTERACTION IN FEATHER FORMATION

Investigators: **Jean-Paul Revel, Leonard Levy***

Previous work by Rawles (1963) and by Dhouailly et al. (1978) has shown that the mesenchyme exerts a powerful influence on feather differentiation, dictating not only their formation but also the kind of feather. Duck mesenchyme can direct chick ectoderm to form duck-like feathers. We decided that this might be an especially good system to study epithelial mesenchymal interactions since epithelium and mesenchyme can be dissociated and reassociated to form functional structures. As a first step we have begun a morphological investigation both by thin-sectioning and freeze-cleaving of developing feather germs on the back and neck skin of 7- to 8-day chick embryos. We have found evidence for cellular extensions arising both from ectoderm and mesenchyme which traverse the basement lamina, thus opening the possibility that there could be direct contact between the cells. At present we have one example which suggests the contacts might be via gap junctions.

References:
Dhouailly, D., Rogers, G. E. and Sengel, P. (1978) Devel. Biol. **65:** 58-68.
Rawles, M. E. (1963) J. Emb. Exptl. Morphol. **11:** 765-789.

*Undergraduate, California Institute of Technology.

132. THE MORPHOLOGY OF BAG CELL NEURONS IN THE ABDOMINAL GANGLION OF APLYSIA

Investigators: **Malcolm E. Finbow, David W. Easter, Leonard K. Kaczmarek, Felix Strumwasser, Jean-Paul Revel**

We have studied the morphology of the bag cell (BC) neurons by freeze-fracture techniques in order to discover the basis of electrical coupling, dye coupling, and the synchrony of firing of these neurons. The BC somata do not appear to make direct contact with one another, being totally surrounded by a complex meshwork of neurites presumably arising from the BC somata themselves. However, numerous large gap junctions are found between the neurites surrounding the somata. These junctions are similar to gap junctions found in other molluscs with large particles located on the P-face and corresponding E-face pits. Only a few gap junctions of small size are found between the BC neurites in the connective region of the ganglion. It would seem, therefore, that the gap junctions between the neurites of the BC body cluster are responsible for the synchronous firing of these neurons.

133. GAP JUNCTIONS BETWEEN NERVE FIBERS AND GLIA

Investigators: **Nancy L. Shinowara, Jean-Paul Revel**

Examination of freeze-etched preparations and sections of trout spinal cord reveals many gap junctions between unmyelinated nerve fibers and associated astrocyte processes, identified by the presence of fibrils, and densely-particulate P-faces. One glial process was found which made gap junctions with two separate nerve fibers. In this instance there was a potential direct pathway for small metabolites and ions between the two neurons. Gap

junctions were also observed between astrocytic processes and the outer layer of myelin in myelinated fibers.

134. THE STUDY OF ADHESION AND SPREADING OF NOVIKOFF HEPATOMA CELL

Investigators: **Chung Wang, Jean-Paul Revel**

Novikoff hepatoma cells usually will not adhere and spread on artificial substratum. However, we have succeeded in making the cells adhere and spread on glass surfaces as well as on Falcon dishes by coating the surface with fibronectin. We have done morphological studies of the attachment process by scanning electron microscopy. The process can be roughly separated into three events: (1) attachment at the contact point, (2) growth of filopodia and lamellipodia, and (3) flattening of the cell.

After the cells have attached firmly, their plasma membrane can be removed at various stages of cell spreading by use of 1% Triton X-100, revealing the insoluble cytoskeleton network which can be examined by both scanning and transmission electron microscopy.

The molecular organization of the attachment plaque and the molecular mechanism of in vitro cell adhesion and spreading is under investigation.

135. HEMOCYANIN COUPLED TO PROTEIN A AS A VISUAL MARKER FOR IMMUNOCHEMICAL ELECTRON MICROSCOPY

Investigators: **Marcia Miller*, Cathy Strader**, Jean-Paul Revel, Michael A. Raftery**, Raymond L. Teplitz***

Protein A coupled to hemocyanin (Hcy/A) provides a versatile immunochemical marker for electron microscopy that can be used with antisera from a variety of animal species and that is easily identifiable in a number of preparative techniques for both scanning and transmission electron microscopy. Hemocyanin from Busycon canaliculatum, purified and concentrated as previously described (Smith and Revel, 1972), was coupled to protein A, obtained from Pharmacia, using glutaraldehyde. The labeling activity and specificity of the Hcy/A conjugate was tested with several indirect labeling preparations. In parallel experiments with hemocyanin conjugated to goat anti-rabbit IgG (Hcy/Ig), Hcy/A was tested for the detection of antibodies to an embryonic chick erythrocyte antigen. Hcy/A was found to provide high specific labeling, as occurs with Hcy/Ig, with the added advantage of very low background labeling on the surrounding substrate surface. The Hcy/A was also used to detect antibodies to acetylcholine receptors on acetylcholine receptor-enriched membrane fragments. Hcy/A was also found useful in the detection of anti-human blood group antibodies. The size and distinctive shape of the hemocyanin molecule allows the detection of the Hcy/A conjugates in both thin sections and replicas for the TEM and conventional preparations for the SEM.

Reference:

Smith, S. B. and Revel, J.-P. (1972) Devel. Biol. **27:** 434.

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

136. CYTOCHALASIN B-INDUCED CHANGES IN THE DISTRIBUTION OF THE TRITON-INSOLUBLE CYTOSKELETON

Investigators: **Paul B. Bell Jr., Jean-Paul Revel**

Scanning electron microscopy of BHK cells that were stabilized by the reversible protein cross-linking reagents dimethyl 3,3'-dithiobispropionimidate and dithiobis (succinimidyl) propionate and extracted with Triton X-100 reveals that the cytochalasin-induced arborization of the cells is accompanied by a redistribution of the cytoskeleton. Treatment with cytochalasin B is followed within 5 min by the appearance of small foci of condensed cytoskeletal fibers. By 90 min the bulk of the cytoskeleton has become aggregated into a large mass located to one side of the cell nucleus. Small foci of cytoskeletal material located at the cell periphery remain attached to the perinuclear mass by thin strands. The nucleus of control cells is encased by large numbers of cytoskeletal fibers, but after 90 min in cytochalasin the nucleus is only sparsely covered with cytoskeletal fibers and it remains attached to the perinuclear cytoskeletal mass by only a few strands. This may account for the ease with which cytochalasin-treated cells can be enucleated by centrifugation. Whole cells and cross-linked cytoskeletons were reduced by mercaptoethanol and run on SDS-PAGE. Although much material is solubilized by the detergent, no decrease in the amount of Triton-insoluble actin, desmin, intermediate filament protein or tubulin was found in cells that were extracted following cytochalasin treatment. Since the Triton-insoluble proteins presumably represent the assembled cytoskeletal elements of the living cells, we suggest that cytochalasin causes changes in the distribution rather than in the state of the cytoskeletal elements. Transmission EM of thin sections of Triton-

extracted cells confirms that the microfilaments, intermediate filaments, and microtubules are not solubilized but become reorganized in cytochalasin-treated cells.

PUBLICATIONS

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Griepp, E. B., Shinowara, N. L. and Revel, J.-P. (1979) Proteolysis and gap junction formation in beating embryonic heart cell cultures. Exptl. Cell Res. Submitted for publication.

Kaczmarek, L. K., Finbow, M., Revel, J.-P. and Strumwasser, F. (1979) The morphology and coupling of Aplysia bag cells within the abdominal ganglion and in cell culture. J. Neurobiol. In press.

Lazarides, E. and Revel, J.-P. (1979) The molecular basis of cell movement. Scientific American **240:** 100-113.

Revel, J.-P. (1978) Morphological and chemical organization of gap junctions. Ninth International Congress on Electron Microscopy, Toronto, Canada.

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Revel, J.-P., Griepp, E. B., Finbow, M. and Johnson, R. (1978) Possible steps in gap junction formation. Zoon **6:** 139-144.

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Solursh, M. and Revel, J.-P. (1978) A scanning electron microscope study of cell shape and cell appendages in the primitive streak region of the rat and chick embryo. Differentiation **11:** 185-190.

Strader, C. D., Revel, J.-P. and Raftery, M. A. (1979) Demonstration of the transmembrane nature of the acetylcholine receptor by labeling with anti-receptor antibodies. J. Cell Biol. Submitted for publication.

Yancey, S. B., Easter, D. and Revel, J.-P. (1979) Cytological changes in gap junctions during liver regeneration. J. Ultrastruct. Res. In press.

Yee, A. G. and Revel, J.-P. (1978) Loss and reappearance of gap junctions in regenerating livers. J. Cell Biol. **78:** 544-564.

Professor Emeritus: Anthonie Van Harreveld
Research Staff: Shian Y. Lee

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137. EFFECTS OF L-PROLINE ON SPREADING DEPRESSION

Investigator: **A. Van Harreveld**

There is evidence that L-proline can act as an L-glutamate antagonist in the central nervous system. It also can prevent spreading depression (SD) in the chicken retina (Van Harreveld and Fifkova, 1973). The latter effect was found to be a biphasic one. At low concentrations (2 to 2.5 mM) L-proline had a marked inhibitory effect on SD. At higher concentrations (5 to 7 mM) the depression was less pronounced, to increase again at still higher concentrations (10 mM and higher). This complicated effect can be explained by the concept that the retina can under appropriate circumstances entertain two mechanisms of SD, one caused by a release of glutamate from the intracellular compartment, the other by a release of potassium ions (Van Harreveld, 1978). It was postulated that the inhibiting effect of L-proline at low concentrations (2 mM) is due to the competition of released glutamate and L-proline for glutamate receptors on dendritic (and somatic) membranes. It was furthermore postulated that the binding of L-proline to the glutamate receptors causes at 5 to 7 mM a moderate release of potassium into the extracellular space, promoting K^+-based SDs. This is supported by the observation that lowering of the K^+ concentration in the bathing solution depresses the incidence of SD at 5 to 7 mM L-proline. A more marked K^+ release at higher L-proline concentrations (5 to 10 mM) would prevent SD.

References:

Van Harreveld, A. (1978) J. Neurobiol. **9:** 419-431.

Van Harreveld, A. and Fifkova, E. (1973) J. Neurochem. **20:** 947-962.

138. CORRELATION OF ANTI-SPREADING DEPRESSION PROPERTIES AND AMNESTIC POTENCY OF SOME PROLINE ANALOGS

Investigator: **A. Van Harreveld**

Rall and Rinsel (1973) proposed swelling of synaptic spines as a mechanism for facilitation of synaptic conduction, as occurs, for instance, during conditioning. It has furthermore been postulated that swelling of dendritic spines observed during spreading depression (SD) is caused by a glutamate release from the intracellular compartment. These considerations led to experiments in which

the effect of L-proline on one-trial conditioning in chicks was investigated. This compound, which is a glutamate antagonist, had indeed an amnestic effect on conditioning in chicks (Van Harreveld and Fifkova, 1974; Cherkin et al., 1976).

The effects of a number of proline analogs on SD in the chicken retina were investigated, and compared with their effects on conditioning in chicks. The latter part of the investigation was carried out by Dr. A. Cherkin and his co-workers at the Veterans Administration Hospital, Sepulveda, California. The effects of the following compounds were examined: L-proline, D-proline, L-pyroglutamate, DL-3,4-dehydroproline, L-4-hydroxyproline, all of which are characterized by a five-membered ring structure; L-baikiain and DL-pipecolic acid which have six-membered rings, and L-azetidine-2-carboxylic acid with a four-membered ring structure. The racemic compounds were applied in twice the concentrations as those of the L- and D-enantiomeres. Of the compounds with five-membered rings, D-proline and L-pyroglutamate had no effect on SD. The other amino acids had the same biphasic effect on SD as L-proline (see preceding abstract). L-baikiain exhibited the biphasic effect on SD, whereas DL-pipecolic acid had no effect. The difference between these compounds is the presence of a double bond in the 4-5 position in L-baikiain. L-azetidine-2-carboxylic acid did not show the biphasic effect. All the analogs exhibiting the biphasic effect on SD typical for L-proline also had amnestic properties, in contrast to the compounds which showed no effect or no biphasic effect (1-azetidine-2-COOH). These observations support the postulate that a glutamate action is involved in the one-trial conditioning investigated.

References:

Cherkin, A., Eckardt, M. J. and Gerbrandt, L. K. (1976) Science **193:** 242-244.
Rall, W. and Rinsel, J. (1973) Biophys. J. **13:** 648-688.
Van Harreveld, A. and Fifkova, E. (1974) Brain Res. **81:** 455-467.

139. EFFECT OF L-PROLINE ON THE CRUSTACEAN NEUROMUSCULAR JUNCTION

Investigator: **A. Van Harreveld**

Glutamate is generally considered as the excitatory transmitter compound of the crustacean neuromuscular junction (Takeuchi and Takeuchi, 1964). A glutamate antagonist such as L-proline can therefore be expected to have an effect on the transmission in this synapse. The effect of L-proline on the "fast" and "slow" contraction of the closer muscle of the claw in Procambarus clarkii was investigated. The claw was perfused with a physiological solution. During a series of stimuli a 1 ml bolus of physiological solution containing the intended concentration of L-proline was passed through the claw. The fast contraction elicited by single shocks was inhibited by L-proline (threshold 2 to 3 mM). Also the slow contraction (elicited by 15 sec faradic stimulation at various frequencies) was inhibited. The inhibiting effect of a given concentration of L-proline was dependent on the frequency of stimulation.

Reference:

Takeuchi, A. and Takeuchi, N. (1964) J. Physiol. **170:** 296-317.

140. A HISTOCHEMICAL METHOD FOR THE ELECTRON MICROSCOPIC LOCALIZATION OF CHLORIDE

Investigators: **A. Van Harreveld, Shian Y. Lee**

There is abundant evidence that under appropriate conditions movements of sodium chloride occur between the intra- and extracellular compartments. A histochemical method for chloride suitable for investigation with the light microscope has been used previously for the demonstration of such ion shifts. The procedure consists of freeze-substitution of brain tissue in a solvent (usually acetone) saturated with silver nitrate at -20°C. This method failed when adapted to electron microscopy because the solubility of NaCl in acetone containing some water released during the substitution caused chloride movements in the very surface layer of the frozen tissue which only is suitable for electron microscopy.

To circumvent this difficulty a method was investigated in which the tissue is freeze-dried and then infiltrated with water-free acetone containing a silver compound of crown ether (Benzo-15-Crown-5). Minute sodium chloride crystals seem to be converted to AgCl by this method. These crystals can be made more visible by applying a procedure in which silver is deposited on the crystals, as in photography.

PUBLICATIONS

Cherkin, A. and Van Harreveld, A. (1978) L-proline and related compounds: Correlation of structure, amnesic potency, and anti-spreading depression potency. Brain Res. **156:** 265-273.

Fifkova, E. and Van Harreveld, A. (1978) Changes in dendritic spines of the dentate molecular layer during conditioning. Soc. Neurosci. Abstracts, 257.

Van Harreveld, A. (1978) Two mechanisms for spreading depression in the chicken retina. J. Neurobiol. **9:** 419-431.

Van Harreveld, A. (1979) Effects of L-proline and some of its analogs on retinal spreading depression. J. Neurobiol. **10:** 355-365.

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CELLULAR NEUROBIOLOGY

Jeremy P. Brockes

A. James Hudspeth

Henry A. Lester

Felix Strumwasser

Assistant Professor: Jeremy P. Brockes
Graduate Students: Karl J. Fryxell, Greg E. Lemke
Research Staff: David R. Balzer Jr., Don J. Nishiguchi
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Summary: We are interested in the molecular basis of cellular interactions that occur in the nervous system. Our approach is to set up these interactions between purified cells growing in tissue culture. This should allow us to establish assays for the purification and characterization of the molecules involved. Much of our work is concerned with the Schwann cell--the principal glial cell in the peripheral nervous system which is most familiar as the cell that generates the peripheral myelin sheath.

We find that the major peripheral myelin proteins are not made by the Schwann cell unless it is triggered by contact with an appropriate axon. The production of these proteins may therefore serve as an assay for the interaction with the nerve--an interaction that we are trying to establish under defined conditions in order to determine the nature of the neuronal signal. It seems that not all of the characteristic components of myelin have to be induced by the nerve since the complex glycolipid sulfatide is made by Schwann cells in isolation.

There is currently considerable interest in factors controlling the proliferation of glial cells since this is an important aspect of normal development in the nervous system, and also of the response to axonal injury in the mature animal. We have observed that purified Schwann cells grow and divide very slowly in culture unless provided with a protein present in pituitary and brain. This activity is restricted to the nervous system and appears to be both novel and potent. For these reasons and also because of the availability of a convenient assay, we have undertaken the purification and characterization of this component. This may allow us in the future to investigate its possible role as a circulating growth factor/hormone or as a local signal exchanged between cells in the nervous system.

In all of our studies we make considerable use of immunological methods, particularly of cell surface immunology. We hope to bring these methods to bear on new projects involving the effect of steroid hormones on developing neurons in the rodent and avian brain.

141. PARTIAL PURIFICATION OF A BOVINE PITUITARY GROWTH FACTOR FOR CULTURED RAT SCHWANN CELLS

Investigators: **Jeremy P. Brockes, Herman J. Gordon, Greg E. Lemke, Don J. Nishiguchi**

Schwann cells maintained in culture in 10% fetal calf serum are stimulated to divide by a protein present in the bovine pituitary and brain (Brockes et al., 1979). This activity can be conveniently assayed by determining the incorporation of [^{125}I]iododeoxyuridine into the DNA of purified Schwann cells growing in microwells. After fractionation of crude extracts of pituitary by ammonium sulfate precipitation, the activity was purified about 3000-fold by cation exchange chromatography on carboxymethylcellulose and phosphocellulose followed by gel filtration on AcA Ultrogel. After ion exchange chromatography, which removes inhibitory activity in the crude extracts, the active fractions give a 50- to 100-fold stimulation of IUdR incorporation at plateau, and a dose response curve that is approximately linear with log protein concentration. The most purified preparations give plateau stimulation of IUdR incorporation at a concentration of about 200 ng/ml. They have no effect on the proliferation of oligodendrocytes (identified with fluorescent antibodies to galactosylcerebroside) in cultures of the rat corpus callosum. The activity elutes from the AcA column at a position corresponding to an apparent molecular weight of approximately 50,000 daltons. The AcA fraction has a simplified protein composition on SDS gels, but is not homogeneous. About 1 mg of protein was obtained from a preparation of 5 kilos of bovine pituitaries. The activity is currently being purified from 20 kilos of pituitaries.

We hope to obtain enough of this material to characterize the molecule, and raise antisera to study its distribution by radioimmunoassay and immunofluorescence. We are particularly interested in the function of this factor; for example it may be released into the circulation by the pituitary, or be of significance for glial division in the brain during development or after lesioning.

Reference:

Brockes, J. P., Fields, K. L. and Raff, M. C. (1979) Brain Res. **165:** 105-118.

142. PRELIMINARY STUDIES ON A BOVINE BRAIN GROWTH FACTOR FOR CULTURED RAT SCHWANN CELLS

Investigator: **Greg E. Lemke**

The Schwann cell mitogenic activity that we are presently isolating from bovine pituitary can be similarly extracted from bovine brain. Acidic extracts of whole brain contain mitogenic activity at a level approximately 30% of that of pituitary crude extracts. If, on the other hand, the brain is dissected into various anatomical areas and extracts prepared from them, one finds a regional variation of specific activity throughout the brain; one region, the caudate nucleus, shows an even higher level of activity than that of the pituitary. The level varies from approximately 25% of the specific activity in pituitary extracts for some brain areas (e.g., medulla, thalamus) to approximately 120% of that specific activity for the caudate nucleus. The pituitary mitogen appears to be equally distributed among anterior, posterior, and intermediate lobes.

Preliminary biochemical characterization of the brain activity presently under way, as well as an eventual analysis with antisera raised against the pituitary factor, should tell us whether or not the two are the same molecule. The biological significance of the regional variation of the activity in the brain remains open to speculation.

143. ASSAYS FOR THE PERIPHERAL MYELIN PROTEINS IN SCHWANN CELLS AT VARIOUS DEVELOPMENTAL STAGES

Investigators: **Jeremy P. Brockes, Don J. Nishiguchi, Martin C. Raff***

Over half of the protein in peripheral rat myelin is present as the glycoprotein P_0 (molecular weight approximately 2.5×10^4 daltons) and the other major components are the basic proteins P_1 and P_2. These proteins could either be normal components of the Schwann cell membrane which are incorporated into myelin, or they could be induced as a consequence of the interaction with myelinated axons that initiates myelination. We have used both immunochemical and immunofluorescence assays to investigate the presence of these components. These have depended on making a rabbit antibody to P_0 (excised from preparative SDS gels) and on a gift of rabbit antibody to the basic proteins (from Dr. D. McFarlin, National Institutes of Health).

The antiserum to P_0 did not react detectably with polyacrylamide gels containing central rat myelin, or with P_1 and P_2 in peripheral myelin; it did react with P_0 in peripheral myelin and in extracts of adult and neonatal sciatic nerve. When reacted with frozen tissue sections using indirect immunofluorescence, it did not stain central myelin, but did stain myelin in adult sciatic nerve, the few myelinated fibers in cervical sympathetic trunk, and occasional areas in neonatal sciatic nerve where Schwann cells had presumably begun to myelinate. Antiserum to basic protein reacted with both of the basic protein bands in central and peripheral myelin, but not with P_0; P_1 and P_2 were detectable in adult and neonatal sciatic nerve. In indirect immunofluorescence assays, the antiserum stained both central and peripheral myelin, the few myelinated fibers of sympathetic trunk, and myelinating regions of neonatal sciatic nerve.

Cultured secondary rat Schwann cells showed no detectable reaction with either reagent, using either technique. We conclude that these three proteins are probably expressed as a consequence of the neuron-Schwann cell interaction that initiates myelination. Furthermore, their synthesis in myelinating co-cultures of neuron and Schwann cell should be an excellent assay for this interaction.

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144. INTERACTION OF CULTURED RAT SCHWANN CELLS WITH ARTIFICIAL FIBERS

Investigators: **A. James Hudspeth, Richard A. Jacobs, Jeremy P. Brockes**

It has been claimed that cultured Schwann cells will wrap around and myelinate 20 to 30 μM diameter fibers of tungsten, glass, or nylon (Ernyei and Young, 1966). The evidence for this was based on polarized light microscopy and a neuroanatomical stain for myelin, and it has not been generally accepted. We have reinvestigated this question using purified Schwann cells, and more decisive criteria for myelination. In preliminary experiments we have plated cells onto a spun-bonded nylon mesh with fibers of approximately 20 μM diameter. Some of the cells which stick to the mesh flatten down upon the fibers and enfold them over their entire circumference. We have examined four such cells in the electron microscope after embedding and sectioning. They show a limited area of membrane overlap (mesaxon) but no evidence of membrane compaction or myelin formation. Immunofluorescent staining of cells on the mesh has not provided

any evidence for induction of P_0, the major protein of peripheral myelin. We are continuing these studies with fibers of smaller diameters. The system may be favorable for investigating those features of the neurite-Schwann cell interaction that are dictated solely by geometry, and for possible derivatization of the fibers with neuronal surface components that may be implicated in this interaction.

Reference:

Ernyei, S. and Young, M. R. (1966) J. Physiol. **183:** 469-480.

145. SYNTHESIS OF SULFATIDES BY SCHWANN CELLS

Investigator: **Karl J. Fryxell**

Sulfatides are sulfolipids composed of one molecule each of sphingosine (an unsaturated amino alcohol), galactose, sulfate, and a fatty acid, all covalently linked. Sulfatides are found primarily, perhaps exclusively, in myelin, both central and peripheral, and make up about 1 to 4% of its dry weight.

We find that both rat Schwann cells (SC) and RN2 (a peripheral rat neurinoma with glial characteristics) incorporate significant amounts of $^{35}SO_4$ into lipid; rat fibroblasts do not (i.e., <1% of the incorporation by SC per mg protein). Thin-layer chromatography of SC lipids shows three bands of radioactivity. On each of two different solvent systems, the two most prominent bands correspond precisely in mobility to the two bands of authentic sulfatide from ox brain. RN2 lipids show only one band of radioactivity, whose mobility is different from both the sulfatide standard and the SC bands.

Dawson et al. (1977) were unable to demonstrate any sulfolipid synthesis by RN22 (a subclone of RN2); Lucas et al. (1977) found that RN2 did synthesize detectable amounts of sulfolipid, which appeared to co-migrate with sulfatide in their solvent system. Using a similar solvent system, we find that the mobilities are distinguishable when both standard and unknown are run in the same track. On another solvent system, the differences are much greater. The identity of the sulfolipid synthesized by RN2 is unknown.

Mirsky et al. (1979) found that purified SC lose the ability to be labeled by antibody directed against sulfatide after a few days in culture. Our results show, however, that cultured SC do not stop synthesizing sulfatide entirely. Thus sulfatide, in contrast to the myelin proteins (Biology 1979, No. 143), is a characteristic component of the myelin membrane that is synthesized in detectable amounts by SC in the absence of neurons. Sulfatide synthesis may provide a practical assay for myelination in vitro.

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146. CHEMICAL STUDIES OF A SCHWANN CELL ANTIGENIC MARKER

Investigator: **Karl J. Fryxell**

Rat neural antigen 1 (RAN-1) is found only on Schwann cells (SC) (Brockes et al., 1977) and a minority of cells in the meninges (K. L. Fields, unpublished). It may be significant that both of these cell types are probably derived from the neural crest. RAN-1 was originally defined by making a polyspecific mouse antiserum against a rat neural tumor line (33B), and then extensively adsorbing the antiserum with rat liver, spleen, and thymus (Fields et al., 1975).

Present evidence strongly suggests that RAN-1 is a membrane protein; attempts to detect it after solubilization, however, have not been successful (Fields, 1977). Whether this is due to proteolysis or to the loss of antigenicity in the presence of detergents is unclear. We have decided to try a different approach--by running solubilized membrane preparations on SDS polyacrylamide gels, fixing the proteins and washing, we can react the separated proteins with antibody in the absence of detergent (Burridge, 1976). Antibody binding is then detected by reaction with [^{125}I]Staphylococcus aureus protein A and autoradiography.

With liver, spleen, and thymus-adsorbed antiserum, we find several bands of immunoreactivity among the membrane proteins of SC or 33B. If the antiserum is further adsorbed with B28 (a rat neural cell line that lacks RAN-1), then SC show only one prominent band, with an apparent (unreduced) molecular weight of approximately 170,000 daltons. This band is clearly absent from fibroblast and B28 membrane proteins. However, 33B has several weak bands of immunoreactivity remaining, and it is unclear if the 170 K band is present in 33B.

At present we intend to try adsorbing the antiserum with fibroblasts to eliminate any remaining nonspecific

bands of reactivity in 33B. We also plan to test membrane proteins from nerve and myelin fractions for immunoreactivity, and to further define the relation between 33B and SC proteins using two-dimensional electrophoresis. The identification of RAN-1 may allow structural studies of the molecule(s) and the production of antisera that are more potent and have a wider phylogenetic range of reactivity. This in turn might allow RAN-1 to be used as a developmental marker.

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147. LONG-TERM CULTURE OF PURIFIED DORSAL ROOT GANGLION NEURONS

Investigators: **Don J. Nishiguchi, Karl J. Fryxell**

A major, long-term goal of this laboratory is to develop a quantitative assay for myelination in vitro and to exploit it for molecular studies of the early events in myelination. Such an assay will require a cell culture system in which myelination occurs as quickly and extensively as possible, and in a reproducible manner. It would also be desirable to minimize the amount of cellular heterogeneity in these cultures, and to provide any reagents that are added with good access to the cells. Although myelination has been observed in explant cultures, we feel that dissociated cell cultures will be necessary to meet the above criteria. Methods for preparing and growing purified Schwann cells (SC) are already well worked out (Brockes et al., 1979). We have begun work on the culture of purified populations of dissociated neurons, as the next step in the development of a defined myelinating culture system.

The dorsal root ganglion (DRG) is a promising source of such neurons, because: (1) the neurons can be dissocated with relatively good survival, perhaps because there are no synapses within the DRG to hold the cells together; (2) in the presence of nerve growth factor the neurons grow processes quite actively; (3) the DRG has fewer types of non-neuronal cells than do central nervous system cultures, simplifying the elimination of non-neuronal cells; and (4) the DRG produces a higher proportion of myelinated axons than other peripheral ganglia do.

When chick DRG cells are grown in a serum-free medium (Bottenstein and Sato, 1979), only the neurons survive. We find, however, that rat DRG non-neuronal cells survive and slowly increase in number in this medium. The non-neuronal cells can be killed by shifting for a few days to a serum-based medium that also contains the pituitary SC growth factor (Biology 1979, No. 141) and the anti-mitotic agent cytosine arabinoside. Of the culture substrates that we have tried, a collagen gel (Bornstein, 1958) seems to be the best for long-term adhesion and survival of the DRG neurons. We hope to add purified SC to such neurons and measure the level of P_0 as an index of myelination (Biology 1979, No. 143).

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148. AN IMMUNOLOGICAL APPROACH TO SEXUAL DIFFERENTIATION OF THE RODENT BRAIN

Investigators: **Jeremy P. Brockes, Mark Gurney**

There are characteristic differences in the behavior and physiology of male and female rats (and of other species, for that matter). Neuroanatomical studies of certain regions of the brain, such as the hypothalamus and preoptic area, have also revealed differences between the two sexes. The newborn rat brain is thought to be "wired" in the female configuration in genetically female and male animals. During the first week post-gestation, the brain is switched to the male configuration if it is exposed to circulating testosterone. Thus a female can be irreversibly masculinized by injecting androgen during this period.

There are several hypotheses about the mechanism of androgen action, but most of them predict that there should be some molecular difference(s) between males and females in these areas. We are attempting to uncover such differences by using a cross-sex syngeneic immunization protocol which has been effective in defining sex-specific antigens in non-neural tissue. The hypothalamus and preoptic area have been dissected from 4-day-old rats (at 4 days of age, androgen treatment exerts a maximal effect on the suppression of both lordosis behavior and cyclic gonadotropin secretion) and used to immunize adult

rats of the opposite sex in the same inbred strain. We are currently investigating the resulting sera by (1) reaction with solubilized immunogen in polyacrylamide gels, (2) cytotoxicity testing on cell suspensions from the hypothalamus, and (3) indirect immunofluorescence on tissue sections, and on cultured cells exposed to hormone in vitro.

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Associate Professor: A. James Hudspeth
Graduate Students: David P. Corey, Ruth A. Eatock, Herman J. Gordon
Research Staff: Norma Crippen, Richard A. Jacobs

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Summary: Hair cells are specialized epithelial cells which are the primary receptors of the vertebrate inner ear and lateral line organ. Each cell is a mechanoreceptor which produces electrical signals in response to movements of its hair bundle, a cluster of large microvilli (stereocilia) and a single true cilium (kinocilium) projecting from the cellular apex. The nature of the stimulus that evokes hair bundle displacement determines the modality to which a given hair cell is sensitive: sound, vibration, angular acceleration, linear acceleration, or water movement. The electrical response induced in the cell by appropriate stimuli modulates the release of a chemical transmitter from synaptic sites on the basal and lateral surfaces of the hair cell, and thereby controls the firing rate of the postsynaptic neurons that convey the signal into the central nervous system.

We are interested in the transduction process by which mechanical stimulation evokes an electrical response, the receptor potential, from a hair cell. We have developed an in vitro preparation of hair cells from the sacculus of the frog's inner ear with which it is possible to record receptor potentials intracellularly while the hair bundles of single cells are stimulated with a fine probe. By this means, we are able to study how a cell responds to stimuli of known amplitude, direction, and velocity. We are employing this preparation not only to study the basic electrophysiology of the hair cell's membrane, but also to investigate the physiological effects of treatments which produce permanent damage to hair cells and consequent deafness or vertigo: overstimulation, or auditory trauma, and exposure to aminoglycoside antibiotics.

149. IONIC BASIS OF THE RECEPTOR CURRENT IN A VERTEBRATE HAIR CELL

Investigators: **David P. Corey, A. James Hudspeth**

The apical surfaces of hair cells in virtually all vertebrate species face an unusual solution, endolymph, with high K^+ and low Na^+ concentrations. This has suggested to some that K^+ carries the receptor current in these hair cells. Yet large intracellular receptor potentials can be recorded from saccular hair cells in vitro when a standard saline solution bathes both apical and basal surfaces, and thus when the electrochemical gradient for K^+ is near zero. We have investigated the ionic basis of the receptor current in two ways: by voltage-clamping single cells to measure the reversal potential of the receptor current, and by superfusing the apical surfaces of hair cells separately from the basal surfaces to learn which ions can maintain the extracellularly-recorded receptor potential.

The reversal potential with normal saline at the apical surface is -2 mV, which is not the reversal potential for either K^+ or Na^+, but which could result with a transduction channel permeable to both ions. We consequently tested a number of monovalent cations by superfusing the apical surfaces of hair cells while recording receptor potentials extracellularly. The response is

maintained when endolymph at the apical surface is replaced with 130 mM Li^+, Na^+, K^+, Rb^+, Cs^+, or NH_4^+; it is smaller but measurable with ions as large as tetramethylammonium, triethanolamine, and tris(hydroxymethyl)aminomethane. The channel is thus relatively nonspecific; if it is in fact a pore, the largest of these ions would require a minimal pore diameter of about 0.6 nm. In vivo, with endolymph on the apical cell surface, K^+ must carry most of the receptor current.

In addition, the voltage dependence of the receptor current enables us to infer something about the structure of the channel itself. The receptor current increases more than proportionally with voltage, consistent with the major barrier to ion permeation being near the middle of the membrane; the best fit to the data occurs for a model with a barrier 40% of the way through the membrane.

This work suggests that the hair cell's transduction channel is a water-filled pore of about 0.6 nm internal diameter and of low ionic selectivity. There is probably a single predominant barrier to cation permeation--a "tight spot"--occurring near the middle of the pore.

150. ADAPTIVE SHIFT OF THE OPERATING RANGE OF HAIR CELLS

Investigators: **David P. Corey, A. James Hudspeth, Ruth A. Eatock**

A fundamental property of most sensory receptors is that they adapt to large or maintained stimuli. Photoreceptors adapt chemically so as to decrease the electrical effect of each successive photon absorbed; some touch receptors adapt by a purely mechanical relaxation. We have found that hair cells, which are sensitive to displacement of their hair bundles over very small distances, adapt to maintained displacements by shifting the position of their sensitive range. They are thereby able to maintain a high sensitivity to small displacements while remaining responsive over a wide range of bundle positions.

The operating range of single cells has been measured by moving individual hair bundles over calibrated distances while measuring the intracellular receptor potential, and more recently by voltage-clamping single cells and measuring the receptor current directly. With 10 Hz stimuli, the operating range (defined as the hair bundle displacement that brings the receptor current from its minimal to its maximal value) is only 0.3 μm, or 2° of hair bundle bend. If large displacements are presented (up to 4 μm), the transduction mechanism saturates: the cell is not responsive to additional small stimuli. Over several seconds, however, the responsiveness returns, and we find that the operating range is then centered at the new, displaced bundle position. The tissue may be fixed at this point and the individual cell identified in the scanning electron microscope to confirm that the hair bundle has actually been displaced and that the measured adaptation is not an artifact of slippage between the hair bundle and the stimulus probe.

The time course of adaptation has been determined by stimulating large clusters of hair cells en masse while measuring the extracellular receptor current. While less direct than intracellular recording, this method has the advantages of better time resolution and lower electrical noise. The operating curve is determined by presenting fast displacement pulses of various amplitudes; the operating range for a cluster of several hundred cells is about 0.4 μm. Adaptation is measured by presenting step displacements of various amplitudes and durations, and determining the operating curve at various times during and after the adapting step. The time course of the shift in operating range is approximately exponential, with a time constant for small shifts of 10 to 50 msec. The sigmoidal shape of the operating curve is largely unaffected by the adaptation process.

151. EFFECT OF AMINOGLYCOSIDE ANTIBIOTICS ON TRANSDUCTION BY VERTEBRATE HAIR CELLS

Investigator: **A. James Hudspeth**

The aminoglycoside antibiotics--streptomycin, gentamicin, and their cogeners--exert a medically important deleterious effect on the hair cells of both the auditory and the vestibular systems. Protracted exposure to the drugs produces structural abnormalities of the mechanosensitive hair bundles at the apices of hair cells and, eventually, death of these receptor cells.

The in vitro preparation for intracellular recording from hair cells affords an opportunity for investigating the physiological effects of aminoglycoside drugs. Application of streptomycin and related drugs to the saline solution bathing the hair cells blocks their mechanoelectric transduction process within minutes; concentrations of approximately 100 μM produce complete blockage of responses. If the drug is removed from the bathing solution promptly, the hair cell's response returns to essentially normal levels. Exposure to blocking doses of

streptomycin for more than a few minutes, however, produces irreversible effects.

The rate of blockage by streptomycin may be tested with more precision by iontophoretic application of the drug directly to the surface of a cell whose response is recorded with a microelectrode. Streptomycin totally blocks the response within 200 msec of its iontophoretic application; the response returns to its original level within 500 msec of the cessation of iontophoresis.

These observations suggest that aminoglycosides may have two separable effects on transduction by hair cells: a rapid, essentially reversible blockage of the response, and a slow, irreversible effect that culminates in cell death.

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Graduate Student: Mauri E. Krouse
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Summary: Many small molecules, both natural and artificial, act on biological membranes by controlling ionic channels. Our particular interest concerns the channels associated with the acetylcholine receptor in the postsynaptic membrane of the nicotinic synapse, where impulses are transferred from a nerve to a muscle fiber or (in some fishes) to an electroplaque. This synapse is a highly efficient electrochemical machine, specialized to function on a millisecond time scale. The challenge is to understand the electrical and chemical aspects of channel functioning on this same time scale.

In our experiments, we monitor the number of open channels in the membrane while using light flashes to manipulate (a) the concentration of drugs near receptors or (b) the structure of the drug-receptor complex. Three known classes of drug interact with acetylcholine receptors: (1) Agonists, such as acetylcholine itself, open channels. (2) Antagonists prevent channels from opening. Curare, for instance, seems to compete with acetylcholine for a binding site on the receptor; and the elapid α-toxins, like α-bungarotoxin, irreversibly block this site. (3) The "open channel blockers" (of which local anesthetics seem to be good examples) reduce membrane excitability by binding within open channels, much like a plug in a drain.

152. ACh-RECEPTOR CHANNELS BEGIN TO OPEN WITHIN 10 μSEC AFTER AGONIST IS APPLIED

Investigators: **Mauri E. Krouse, Henry A. Lester, Menasche M. Nass, Jeanne M. Nerbonne, Norbert H. Wassermann*, Bernard F. Erlanger***

We have improved (by a factor of 10) our estimate of how rapidly acetylcholine (ACh)-receptor channels begin to open after agonist appears nearby. Isolated Electrophorus electroplaques are arranged for transcellular recording. The innervated face is bathed in a solution of cis-Bis-Q (3,3'-bis-[α-(trimethylammonium)methyl]-azobenzene) which has no effect on receptors. Trans-Bis-Q is a potent agonist; a concentration of 350 nM activates half the receptors at the resting potential. Roughly half the cis-Bis-Q molecules are photoisomerized to the trans configuration within 1 μsec by a pulse of 440 nm light from a dye laser. Our methods can detect a depolarization caused by activation of 0.5-1% of the receptor channels. At 23°C and at a trans-Bis-Q concentration of 1 μM, this level of activation occurs within 10 μsec after the flash. This latency has a Q_{10} close to 3, and at lower [trans-Bis-Q] the latency increases as the inverse of the concentration. Thus, the minimum latency has the same concentration and temperature dependence on a μsec time scale as the channel opening rate, measured in relaxation experiments,

has on a millisecond time scale. The data show that if there is an absolute delay involved in activation of receptor channels by agonist, this delay is less than 10 μsec at 23°C. Our agonist concentration jumps are still much smaller than the local ones that occur when the presynaptic nerve terminal releases a quantum of ACh into the synaptic cleft.

Further information is obtained from a second flash, which photoisomerizes trans-Bis-Q molecules bound to receptors (as well as Bis-Q molecules in solution). Since the cis configuration is a much weaker agonist, channels close rapidly and the electroplaque repolarizes. The observable latency for this repolarization is even less than for the depolarization due to the first flash.

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153. FURTHER STUDIES ON A COVALENTLY BOUND PHOTOISOMERIZABLE AGONIST

Investigators: **Henry A. Lester, Mauri E. Krouse, Menasche M. Nass, Norbert H. Wassermann*, Bernard F. Erlanger***

After disulfide bonds are reduced with dithiothreitol, trans-3-(α-bromomethyl)-3'-[α-trimethylammonium)methyl] azobenzene (trans-QBr) alkylates a sulfhydryl group on receptors. The membrane conductance induced by this "tethered agonist" shares many properties with that induced by reversible agonists. Equilibrium conductance increases as the membrane potential is made more negative; the voltage sensitivity resembles that seen with 50 μM carbachol. Voltage-jump relaxations follow an exponential time course; the rate constants are about twice as large as those seen with 50 μM carbachol and have the same voltage and temperature sensitivity. With reversible agonists, the rate of channel opening increases with the frequency of agonist-receptor collisions; but with tethered trans-QBr, this rate depends only on intramolecular events. In comparison to the conductance induced by reversible agonists, the QBr-induced conductance is at least tenfold less sensitive to competitive blockade by tubocurarine and roughly as sensitive to "open-channel blockade" by QX-222.

Light-flash experiments with tethered QBr resemble those with the reversible photoisomerizable agonist, Bis-Q: the conductance is increased by cis → trans photoisomerizations and decreased by trans → cis photoisomerizations. As with Bis-Q, light-flash relaxations have the same rate constant as voltage-jump relaxations. Receptors with tethered cis-QBr have a channel duration severalfold briefer than with the tethered trans isomer. By comparing the agonist-induced conductance with the cis/trans ratio, we conclude that each channel's activation is determined by the configuration of a single tethered QBr molecule. The QBr-induced conductance shows slow decreases (time constant several hundred msec) which can be partially reversed by flashes.

The similarities suggest that the same rate-limiting step governs the opening and closing of channels for both reversible and tethered agonists. Therefore, this step is probably not the initial encounter between agonist and receptor molecules.

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154. LIGHT-ACTIVATED COMPETITIVE ANTAGONIST

Investigators: **Menasche M. Nass, Jeanne M. Nerbonne, Mauri E. Krouse, Henry A. Lester, Norbert H. Wassermann*, Bernard F. Erlanger***

Curare and other related drugs act as competitive antagonists of the nicotinic acetylcholine receptor. Although equilibrium binding constants for antagonists can be obtained experimentally, there are, at present, no methods for direct kinetic measurements of antagonist binding rates. Therefore, we are developing a technique to produce rapid (<1 msec) increases and/or decreases in the concentration of an antagonist in solutions bathing voltage-clamped Electrophorus electroplaques. The kinetics of antagonist action can then be measured directly from the changes in the kinetics and equilibria of agonist-induced currents.

Both cis and trans-2-Bis-Q (2,2'-bis-[α-(trimethylammonium)methyl]azobenzene) are competitive antagonists at the electroplaque and neither isomer displays open-channel blockade at concentrations <1 μM. Agonist-induced currents are decreased in the presence of cis and/or trans-2-Bis-Q: the apparent equilibrium dissociation constant for carbachol is increased by 50% at 0.15 μM cis-2-Bis-Q or at 0.50 μM trans-2-Bis-Q. Thus, cis-2-Bis-Q is approximately threefold more potent than the trans isomer.

The photochemistry of 2-Bis-Q is virtually identical to that of Bis-Q. A visible light flash in the cis photostationary state results in an increase in the agonist-induced currents. The time course of the increase depends on the temperature and on the nature and

concentration of the agonist. For acetylcholine, the flash-induced conductance increase follows an exponential time course with a relaxation rate constant ranging from 0.5 to 1 times the voltage-jump relaxation rates. UV light flashes in pure trans-2-Bis-Q or the trans photostationary state produce a net decrease in agonist-induced currents. The latter experiments are presently limited by the low energy output of the flash lamp in the ultraviolet.

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155. LIGHT-ACTIVATED BLOCKER OF ACETYLCHOLINE-RECEPTOR CHANNELS

Investigators: **Mauri E. Krouse, Henry A. Lester, Menasche M. Nass, Jeanne M. Nerbonne, Bernard F. Erlanger*, Norbert H. Wassermann***

Open channel blockers such as procaine alter the kinetics and equilibria of agonist-induced currents. To quantitate these effects, we employ EW-1 (N-p-phenylazophenyl carbamyl choline)--a photoisomerizable azobenzene derivative. The cis isomer of EW-1 probably enters open nicotinic receptor channels of Electrophorus electricus electroplaques thus interrupting the flow of ions through these channels. The trans isomer, at concentration less than 25 μM, has a minimal blocking effect on open channels.

The decay of the postsynaptic currents (PSC) can be accelerated by the cis isomer of EW-1 (which we produce from the trans isomer by a light flash [300-500 nm]). This increase in PSC decay rate is directly proportional to the light intensity, suggesting that one cis molecule is sufficient to block the open channel. In voltage-jump experiments, a light flash produces a relaxation to a smaller level of agonist-induced conductance. This flash-induced relaxation has an exponential time course with a rate constant greater than that of the voltage-jump relaxation rate constant. The flash-induced effect on agonist-induced current at a given [trans EW-1] is greater than the effect induced by the same concentration of nearly pure cis EW-1. At neutral pH, cis EW-1 thermally isomerizes to the trans isomer with a time constant of about 2 min; the time constant decreases at more acidic pH. However the blockade induced by a light flash decreases with a time constant of approximately 0.3 sec. These results suggest (1) that trans EW-1 accumulates near receptors, and (2) that a light flash liberates cis EW-1, resulting in [cis EW-1] transiently in excess of that in bulk solution.

The decrease in the agonist-induced conductance after a light flash is directly proportional to the channel lifetime induced by the agonist. Our results support the hypothesis that one cis EW-1 molecule can block the open channel and that this blocking does not depend on how the channel was opened.

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156. PHOTOCHEMICALLY LABILE PRECURSORS OF CYCLIC NUCLEOTIDES AND CALCIUM

Investigator: **Jeanne M. Nerbonne**

Cyclic nucleotides and calcium are second messengers in the intracellular regulation of numerous biochemical processes. We would like to have additional kinetic information about the storage, action, and metabolism of cyclic nucleotides and calcium. We are therefore developing techniques to permit rapid, efficient, and (wherever possible) reversible alterations in intracellular concentrations of cAMP, cGMP, and calcium through the use of photolabile precursors.

The o-nitrobenzyl ester of cAMP (Engels and Schlaeger, 1977) has been prepared and its photochemical properties examined. Irradiation of the ester in alcoholic solution ($\lambda > 300$ nm) yields free cAMP under conditions that yield no thermal hydrolysis. The ester, unlike cAMP, is lipophilic and is expected to traverse cell membranes easily. This compound is currently being assayed for its ability to induce afterdischarge in the bag cells of Aplysia californica, an effect attributed to cAMP. Several other cAMP derivatives, which are expected to have more favorable photochemical characteristics (absorption spectra and quantum yields) have been designed and their syntheses are under way.

The synthesis of 4,4'-bis-(α,α'-iminodiacetic acid) azotoluene, designed as a possible photolabile calcium chelator, has very recently been completed. The photochemical behavior of this compound and its chelating properties are under investigation.

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Summary: Research accomplishments in the last year have included completion of the amino acid sequencing of the neuropeptide ELH (egg-laying hormone) of Aplysia californica, as well as those of two other peptides, from the atrial gland, which are involved in activation of the bag cell (BC) neurons, the source of ELH. This neuroendocrine system is probably the most completely described system, physiologically and biochemically, in the animal kingdom to date. Work on the neuronal circadian oscillator system in the eye of Aplysia has included completion of a study of the X-ray abolition of the circadian oscillation in the absence of other observable effects on four membrane functions and one "coupling" function.

Arlene Chiu (graduate student) and Eri Heller (senior research fellow) have purified ELH to a homogeneous product, by a two-step procedure, which provides 100-fold enrichment with a 36% yield. Michael Hunkapiller (senior research fellow in Professor Hood's laboratory) has shown that ELH contains 36 amino acids; isoleucine is the amino terminus and a lysine, possibly modified, is the carboxy terminus. Chiu and Viele (research assistant) have made antibodies in rabbits to ELH (as coupled to thyroglobulin). By the use of the second antibody technique (conjugated to fluorescein) they have demonstrated that ELH is contained in primary cultures of BCs. Heller has purified two peptides, from the atrial gland (AG), a portion of the reproductive tract. Hunkapiller has also sequenced these two peptides which have similarities to each other but are both quite unlike ELH. Kaczmarek (senior research fellow) has shown that the two AG peptides activate afterdischarge in BC neurons in the intact BC cluster at near 100 nanomolar concentrations. We have evidence that these effects are presynaptic to the BCs and assume that the functional significance of the AG peptides is likely to be a hormonal link between copulation and induction of BC discharge which releases ELH, causing egg-laying and correlated behaviors.

The hypothesis that afterdischarge (or natural seizure) in BCs is causally related with the observed rise in cAMP (Kaczmarek et al., 1978) has been strengthened by

experiments with 8-benzylthio cAMP (8-BT). Kaczmarek and I have shown that 8-BT induces "afterdischarge" in isolated BCs in cell culture. Kent Jennings (graduate student) finds that within the first 2 min of afterdischarge in the intact BC cluster, phosphorylation of a 22,000 dalton protein is predominant. There is additional evidence, from work with crude membrane preparations, that these effects are likely to be mediated by the cAMP-dependent protein kinase enzyme.

Dissection of the neuronal circadian oscillator system in the eye of Aplysia has been approached by the use of X-rays on intact eyes and by electrical recording from isolated cells in cell culture. Woolum (senior research fellow) has completed studies on the effects of 50 KVP X-rays on several functions in the eye. X-ray doses between 8 and 24 krads proportionally decrease the amplitude of the circadian rhythm of CAP frequency. 40 krad doses abolish the CR without affecting any of four membrane functions and one "coupling" function that we have been able to measure, including photoreception. We argue that it is unlikely that the membrane is a unique site of genesis of the CR and believe that the oscillator mechanism itself, rather than "coupling," is sensitive to X-rays. Katherine Lee (graduate student) reports that there is a circadian variation of the synthesis of aqueous-insoluble proteins in the eye of Aplysia.

Viele and I have produced cultures of dissociated cells from the eye. We have concentrated on recordings from photoreceptors (PRs) which can be obtained totally disconnected from other cells. Intracellular recordings from disconnected PRs indicate that they remain responsive to light over at least the first three days of cell culture. While sensitivity to light appears to be quite high in cell culture, we find no evidence for circadian variations in sensitivity of PRs to light implying that these cells are not part of the circadian oscillator system in the eye. John Scotese (research assistant) is investigating, by scanning and transmission electron microscopy, the structure of photoreceptors and other cells of the eye in cell culture, in order to enhance identification of cell types.

The role of the eyes in driving circadian locomotion in Aplysia is being investigated by Mary Nousek (research assistant). Aplysia with bilateral optic nerve sections perform in constant dim red light as animals without eyes. There is a large reduction (~80%) in the circadian component of the power spectrum implying that neural control (vs. hormonal) by the eyes is overriding in this system.

Reference:

Kaczmarek, L. K., Jennings, K. R. and Strumwasser, F. (1978) Proc. Nat. Acad. Sci. USA **75**: 5200-5204.

157. THE NEUROPEPTIDE EGG-LAYING HORMONE OF APLYSIA: PURIFICATION, AMINO ACID SEQUENCE AND ANTIBODIES

Investigators: **Arlene Y. Chiu, Michael W. Hunkapiller, Felix Strumwasser**

When the bag cell (BC) neurons of Aplysia californica are electrically stimulated to produce an afterdischarge, they release a number of peptides, one of which causes egg laying and related behavior. We have purified this egg-laying hormone (ELH) by a two-step procedure to yield a homogeneous product with biological activity. Direct microsequence analysis of ELH revealed a single amino acid sequence of 36 residues with a calculated molecular weight of 4385 and a calculated pI of 9.7. These results are compatible with previous reports on the size of ELH from SDS gels and gel filtration studies and on the apparent pI of the molecule on isoelectric focusing gels. The amino acid composition of ELH after acid hydrolysis shows good agreement with the sequence data and we report that the amino acid sequence of ELH is: N-Ile-Ser-Ile-Asn-Gln-Asp-Leu-Lys-Ala-Ile-Thr-Asp-Met-Leu-Leu-Thr-Glu-Gln-Ile-Arg-Glu-Arg-Glu-Arg-Tyr-Leu-Ala-Asp-Leu-Arg-Gln-Arg-Leu-Leu-Glu-Lys-OH.

In the purification procedure, the supernatant of a homogenate of BC clusters is subjected to cation exchange chromatography on Sephadex SP C25 followed by gel filtration on BioGel P-6. This results in a 100-fold enrichment of ELH from BC homogenates and a 36% recovery of purified, radiolabeled marker ELH. We calculate, therefore, that a sexually mature animal contains 19 to 40 μg of ELH in a pair of BC clusters.

The egg-laying behavior triggered by ELH includes suppression of feeding and locomotion, head weaving movements, and extrusion and deposition of the egg strand. It is known that neuronal targets respond to the in vitro application of ELH and it is likely that there are also non-neuronal targets. Testing of specific ELH fragments is in progress to locate the active site(s) on the molecule (work in collaboration with R. E. Miller of Monsanto Co.). We have also generated antibodies specifically directed against ELH coupled to thyroglobulin. This will enable us to localize, by immunohistochemistry, sites of hormone

storage within cell bodies and processes of the BC clusters and abdominal ganglion.

158. AFTERDISCHARGE IN BAG CELL NEURONS IS INITIATED BY PEPTIDES FROM THE ATRIAL GLAND OF APLYSIA

Investigators: **Eri Heller, Leonard K. Kaczmarek, Michael W. Hunkapiller, Leroy E. Hood, Felix Strumwasser**

The neuropeptidergic bag cells in the abdominal ganglion of Aplysia are able to initiate egg laying and its associated behavior by secreting egg-laying hormone (ELH) during a long-lasting afterdischarge. This afterdischarge may be triggered by electric stimulation of a presumed afferent pathway from the head ganglia. Copulation may be a natural stimulus for egg laying and it has been shown that the atrial gland in the reproductive tract of Aplysia contains a factor that is able to induce egg-laying behavior when injected into a recipient Aplysia (Arch et al., 1978). We have now found that extracellular application of crude extracts or of purified peptides from the atrial gland produces long-lasting afterdischarges in the bag cells. This effect is also observed with isolated pleuroabdominal nerve preparations which contain asomatic bag cell neurites. Two preliminary lines of evidence suggest that these peptides may act by activating the afferent input from the head ganglia. (1) No effects were observed on isolated bag cells in primary cell culture, (2) in experiments with the entire intact nervous system, in which the abdominal ganglion and the head ganglia were maintained in separate chambers, application of these factors to the head ganglia alone could induce bag cell afterdischarge.

We have purified two peptides, A and B, that are able to induce both egg-laying behavior in recipient animals and bag cell afterdischarges. These peptides were isolated from homogenates of the atrial gland by a combination of ammonium sulfate precipitation, agarose gel filtration, and cation exchange chromatography. Each peptide has 32 amino acid residues. Microsequencing revealed the following sequence for peptide A: H-Ala-Val-Lys-Leu-Ser-Ser-Asp-Gly-Asn-Tyr-Pro-Phe-Asp-Leu-Ser-Lys-Glu-Asp-Gly-Ala-Gln-Pro-Tyr-Phe-Met-Thr-Pro-Arg-Leu-Arg-Phe-Tyr-OH. Peptide B differs from A in only four positions. The first nine residues of B are: Ala-Val-Lys-Ser-Ser-Ser-Tyr-Glu-Lys- while residues 10-32 of B are identical to those of A. Neither sequence resembles that of ELH. The calculated MW of A is 3714 and that of B is 3822. The pI of A is about 8 and that of B is 9. Gel filtration of atrial gland homogenates on agarose in the presence of urea reveals material that can induce egg laying in the excluded volume in addition to those fractions containing peptides A and B. We are currently determining the relative potencies of these differing fractions.

Reference:

Arch, S., Smock, T., Gurvis, R. and McCarthy, C. (1978) J. Comp. Physiol. **128**: 67.

159. THE INDUCTION OF REPETITIVE DISCHARGE IN CULTURED BAG CELL NEURONS BY A cAMP ANALOG

Investigators: **Leonard K. Kaczmarek, Felix Strumwasser**

The cluster of neuropeptidergic bag cells in the abdominal ganglion of Aplysia is able to generate a long-lasting afterdischarge of compound action potentials following brief electrical stimulation or extracellular addition of cAMP analogs. We have now examined the response of individual cultured bag cells to extracellular application of the cAMP analog, 8-benzylthio cAMP. In common with bag cells in an intact cluster, individual cultured bag cells respond to 8-benzylthio cAMP by generating a long-lasting discharge of action potentials. The discharge, in cultured cells, started after a mean delay of 27 min following addition of the cAMP analog and could be blocked by cobalt ions. The onset of the induced discharge was correlated with a marked increase in membrane resistance which resulted in a decrease in the threshold for spikes evoked by depolarizing current pulses. The width of action potentials evoked by a single depolarizing pulse of constant current was augmented following 8-benzylthio cAMP. Frequency-dependent spike broadening was also markedly enhanced. Fully broadened action potentials generated by repetitive current pulses ($\sim$1 pulse/sec) were approximately double the width of controls and were characterized by a prominent shoulder on their falling phase. In a small number of experiments a prominent regenerative hyperpolarizing response was observed following 8-benzylthio cAMP. This response was associated with an increase in membrane conductance and could be blocked by cesium ions (12 mM).

The response of cultured bag cells to 8-benzylthio cAMP qualitatively resembles the response of intact bag cell systems to either cAMP analogs or electrical stimulation. Quantitative differences exist however, particularly in the frequency of firing and mode of onset of discharge

in these two systems. The response of the cultured bag cells is consistent with the hypothesis that cAMP may induce a net decrease in the membrane conductance to potassium ions.

160. PROTEIN PHOSPHORYLATION DURING AFTER-DISCHARGE OF THE NEUROENDOCRINE BAG CELLS IN APLYSIA

Investigators: **Kent R. Jennings, Leonard K. Kaczmarek, Felix Strumwasser**

One proposed mechanism for mediating slow changes in neuronal excitability involves protein phosphorylation by cAMP. A major obstacle in evaluating this mechanism by biochemical means is the very short time course (msec) of "slow" neuronal excitability changes. The bag cell system of Aplysia offers a model system where the long time course of neuronal excitability changes makes it amenable to biochemical analysis. The neuroendocrine bag cells in the abdominal ganglion of Aplysia generate a long-lasting afterdischarge upon brief electrical stimulation of a pleurovisceral connective nerve. Evidence has been presented that activation of adenylate cyclase may be involved in the genesis of afterdischarge (Kaczmarek et al., 1978). Experiments were therefore conducted to determine whether the actions of cAMP might be mediated by a protein kinase. Experimental ganglia were either electrically stimulated to afterdischarge or incubated for 15 min in the presence of 8-benzylthio cAMP. These treatments resulted in 51% (N=9) greater ^{32}P incorporation into TCA-insoluble material from the bag cell somata and surrounding connective tissue, which contains many bag cell neurites. Increased phosphorylation into one protein band was observed with either electrical stimulation or treatment with the cAMP analog when compared with controls. Microdensitometric analysis of the gel autoradiograms showed a 45% (N=10) increase in phosphorylation of this protein. The molecular weight of this phosphoprotein was estimated to be 22,000 daltons by its migration on SDS polyacrylamide gels. The phosphorylation of the 22,000 molecular weight protein in the bag cell region occurs within 2 min of the onset of afterdischarge. We have also observed phosphorylation of two high molecular weight (approximately 120,000 and 125,000 dalton) proteins in the bag cell region.

In an attempt to determine the tissue specificity of these protein phosphorylation events, the bag cell somata were squeezed out from their surrounding connective tissue and phosphorylation of both crude homogenates and membrane preparations of this tissue was studied. It was found that the catalytic subunit of bovine cardiac protein kinase (a gift of P. Greengard) stimulated phosphorylation of approximately six phosphoproteins in the tissue homogenates, including the 22,000 protein. These effects were very pronounced and studies are now being conducted to determine if cAMP-dependent phosphorylation can be demonstrated in membrane fractions prepared from bag cell tissues.

The long time course of the excitability changes observed in the bag cells (30 min) may allow correlation of the time course of protein phosphorylation (in the intact bag cell clusters) with the onset and termination of the afterdischarge.

Reference:

Kaczmarek, L. K., Jennings, K. R. and Strumwasser, F. (1978) Proc. Nat. Acad. Sci. USA 75: 5200.

161. EFFECTS OF X-RAYS ON THE CIRCADIAN RHYTHM IN THE EYE OF APLYSIA

Investigators: **John C. Woolum, Felix Strumwasser**

We have extended the measurements of the effects of X-rays on the circadian rhythm (CR) of frequency of compound action potentials (CAPs) in the eye of Aplysia. Doses of 50 KVP X-rays between 8 and 24 krads decrease the amplitude of the rhythm in proportion to the dose. Doses at or greater than 40 krads completely destroy the rhythm. However, at such doses, every electrophysiological test performed indicates that nerve impulse conduction, synaptic transmission, synchronization of the CAP mediated by electrical coupling, the bursting pacemaker, and photoresponse mechanisms remain intact under conditions in which the CR is not expressed. It therefore seems unlikely that the circadian oscillator is generated by only membrane mechanisms.

Using lead shields we have been able to irradiate one-half of the eye without irradiating the other half. Results of these experiments show that if the anterior (corneal) part of the eye receives 60 krads the rhythm amplitude is decreased slightly whereas if the posterior (optic nerve) part of the eye receives the same dose, the rhythm amplitude is greatly decreased (totally absent in about half of the eyes irradiated in this way). The simplest interpretation of these results is that there are a number of oscillators distributed throughout the eye (many more in posterior region) and that the amplitude of the rhythm is proportional to the number of oscillators

that are active and in phase.

We are currently studying the effects of near ultraviolet radiation on the circadian rhythm. It is hoped that from the wavelength dependence of the sensitivity of the circadian oscillator (action spectrum), information can be obtained about the molecular structures involved in the circadian oscillator.

162. DISSECTION OF A NEURONAL CIRCADIAN OSCILLATOR SYSTEM: INTRACELLULAR RECORDING FROM SINGLE DISCONNECTED PHOTORECEPTORS IN CELL CULTURE

Investigators: **Felix Strumwasser, Daniel P. Viele, John M. Scotese**

The isolated eye of Aplysia californica produces a circadian rhythm (CR) in the frequency of optic nerve compound action potentials (CAPs), under conditions of total darkness, whose period is temperature-compensated. The CR of CAPs is entrainable, in vitro, by light as well as chemical agents. Thus this eye contains all the cellular machinery for a temperature-compensated, entrainable circadian oscillator (CO).

In an attempt to dissect the CO further we have produced primary cultures of disconnected eye cells. We incubate eyes in a neutral protease (2.5% w/v) for 12 hr (15°C), followed by 7 hr (23°C) and gently triturate to dissociate cells. (After this enzyme treatment of intact eyes, the CR is not abolished.) Photoreceptors (PRs) and monopolar (MP) neurons are easily recognized. Our best cultures have yielded approximately 800 single PRs and 1000 MPs from a single eye. These cells as well as others attach to plastic culture dishes, produce webs and, in the case of MPs, grow long neurites in either modified MEM or L15 medium.

PRs can be recognized by a microvillous bush (rhabdomere) which marks the portion of the cell oriented toward the lens. Dimensions of selected PRs, from scanning electron microscopy (EM) of fixed, dehydrated specimens (n=4), reveal a cell body ~30 x 14 μm with microvillous extensions ranging between 4 to 30 μm. Sections of PRs from cell culture viewed with transmission EM exhibit a normal morphology with three distinct parts, including a rhabdomere forming the apical portion, a cluster of pigment granules in the midportion, and a nucleus and a dense population of photic vesicles in the basal portion.

Stable intracellular recordings from isolated PRs have lasted 36 to 48 hr in exceptional cases but can normally be maintained for over 12 hr. PRs respond to light with graded depolarizations and do not produce action potentials. During the light response, there is a conductance increase, as measured by the steady-state membrane voltage obtained in response to constant current pulses. Some preparations exhibit "spontaneous" quantum bumps whose frequency increases (up to ~20/min) during dark adaptation and is suppressed after a light pulse. Light sensitivity in dark-adapted cell cultures extends over at least five decades of intensity with ~25 mV responses to 10^{-3} lux (5 sec pulse). We find, using a programmer to scan the dynamic range of a PR, that light responses, after dark adaptation, are stable and have no circadian components, thus eliminating the PR as a source of the CO in the eye of Aplysia.

163. CIRCADIAN FLUCTUATIONS IN PROTEIN SYNTHESIS IN THE ISOLATED EYE OF APLYSIA CALIFORNICA

Investigators: **Katherine Dai-Li Lee, Felix Strumwasser**

Protein synthesis in isolated eyes of Aplysia californica was measured in 46 eyes at seven different times of the day, while in darknesss, in order to determine whether there were any circadian variations.

After a 2 hr incubation in [^{3}H]leucine, the isolated eyes were rinsed, dissected free of the optic nerve, and homogenized in glass grinders. Each homogenate was centrifuged yielding an aqueous-insoluble pellet. Proteins in the ice-cold supernatant were precipitated by adding trichloroacetic acid (TCA) and bovine serum albumin (BSA) as a carrier.

After the second centrifugation, the pellet was treated again with TCA and centrifuged. The pellet from each centrifugation was dissolved in NaOH, heated for 3 to 5 min at 85°C, and acidified by adding HCl after it cooled down. Samples were then added to aquasol and counted on a scintillation counter.

We find that there are circadian variations in the counts of newly synthesized aqueous-insoluble proteins and in the TCA-supernatant but of opposite phase. The peak of aqueous-insoluble protein synthesis occurs at the time of day when lights would have come on in the home tank and is ~100% higher than the trough of this protein synthesis. The peak of counts in the TCA-supernatant, however, occurs 4 hr after lights-off and is 160% higher than the trough which occurs at the same time as projected lights-on.

These circadian patterns of aqueous-insoluble pro-

tein synthesis and counts in the TCA-supernatant are positively and negatively correlated, respectively, with the circadian rhythm of optic nerve impulses.

Our studies, therefore, support the suggestion of the involvement of protein synthesis in the production of the circadian rhythm of the isolated eye of Aplysia californica by Rothman and Strumwasser (1976).

Reference:

Rothman, B. S. and strumwasser, F. (1976) J. Gen. Physiol. 68: 359-384.

164. MECHANISMS OF CONTROL OF CIRCADIAN LOCOMOTION IN APLYSIA BY THE EYES

Investigators: **Mary M. Nousek, Felix Strumwasser**

It is well known that the isolated eye of Aplysia contains a neuronal circadian oscillator. It has also been shown that the eyes of Aplysia are the dominant oscillator system controlling the free-running circadian locomotor activity of the organism (see Biology 1977, No. 148). The mechanism of this control is currently being investigated.

As a result of the discovery of presumed peptides being released from the isolated eyes of Aplysia in a circadian fashion (Stuart et al., 1979), it is possible to suspect the presence of humoral factor(s) that may be responsible for driving the circadian locomotor activity of the animal. If it can be shown that the locomotor CR of the organism persists in the absence of intact neural connections from the eye to the cerebral ganglion, the case for the existence of a neurohormone would be strengthened.

In the current study, double-optic nerve sections were performed on 34 Aplysia at the site of the cerebral ganglion. Locomotor activity of the animals in constant red light was monitored using a TV camera-video encoder-computer system and data collected continuously for at least 15 days after the animals' recovery from anesthesia. The overall periodicity of movements was analyzed using an ensemble average power spectrum of locomotor activity for the entire population. A check on the condition of the eyes was done postmortem by recording the CAPs produced by the intact eyes in organ culture.

It was found that the power spectrum of locomotor activity at 24 hr is reduced 79% when compared to normal intact animals. This finding is similar to that reported for animals whose eyes have been removed--~76% reduction (Biology 1977, No. 148). In addition eight pairs of eyes were monitored in organ culture from animals 15 to 31 days after surgery. All 16 eyes produced a CR of CAPs in total darkness with an average period of 23.0 hr and are assumed representative of the group. In no case did an eye from an operated animal fail to produce a CR of CAPs. Phase differences between pairs of eyes in these eight animals ranged from a minimum of 0.2 hr to a maximum of 3.2 hr in the first two cycles. It is interesting to compare this to the condition found in recordings from eyes of five single-optic nerve sectioned animals whose phase differences between pairs of eyes from the same animal ranged from 3.2 hr to 15.8 hr.

Our results to date seem to indicate that the control by the eyes over the circadian rhythm of locomotor activity is not mediated by humoral factors released from the eye and imply that neural impulses conducted to the cerebral ganglion are essential.

Reference:

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NEUROBIOLOGY AND BEHAVIORAL BIOLOGY

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Summary: During the past year we have continued our investigation of the physiology of visual perception in primates. We have found evidence for two additional visual areas on the ventral surface of the occipital lobe in the owl monkey (see Figure 1). We have also found a remarkable class of neurons in the Dorsolateral Area (DL) that respond exquisitely to stimuli expanding and/or contracting about an optimal size. The properties of these neurons closely resemble those postulated by Regan and Beverley (1978) on the basis of experiments in human subjects that indicate the existence of special channels for depth perception evoked by changing stimulus size.

Reference:

Regan, D. and Beverley, K. I. (1978) Vision Res. **18:** 209-212.

165. INTERHEMISPHERIC CONNECTIONS OF VISUAL CORTEX IN THE BUSH BABY AND THE OWL MONKEY

Investigators: **William T. Newsome III, John M. Allman**

We have anatomically mapped the pattern of degenerating axonal terminals within visual cortex produced by surgical section of the splenium of the corpus callosum in the bush baby, Galago senegalensis, and the owl monkey, Aotus trivirgatus. Previous studies of both these species have shown (1) that the boundaries of striate cortex (V-1) and an extrastriate visual area (MT) can be identified reliably by architectonic criteria and (2) that the boundaries of both areas physiologically represent the vertical meridian and the far periphery of the visual field. Our goals have been (1) to assess the degree of congruence between degenerating callosal terminals and the identified boundaries of V-1 and MT and (2) to gain information concerning the existence and organization of as yet unknown extrastriate visual areas. The data are illustrated in Figure 1. In both the owl monkey and the bush baby a discrete band of degeneration corresponds precisely with the vertical meridian representation of the V-1-V-2 border, and the region of far periphery representation at the V-1-prostriate border is free of degeneration. In the bush baby and to a lesser extent in the owl monkey, an increased density of axonal terminals corresponds to the vertical meridian representation of MT. The region of periphery representation in MT in the bush baby is free of degeneration. Over much of areas DL and DI in the owl monkey and where the central representations of V-2, DL, and MT are in close proximity in both the owl monkey and the bush baby, the pattern of callosal terminals is complex and has little value for determining precise areal boundaries. A discrete band of degeneration on the ventral hemispheric surface of the owl monkey corresponds closely with the electrophysiologically defined vertical meridian representation corresponding to the border between two new visual areas: the ventral posterior (VP) and ventral anterior (VA).

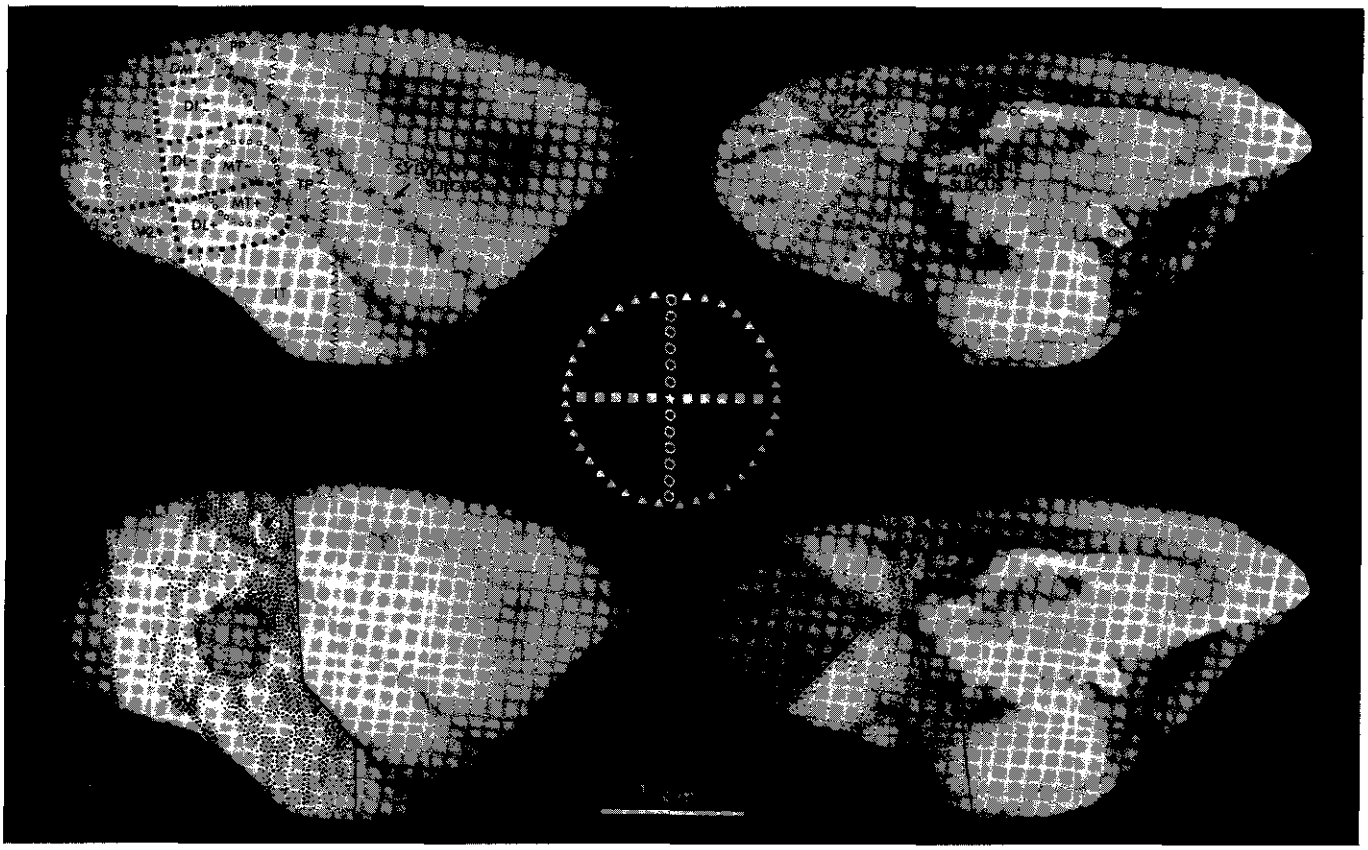

Figure 1. Topographic organization of visual cortex in owl monkeys as revealed by electrophysiological and callosal mapping. At the top are a dorsolateral and a ventromedial view of the right hemisphere of the brain. The visual field symbols on the perimeter chart are superimposed on the cerebral cortex to illustrate the representation of the visual field in the individual visual areas. The open circles symbolize representations of the vertical meridian, filled squares denote representations of the horizontal meridian, and the filled triangles locate representations of the periphery of the visual field. Pluses symbolize the upper visual field, minuses the lower field. Dashed lines are borders where visual field locations are represented other than the ones which are illustrated by symbols in the perimeter chart. The row of Vs represents the approximate anterior border of visual cortex, and dotted lines broken by question marks are uncertain borders. (DI, Dorsointermediate Area; DL, Dorsolateral Area; IT, Inferotemporal Cortex; M, Medial Area; MT, Middle Temporal Area, ON, Optic Nerve; OT, Optic Tectum; PP, Posterior Parietal Cortex; VA, Ventral Anterior Area; VP, Ventral Posterior Area; TP, Temporal Parietal Cortex; X, Optic Chiasm.) The bottom figures show the pattern of callosal degeneration over the expanse of cortex for which the topographic organization is shown at the top. The shaded area at the occipital pole is striate cortex (V-1), the shaded area on the lateral surface in extrastriate cortex is MT. The large dots signify heavy and moderate degeneration, the small dots denote light degeneration. The solid black line shows the anterior extent of the reconstruction.

166. VISUAL RESPONSE PROPERTIES OF SINGLE NEURONS IN THE DORSOLATERAL CRESCENT (DL) IN THE OWL MONKEY

Investigators: **Steven E. Petersen, James F. Baker, Kathleen S. Rockland, John M. Allman**

The Dorsolateral Area (DL) is one of the five visual field representations that adjoin the anterior border of V-2 and collectively constitute the third tier of visual areas in the owl monkey. DL wraps around the Middle Temporal area (MT) and relates to it topographically much as V-2 does to V-1. We have quantitatively studied the response properties of 54 neurons in DL to stimuli of different size, direction of movement, and orientation. 23 of the 54 neurons showed a striking size selectivity. Many of these cells responded only to a narrow range of preferred sizes, and the preferred sizes were often much smaller than the receptive field. An example is illustrated in Figure 2. Other units exhibited spatial summation up to a certain stimulus size, but were decreasingly responsive to larger stimuli. Neurons with similar size preferences were encountered sequentially in penetrations made normal to the brain surface. We also have observed qualitatively that many of these neurons were very responsive to expanding and/or contracting stimuli around the preferred size. The response properties of size-selective cells were largely independent of stimulus

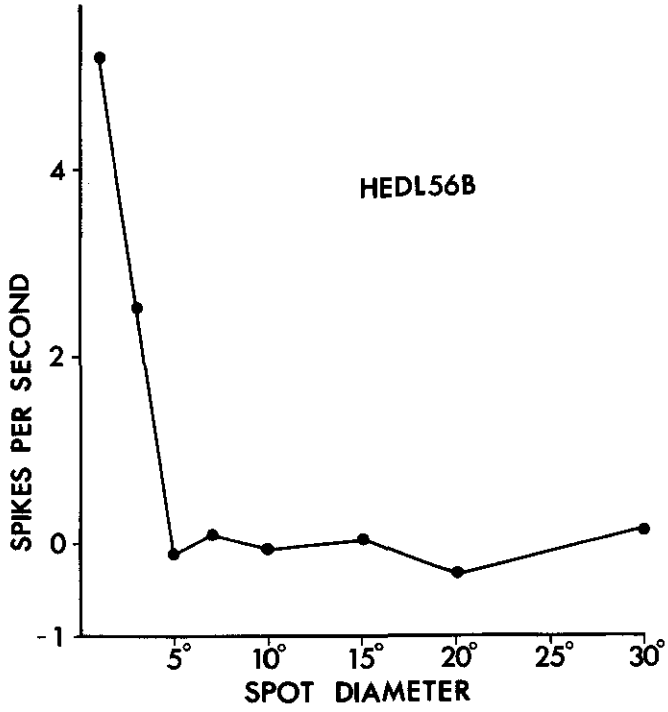

Figure 2. Response of a neuron in DL as a function of spot diameter. The stimulus was a dark spot moving horizontally from right to left at a velocity of 25 degrees per second. These parameters were found to be optimal for this cell in previous tests. The spot sizes were presented in pseudorandom order. Each value was the average of the responses to five stimulus presentations at that diameter. The response was measured relative to the spontaneous activity of the neuron. The neuron's receptive field was a broad oval 16 by 18 degrees that was very much larger than the preferred stimulus size.

intensity and contrast. The properties of these neurons closely resemble those postulated by Regan and Beverley (1978) on the basis of experiments in human subjects that indicate the existence of special channels for depth perception evoked by changing stimulus size.

DL neurons differ from cells in other extrastriate areas in their directionality and tuning to moving bars and spots. Directionality was quantified by comparing the response in the best direction to response in the opposite direction. Tuning was measured by comparing the response in the best direction to the responses in the surrounding directions within ±90 degrees. Figure 3 illustrates the distribution of neurons in DL, MT, and the medial group of third tier areas (Dorsointermediate, DI; Dorsomedial, DM; and Medial, M) as a function of the directionality and tuning indices. DL is intermediate between MT and the medial group in directionality and less sharply tuned than either. These data strongly suggest a differentiation of function among these areas.

Reference:

Regan, D. and Beverley, K. I. (1978) Vision Res. **18:** 209-212.

167. PHYSIOLOGY OF PERCEPTUAL RIVALRY

Investigators: **John M. Allman, Joel Myerson, Francis M. Miezin**

Binocular rivalry, in which the perceived stimulus changes although the physical stimulus stays the same, may provide a valuable opportunity to discover neural activity changes that co-vary with changes in perception. When exposed to rivalry-inducing stimuli such as gratings moving in opposite direction for each eye, human subjects report that the perceived scene alternates abruptly every few seconds from what one eye sees to what the other eye sees so that the bars periodically appear to reverse their direction of movement. Previously (Allman et al., 1977)

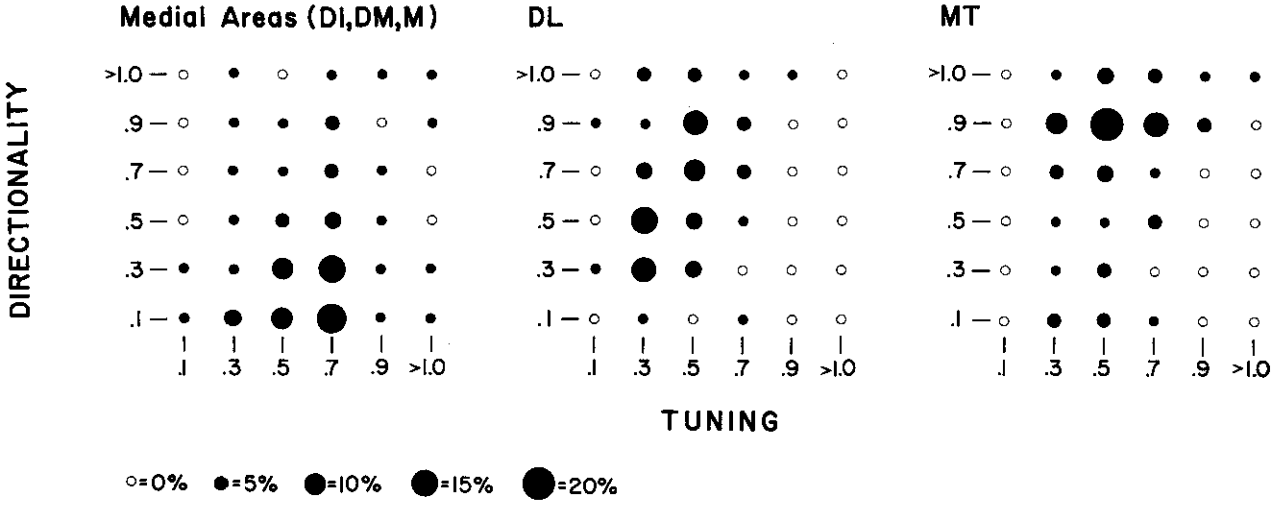

Figure 3. Two-dimensional dot array for the directionality index vs. the tuning index. Dot sizes are proportional to the percentage of neurons within each area or group of areas having indices within ±0.1 of the values indicated.

we reported that the rate of perceived alternation in direction for both humans and for a monkey, trained to report changes in direction of movement by tapping one of two keys, was an increasing function of the velocity of the bar gratings. This similarity in the psychophysics of binocular rivalry suggests a similarity in the physiological mechanisms of perception in monkey and man. This past year we have trained a second monkey, verified the psychophysical similarity, and have just begun recording from visual neurons in striate cortex while the monkey is experiencing binocular rivalry and reporting its perceptions.

Reference:

Allman, J. M., Myerson, J. and Miezin, F. M. (1977) Soc. Neurosci. Abstracts **3:** 551.

168. ORGANIZATION OF THE VISUAL SYSTEM IN A STREPSIRHINE PRIMATE

Investigators: **John M. Allman, C. B. G. Campbell*, EveLynn McGuinness**

The neural apparatus for the analysis and synthesis of visual information consists largely of a set of discrete topographic representations of the visual field. The components of the set of visual maps, their internal organization, and their interconnections differ among species as presumably do the behavioral capacities of different species. Our goal has been to determine which of the set of visual maps found in different primate species are characteristic of the order as a whole and which are specializations that have evolved only within certain groups of primates. Most of the information on the organization of the visual system in primates comes from New and Old World monkeys. Our goal has been to compare the organization of the visual system in these better known species with a representative of the strepsirhine primates, a group that has retained many characteristics of the ancestral primates. To this end we have been mapping the organization of the visual system in the lesser bush baby, Galago senegalensis. The primary visual cortex (V-1), the second visual area (V-2), and the middle temporal visual area (MT) in Galago are very similar to those found in monkeys. However the third tier of cortical visual areas appears to be composed of only two or possibly three areas in Galago as compared to five in New World monkeys. One of these areas corresponds to the Dorsolateral Area (DL) in the owl monkey. A second third tier area, the Dorsal Area, lies medial to DL in Galago and occupies the location of three third tier areas, DI, DM, and M in the owl monkey (see Figure 1). The Dorsal Area may correspond to one of these areas in the owl monkey; however the other two may have evolved in the line of descent leading to owl monkeys subsequent to its splitting off from the line leading to Galagos.

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169. EVOKED POTENTIALS TO DYNAMIC RANDOM-DOT CORRELOGRAMS IN MONKEY AND MAN: A TEST FOR CYCLOPEAN PERCEPTION

Investigators: **Francis M. Miezin, Joel Myerson, Bela Julesz*, John M. Allman**

Large evoked potentials are elicited in humans by dynamic random-dot correlograms of binocularly identical noise alternating with binocularly negatively-correlated noise. These correlograms contain no monocular cues; thus the evoked potentials are an objective indicator of a cyclopean perception (Julesz, 1971). We tried to elicit evoked potentials to these stimuli in alert macaque monkey so as to compare them with human evoked potentials and to develop a fast, objective test for cyclopean perception in animals. The monkey was trained to sit quietly in a chair placed facing a rear-projection screen. Dynamic random-dot correlograms, alternating at 0.5 Hz, were generated at 60 frames/sec. Stimuli for the left and right eyes were fed into the red and green channels, respectively, of an Advent projection TV set. Stereo separation was achieved by placing red and green Wratten filters over the red and green projection optics. In the cyclopean condition, goggles with red and green filters over the left and right eyes, respectively, were worn by monkey and human observers. After every even reversal, left and right dynamic noise was identical giving the percept of a flat depth plane; after every odd reversal, dynamic noise for one eye was the negative image of the noise for the other eye giving rise to binocular rivalry and an uncertain depth percept. To test for monocular cues, we used three control conditions: (A) placing identically colored filters over both eyes; (B) vertically shifting the green image relative to the red image; (C) placing a 0.5 log neutral density filter in front of the green filter (this delays conduction from that eye thereby reducing the amount of time the two images are correlated). In a fourth control condition (D), 0.5 log neutral density filters were placed over both filters; this delays conduction from both eyes equally so that the images appear as in the cyclopean condition but dimmer.

While the cyclopean condition and control D gave large evoked potentials, controls A and B gave none and control C gave a greatly diminished evoked potential. This proves that the evoked potential was a response to only the cyclopean aspects of the stimulus. Human subjects were told to look at the screen's center whereas the monkey's gaze wandered freely over the entire screen. Nonetheless, the monkey's evoked potentials were as large as those of the human, demonstrating that the method does not depend upon steady fixation of the stimulus. This result testifies to the robustness of evoked potentials to dynamic random-dot correlograms and extends their usefulness as a test for cyclopean perception.

Reference:

Julesz, B. (1971) Foundations of Cyclopean Perception. University of Chicago Press, Chiago, Illinois.

*Head, Sensory and Perceptual Processes, Bell Laboratories, Murray Hill, New Jersey.

170. TWO MACRO-REPRESENTATIONS OF THE FACIAL MUSCLES IN THE PRECENTRAL GYRUS OF MACAQUE MONKEYS

Investigators: **EveLynn McGuinness, David W. Sivertsen*, John M. Allman**

Using a chronic preparation we stimulated with microelectrodes approximately 2000 responsive sites in the face region of motor cortex in six macaque monkeys (four Macaca mulatta, two Macaca fasicularis). Current levels used were always less than 25 μA. The modal threshold for mimetic muscles was between 2.5 and 5 μA. Responses were extremely discrete, the usual response at threshold current levels was a small focus of movement in part of a muscle. Facial muscles cluster together into two macro-representations or domains in the posterior and anterior portions of the face region of the precentral gyrus with tongue movements represented in the intervening portion and along the lateral extent. Eyelid movements are represented at the medial edge of the face representation adjoining the representation of shoulder muscles. There is a diagonal band of jaw responses which begins laterally at the central sulcus and cuts across the surface of the gyrus in the anteromedial direction. Within both anterior and posterior domains there is local re-representation of muscle movements. This micro-representation has been described previously (McGuinness and Allman, 1977; Kwan et al., 1978). Although the domains cannot be characterized as distinct topographical representations of the entire face on the basis of the data we now have, there is a tendency for responses of adjacent muscles to occur together and the representations may be roughly topographical within the limits set by the morphological structure of the muscles themselves. We feel that the basic micro-organization is columnar; however, the columns could be either roughly cylindrical or take the form of narrow curving bands, running mediolaterally across cortex. On the basis of micro-stimulation alone it is difficult to differentiate between patterns produced by stimulation of frequently repeated noncontiguous zones devoted to the same muscle or narrow bands which are intersected by electrode penetrations at different points in cortex. Our results might best be characterized as two complete but not strictly topographical representations of the facial muscles which are roughly mirror-symmetrical around the tongue and possibly the eyelid. Strick and Preston (1978) has reported a dual representation in the hand region of motor cortex in the squirrel monkey. Although the pattern he finds differs from ours in detail (alternation rather than reversal) the basic principle is similar.

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Kwan, H. C., MacKay, W. A., Murphy, J. T. and Wong, Y. C. (1978) J. Neurophysiol. **41:** 1120-1131.

McGuinness, E. and Allman, J. M. (1977) Soc. Neurosci. Abstracts **3:** 274.

Strick, P. L. and Preston, J. B. (1978) Brain Res. **154:** 366-370.

*Undergraduate, California Institute of Technology.

171. BEHAVIORAL SYSTEMS ANALYSIS

Investigators: **Joel Myerson, Francis M. Miezin**

The behaving organism and its environment constitute interacting elements of a feedback system: operant responses act upon the environment and the resultant environmental changes feed back in the form of "reinforcements" and "punishments," modifying the organism's behavior. While historically the study of operant behavior has concentrated on steady-state performance, the analysis of feedback systems in other disciplines attempts to describe the transient response to changes in input as well as the equilibrium, or steady-state, behavior. In fact the equilibrium behavior of a system is implicit in a successful description of the transient response. We have applied the techniques of systems analysis to operant behavior in the hope of producing a quantitative account of responding in both transition and steady-states.

We have assumed a model of the reinforcement

process that is analogous to the kinetics of a reversible chemical reaction (Staddon, 1977): in a choice situation, reinforcing one response alternative increases its future probability at the expense of the probability of a second alternative, and vice versa. This kinetic model may be elaborated into a complete systems analysis by incorporating feedback functions, i.e., mathematical descriptions of the effects of choices on reinforcement rates. We have generated predictions of relative preference for response alternatives when each alternative produces reinforcements according to a separate probablistic schedule. At this "molar" level of analysis, correct predictions of both the form of the acquisition of preference and of asymptotic preference levels followed from our systems analysis (Myerson and Miezin, 1979). When reformulated at a more "molecular" level, i.e., in terms of the probabilities of switching back and forth between alternatives, correct predictions of switching behavior may be derived, as well as predictions at the molar level. The present systems analysis is unique among recent theoretical efforts concerned with operant choice behavior in its ability to describe molecular as well as molar properties of both transition-state and steady-state performance.

References:

Myerson, J. and Miezin, F. M. (1979) Paper presented at the 1979 meeting of the Association for Behavior Analysis, Dearborn, Michigan.

Staddon, J. E. R. (1977) J. Exptl. Anal. Behav. **28:** 163-170.

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Allman, J. M., Campbell, C. B. G. and McGuinness, E. R. (1979) The dorsal third tier area in Galago senegalensis. Brain Res. Submitted for publication.

McGuinness, E. R., Sivertsen, D. W. and Allman, J. W. (1979) Two macro-representations of the facial muscles in the precentral gyrus of macaque monkeys. Soc. Neurosci. Abstracts. In press.

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Myerson, J. and Miezin, F. M. (1979) The kinetics of choice: An operant systems analysis. Psychol. Rev. Submitted for publication.

Myerson, J., Myerson, W. A. and Parker, B. K. (1979) Automaintenance without stimulus-change reinforcement: Temporal control of key-pecks. J. Exptl. Anal. Behav. In press.

Newsome, W. T. and Allman, J. M. (1979) Interhemispheric connections of the visual cortex in the bush baby, Galago senegalensis and the owl monkey, Aotus trivirgatus. Association for Research in Vision and Ophthalmology Abstracts, p. 157.

Petersen, S. E., Baker, J. F., Rockland, K. S. and Allman, J. M. (1979) Visual response properties of single neurons in the dorsolateral crescent (DL) in the owl monkey: Selectivity for stimulus size, direction and orientation. Soc. Neurosci. Abstracts. In press.

Professor: Derek H. Fender
Sherman Fairchild Distinguished Scholar: Bela Julesz**
Visiting Associates: Raymond P. Briggs, Patrice L. French, Itzhak Hadani*, Stanley Klein, Thomas E. Ogden*
Research Fellows: James P. Ary, Mark C. Citron, Michael T. Hyson
Graduate Students: Michael J-W. Chen*, Terrance M. Darcey*, K. Jeffrey Eriksen*, Ross M. Larkin*
Research Staff: Cornelis M. Dekker*

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**Head, Sensory and Perceptual Processes, Bell Laboratories, Murray Hill, New Jersey.

Support: The work described in the following research reports has been supported by the National Institutes of Health, USPHS.

Summary: This group is concerned with information processing in the human visual system. The techniques of Wiener analysis are being developed and applied to the identification of the nonlinear processing of the normal visual system and to the identification of visual abnormality. Three areas are of specific interest: (1) the processes which produce potentials on the surface of the human head and eyes; (2) the processes which control the movement of human eyes; and (3) the processes whereby the images presented to the two retinae generate the percept of depth.

172. IDENTIFYING BRAIN FUNCTION THROUGH EVOKED SCALP POTENTIALS

Investigators: **James P. Ary, Michael J-W. Chen**

In identifying the function of the human brain, there is an enormous gap between the understanding that can be achieved by single-cell studies on lower animals and the understanding that derives from behavioral or psychophysical studies on the intact human. Probably the only realistic way to bridge this gap is by studying evoked scalp

potentials. However, the analysis of scalp potentials is clouded by the fact that sensory inputs pass through many stages of processing, each of which generates a signature in the scalp potentials. The goals of this research are to disentangle these signatures, locate their sources, and identify them with function.

Powerful tools are required for extracting definitive relations from the hodgepodge of scalp potentials. We have applied two complementary techniques for this purpose: white noise nonlinear analysis and computer-fitted source localization. Time-invariant, finite memory systems can be fully characterized by a set of kernels derived by cross-correlating the output with a white noise input. We have measured scalp potentials while white noise-modulating the luminance of a 6° square presented in the right half of the visual field, and have computed first-order kernels. The later peaks in these first-order kernels, 90 msec and beyond, are nearly identical to the transient visually-evoked scalp potential (VESP) except that the signal-to-noise level is predictably better with the white noise stimulus. Also the scalp distributions of these two types of response analysis are very similar. In conjunction with second-order kernels, these data should predict the magnitude and phase of the steady-state VESP at the various frequencies as well as the transient response to arbitrary luminance changes. One clinical application of VESP luminance kernels is in the concise description of the amplitude vs. flash intensity function which Buchsbaum (1975) has used in the diagnosis of psychopathology. Other applications are likely to present themselves as we learn to interpret reliable features in kernels. The primary limitation in isolating the contributors to the VESP and in extracting the information contained therein is the low reliability of the data. We find that the key to realizing the signal-to-noise ratio improvement predicted for white noise analytic methods has been finding the proper stimulus statistics to test optimally the structure of interest. We are determining the optimal stimulus conditions for minimizing the variance of the estimates of higher-order kernels. The sources for prominent features of these kernels can be located by applying volume conduction theory and least squares fitting to equivalent model sources. Having identified the cortical regions that contribute to the response, it is then possible to sense the activity in one region relatively independently of others. We are exploring electrode weighting schemes and correlational algorithms for extracting the general input-output relation of cortical regions of known activity.

This is a preliminary report of work in progress and as yet no written reports are available for distribution.

Reference:

Buchsbaum, M. S. (1975) In: The Biology of the Major Psychoses: A Comparative Analysis, D. X. Freedman (Ed.), Raven Press, Long Island, New York.

173. SPATIO-TEMPORAL COURSE OF EXCITATION IN THE HUMAN BRAIN

Investigators: **Terrance M. Darcey, James P. Ary, Derek H. Fender**

A system has been developed which allows us to follow human sensory information processing via multichannel scalp potential measurements and the subsequent determination of corresponding electrical cortical sources. Scalp potential distributions resulting from certain sensory stimuli are analyzed using a field theoretical approach in combination with realistic assumptions regarding the electrical and geometrical composition of the head. Several volume conduction models of the human head have been developed and compared. These include a homogeneous sphere and a sphere covered by two or four shell layers. The double-shelled sphere has proven adequate for most cases. Electrical sources have been modeled as single or pairs of current dipoles and as dipole sheets of uniform surface density. Two sheet geometries have been considered: a spherical segment and a plane annular sector. Electrical sources are specified in terms of their position, orientation, and extent.

This scheme has been applied successfully to a study of the mapping between the visual field and visual cortex in humans. Several results were forthcoming. Sources were found to depend on stimulus locus in a manner which matched the topography and functional anatomy of the visual cortex. Second, over the 60 to 180 msec response time these sources change from a position near the medial fissure moving into the cortical hemisphere contralateral to the stimulus locus to a depth of one-third of the brain's radius (see Figure 1). The sources then move back toward the medial fissure as 180 msec is approached. During this sequence the orientation of the source also changes from rightward pointing to leftward with the inversion at about 125 msec. Peak amplitudes are largest at 92 and 152 msec when the sources are deep within the hemisphere. Finally, responses from stimulation of small parts

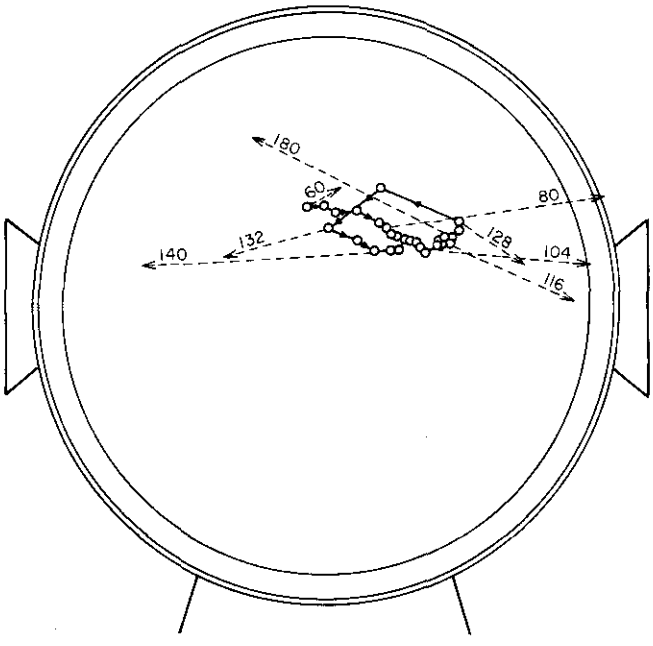

Figure 1. Three-shell model equivalent source series and 95% confidence regions for subject JPA bottom-left quadrant stimulation.

of the visual field add up almost perfectly in agreement with large field responses where the large field is the sum of the constituent small fields.

174. LGN: LATERAL GENICULATE NUCLEUS AND DEEP-SOURCE LOCALIZATION AND IDENTIFICATION THROUGH EVOKED POTENTIALS

Investigators: **James P. Ary, Michael J-W. Chen, Derek H. Fender**

Using the methodology developed in this laboratory, the spatial temporal course of human visual information processing can be followed via a combination of multichannel scalp potential recordings, localization of corresponding electrical sources, and nonlinear system identification theory.

Through a series of experiments, substantial success has been made in isolating LGN activity in multichannel scalp potential recordings. After testing several types of stimuli, we find that a bright xenon flash transilluminating a red and black checkerboard pattern is sufficiently powerful to evoke a reliable response. This stimulus has been delivered to the central six degrees of the right half-field in a random sequence with a decision interval of 4 msec and a flash probability of 6%. In analyzing responses from 3-1/2 min of this type of white noise over 3000 stimulus events, we find reliable peaks in the first-order kernels at latencies of 40, 52, and 58 msec. These early peaks, from our reckoning, are very likely from LGN and other deep sources.

The magnitude distributions of these peaks over the scalp surface are highly correlated and coherent resembling those of simple dipole sources. The late peaks in these first-order kernels, 90 msec and beyond, are nearly identical to the transient visually-evoked scalp potential (VESP) except that the signal-to-noise ratio is predictably better with the white noise stimulus.

The sources for the prominent early peaks have been localized by applying electric field theory and least squares fitting to equivalent sources. Having identified the cortical or thalamic regions that contribute to these responses it may then be possible to identify their corresponding anatomical and physiological components.

Identification of LGN activity through the VESP would also be beneficial in clinical applications because it is the most important link in the retino-thalamo-cortical pathway of the visual system.

This is a preliminary report of work in progress and as yet no written reports are available for distribution.

175. PHOTOPIC RAPID ADAPTATION IN THE ELECTRORETINOGRAM: A WHITE NOISE ANALYSIS

Investigators: **Ross M. Larkin, Stanley Klein, Thomas E. Ogden, Derek H. Fender**

When light enters the eye it initiates a complicated chain of events which eventually leads to the sensation of vision. The electroretinogram (ERG) is a widely used indirect measure of the visual system. One of the most interesting aspects of the visual system, adaptation, is its ability to function over a very wide range of incoming light intensities.

To study rapid adaptation, a new technique was developed which may also be used for looking at other biological systems. Kernels (a form of cross-correlation using white noise stimuli) have proven very useful in the study of the dynamics of photopic rapid adaptation. Using first-order kernels we have probed the wide range of adaptation and compared kernels to flash responses. With the second-order kernel we found evidence that a late (125 msec) wave in the ERG is caused by rapid adaptation. We have also identified the components of highly abnormal ERGs, and obtained basic information about the internal organization of the system. The third-order kernel characterizes suppression-recovery in the photopic ERG.

We have also speculated on the correlation of our results with some of the prevailing views of the organization and operation of rapid adaptation in the photoreceptors of the retina.

176. SACCADIC-EYE-MOVEMENT EVOKED RESPONSES

Investigators: **Michael J-W. Chen, Derek H. Fender**

Eye movements are the results of commands emanating from the brain. It is now widely accepted that the discharge of a class of saccadic-burst neurons located in the brain stem reticular core provides the necessary control signals for the generation of saccadic movements in the form of an intense burst of activity which slightly precedes the onset of the saccade and lasts for a duration equal to that of the saccade. Furthermore, since saccadic eye movements change the locus of the retinal image, there should also be a response from the visual cortex. Thus there are brain activities before, during, and after saccadic eye movements. The first phase of this study is to investigate saccadic-movement evoked responses by using an infrared-ray horizontal eye-movement tracking device and recording the electroencephalogram, as shown in Figure 2. By using cross-correlation methods, eye-movement evoked responses can be extracted.

Figure 2. Electrode helmet and infrared photodiode eye-movement tracking spectacles.

The second phase of this study involves the combination of eye-movement tracking and multichannel scalp recording with computer-fitted source localization procedures to locate the neural control mechanisms in the brain.

This is a preliminary report of work in progress and as yet no written reports are available for distribution.

177. EYE MOVEMENTS IN READING

Investigators: **Michael T. Hyson, Raymond P. Briggs, Patrice L. French, Derek H. Fender**

The goal of this project is to investigate the role of eye movements in reading. We are developing a multiprocessor, real-time computer system, and a high speed plasma display in order to change text on the display in response to a subject's eye motions. The processors should allow the prediction of saccadic motions and alterations of text in 9 msec.

The reading studies have proceeded by studying the discriminability of different letters with different fonts. We have developed a computer routine which collects the reaction times of subjects deciding if pairs of letters are identical or different. From these data we may infer the features that allow discrimination of letters.

We have improved the linearity and stability of the scleral lens eye-movement measuring system so that we can consistently monitor a subject's gaze within 0.1° over a 10° field of view. This allows us to specify which letter on a display is being fixated. We have adapted an infrared photodiode eye-position measuring device for subjects who do not have scleral contact lenses and have made a computer animation of eye motions of a subject reading to allow slow motion study of fixations.

We are now studying eye motions in response to texts in which meaning and structure are highly constrained in order to assess the relative amounts of cognitive control and involuntary control of eye motions. In one experiment we ask the subject to read simple tautologies:

Paul is taller than Fred.

Fred is shorter than Charles.

Who is taller?

Paul Fred Charles

The subject reads the text and fixates his answer. Thus the eye motion indicates which parts of the text are important for answering the question and also measures the total time required to answer different types of questions.

In a short time, we will have programs that will initiate and control the text display on the basis of eye position--the "Eye Button." We will then collect descriptive statistics of eye motions in reading and predict points of regard in order to place a "window" of text at the point where the subject is looking. Varying the size of the window will determine the role of peripheral vision and other cues in reading.

These studies of normals will eventually encompass dyslexic children in order to determine if poor eye motions are related to poor comprehension.

This is a preliminary report of work in progress and as yet no written reports are available for distribution.

178. STUDIES OF EYE TREMOR

Investigators: **Michael T. Hyson, Lawrence Stark*, Derek H. Fender**

Small motions of the eye, <30 sec of arc, are called tremor, a constant movement that serves to refresh the visual image.

We are developing a new device to track these motions using the scleral contact lens. A weak beam of laser light will be reflected off a small mirror attached to the scleral lens, and imaged onto an array of photodiodes. As the eye turns, the mirror will deflect the beam to different positions on the photodiodes, indicating the eye's position. We hope to characterize the velocity, displacement, and spatial distribution of tremor in order to infer characteristics of the oculomotor system.

Stark has shown that voluntary nystagmus is saccadic in nature. We hope to show that tremor also has saccadic character. If this is so, we may infer that the neural structures generating eye motions are fundamentally the same for both the largest and smallest eye motions.

The study of the spatial distribution may show that tremors have a "fractal" character, similar to Brownian motion (see Mandelbrot, 1977). If eye motions are "fractal," they would have the same statistical distribution regardless of scale. This would be a useful property for a pattern recognizer, such as the brain.

This is a preliminary report of work in progress and as yet no written reports are available for distribution.

Reference:

Mandelbrot, B. B. (1977) Fractals, Form, Chance, and Dimension, W. H. Freeman, San Francisco.

*College of Optometry, University of California, Berkeley, California.

179. STUDIES OF MOTION DETECTION IN STABILIZED VISION

Investigators: **Itzhak Hadani, Michael T. Hyson, Derek H. Fender**

The monocular detection of image motion on the retina is an important property of the visual system. Hadani et al. (1978) have suggested that since monocular retinal images occurring at successive times are also seen at different points of view, due to tremor and other eye motions, they could be used to infer spatial location and depth.

We therefore tested this conjecture under stabilized and nonstabilized conditions using random dot stereograms containing bars and squares. If presented binocularly, such pairs give rise to planes in depth. However, we presented them to one eye alternately. This gives the impression of a jumping bar or square, and indeed gives rise to monocular depth impressions that vary depending on whether the image is stabilized, its rate of presentation, and the magnitude of displacement, showing that eye motions do affect depth impressions. In general, displacements and rates of presentation had to be higher under stabilized conditions.

We also found that motion detection in normal vision is highly acute. Absolute motions could be detected down to 35 inches of arc. Differential motion could be detected down to 2 sec of arc with 80% reliability and 5 sec of arc with 100% reliability.

This is a preliminary report of work in progress and as yet no written reports are available for distribution.

Reference:

Hadani, I., Ishai, G. and Gur, M. (1978) Visual Stability and Space Perception in Monocular Vision: A Mathematical Model. Technion Research and Development Foundation, Israel.

180. BINOCULAR HYSTERESIS AND FUSION WITH VOLUNTARY EYE MOTIONS

Investigators: **Michael T. Hyson, Bela Julesz**

For this work we fit subjects with scleral contact lenses which move with their eyes. A stalk attached to each lens carries a mirror, used to "stabilize" retinal images, and a small light bulb, used in monitoring eye movements.

A "stabilized" image stays at the same place on the retina regardless of where the subject looks. The "stabilization" is done by projecting an image onto the mirror attached to the lens and focusing the reflected

image back onto the subject's retina. As the eye turns, the image is deflected just enough so that it still lands on the same retinal location.

To monitor eye motions, the light bulbs on each lens are made to cast shadows onto photocells, whose voltage output changes with the amount of light reaching them. The turning of the eyes causes the shadows to move, changing the photocells' output voltages, which are recorded for later analysis.

One function of the brain's eye-movement control system is to point both eyes at the same point in space. This allows the extraction of binocular depth information by the visual system. Fender and Julesz (1967) found that if the images to the two eyes are stabilized on the retinas, the left eye and right eye images can be misaligned horizontally up to 2° before the sensation of depth in a random dot stereogram is lost. To regain the depth, the images must be almost realigned, thus showing that the detection of depth and the tolerance of the system for alignment errors depend on its history of stimulation and thus shows hysteresis.

We have extended this idea to normal vision with voluntary eye motions. We misalign the images while monitoring the motions of the eyes. The subject maintains his depth percepts during this misalignment, even though the images now fall on new parts of the retina. This suggests that the brain is dynamically altering its internal frame of reference during this process. We call this a "cortical shift," representing cooperative processing among different areas of the brain. We have found that the subject can tolerate misalignment of up to 4°, somewhat more than under stabilized conditions.

We have now observed that fusion is maintained in normal conditions by a combination of vergence motions and a shift of the cortical frame of reference. Eye motion accounts for about 2.5° of the tolerated misalignment and the cortical shift about 1.5°.

We have also found a region of instability where fusion becomes temporarily impossible. Further study of this process will lead to an understanding of the dynamics of coordination between muscular and cortical fusion mechanisms.

This is a preliminary report of work in progress and as yet no written reports are available for distribution.

Reference:

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Professor: Masakazu Konishi
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Research Staff: Eugene Akutagawa, Marilyn S. Cunha, William L. Fromm, Jamie P. Leverette
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181. INNATE PREDISPOSITION IN BIRD SONG LEARNING

Investigators: **Masakazu Konishi, Eugene Akutagawa**

It has long been known that young songbirds selectively learn the song of their own species. This led to the hypothesis that young birds are endowed with an inborn ability to recognize the conspecific song. A rigorous test of this hypothesis requires birds uninfluenced by sounds other than their own. Gene Akutagawa has developed a technique to raise white-crowned sparrows from the egg in total sound isolation. So far we have obtained only one male among those raised in this manner. This bird learned the song of its own species in a choice situation involving the songs of five sympatric species.

182. FUNCTIONAL ORGANIZATION OF THE AUDITORY FOREBRAIN IN BIRDS, WITH AN EYE ON SONG LEARNING

Investigators: **Hans-J. Leppelsack, Daniel Margoliash**

One of the remarkable facts about songbirds is that they can be successfully taught to sing a variety of artificial, computer-generated song facsimiles. For example, a white-crowned sparrow, whose natural song may consist of two or three simple phrases composed of whistles and trills, will accept computer-generated versions of the songs in which the order of the phrases has been scrambled, and in which the direction of each frequency modulation (FM) in the trill section has been reversed.

The availability of birds of the same species that sing simple, yet significantly different songs, encourages one to search in the brain for units whose response properties specifically reflect the song learning experience. To this end, we have conducted preliminary experiments on the functional organization of the primary avian auditory center in the forebrain, Field L.

Whereas auditory centers in mammals can be located on the cortex and thus primarily extend in two dimensions, the analogous areas in birds (e.g., Field L) are located in the striatal parts, and thus the depths of the forebrain. A three-dimensional tonotopic organization clearly exists for some areas. In Field L, higher frequencies are represented more rostrally and ventrally. A certain subcenter of this nucleus, which lies medially, does not show as clear a tonotopy.

The functional organization indicated by these findings was used to localize units with song-related responses. In the medial area of Field L a higher percentage of units responding to FMs exist. Initial observations indicate the preferred direction of FM for the majority of units may be correlated with the direction of FM in the individual's song. Future experiments will attempt to ascertain definitively if this area indeed reflects the song learning experience.

183. CHRONIC NEUROPHYSIOLOGICAL RECORDINGS FROM SINGING BIRDS

Investigator: **James S. McCasland**

The system of brain nuclei which produces bird song exhibits many interesting features that make it a particularly suitable model system for studying some of the fundamental questions in behavioral neurobiology. Among these features are long-term information storage and retrieval, lateralization, sexual dimorphism, and adaptive plasticity following damage to the system. In recent years much has been learned about the neuroanatomical, hormonal, and behavioral aspects of bird song, but as yet little is known about the actual neuronal mechanisms of song production.

The purpose of this project is to obtain chronic recordings from neurons which are involved in producing song. Preliminary data from recordings of this type are available for nucleus RA, a telencephalic nucleus which sends efferent fibers to the syringeal motor nucleus (nXIIts) that controls vocalization. Multiple unit activity in RA of the zebra finch shows an increased level beginning about 50 msec before the onset of song, and a sharply decreased level (possibly due to inhibition from other song system nuclei) which coincides with the cessation of song. The neural activity shows little

modulation during song production.

The songs of many zebra finches consist of a variable number of repeated subunits, separated by intervals ranging from tens of milliseconds to half a second or more. The decrease in neural activity at the end of a song subunit anticipates the termination of song, i.e., the decrease occurs at the end of a given subunit if and only if the next subunit will not begin sooner than 50 msec. For longer delays, the decrease begins at the end of a subunit and lasts until 50 msec before the beginning of the next subunit. At the end of the song, multiple unit activity gradually recovers, reaching the baseline level after 1-2 sec. Thus the decreased activity represents a neuronal correlate of the bird's "decision" about further song production.

This preparation provides a unique opportunity to investigate neural correlates of song lateralization (differential activity in the left and right sides of the brain during song production), "command center" nuclei for song production, and single unit correlates of song.

184. CELLULAR ANALYSIS OF HORMONE-INDUCED SEXUAL DIFFERENTIATION IN THE ZEBRA FINCH SONG SYSTEM

Investigator: **Mark Gurney**

Male zebra finches sing a brief song phrase to the female during courtship. Castration of an adult male reduces the bird's frequency of singing; testosterone replacement reinstates the behavior. Testosterone treatment of female zebra finches does not activate song nor induce other elements of courtship behavior.

We find that correlative changes of brain and behavior in zebra finches are organized by sex hormones during development. Newly hatched zebra finch chicks were subcutaneously implanted with silastic pellets containing either 50 μg of dihydrotestosterone or 50 μg of 17 β-estradiol. Testosterone treatment activates song in adult females which were implanted with estradiol when chicks, but fails to activate song in those females which had received dihydrotestosterone. The singing females approach a sexual partner with pivoting movements, straighten to an erect posture, fluff their throat feathers, and rapidly repeat their song phrases in a behavioral sequence which closely resembles that of the male.

In zebra finches, brain nuclei of the efferent pathway for control of song show dramatic sex differences in their volume (Nottebohm and Arnold, 1976). We find that 17 β-estradiol treatment of genetically female chicks organizes male-like cytoarchitectonic differentiation of the telencephalic song nuclei RA, HVc, MAN, and X. Dihydrotestosterone induces masculinization of the brain stem song nuclei nXII and DM. Dendritic field spread, soma size, and the consequent volume of the brain nucleus is larger in males than females at all levels of the song system. The exposure of female chicks to either androgen or estrogen supports growth of the hormone's respective target neurons in either the brain stem or telencephalic song nuclei. These neurons reach a size identical with that of the equivalent cell type in a normal male. Our anatomical comparison of normal adult male and female song systems reveals that all cell types and all identified connections are present in both sexes. Thus, the specification of cellular identity--i.e., position, number, dendritic morphology, and efferent synaptic projection--is expressed independently of the hormonal environment. Rather than selecting pathways of anatomical differentiation, androgens and estrogens exert a similar pleiotrophic effect on their respective target neurons. Although in the female song system we identify all the cell types and connections present in that of the male, testosterone does not activate song. Thus, 17 β-estradiol may also exert a specific inductive effect on the telencephalic song neurons which renders them physiologically competent to respond to testosterone in the adult.

Reference:

Nottebohm, F. and Arnold, A. (1976) Science **194:** 211-213.

185. CAN TOYS BE USEFUL?

Investigator: **Daniel Margoliash**

The elucidation of a limited set of pertinent stimulus parameters is fundamental to an understanding of the information processing in sensory systems. The work of Hubel and Wiesel (1962) on cat visual cortex serves as an excellent example. They discovered that bars of light of various dimensions and orientations, moving in various directions, are highly relevant stimuli, both in terms of the response properties of individual cortical cells, and in terms of the arrangement of those cells within visual cortex. These notions have revealed basic principles of organization in the visual system.

Equivalent notions are only now emerging for studying the responses of auditory units in the central nervous system. In recent years, several preparations have

emerged which enjoy the advantage of having a well defined, limited stimulus parameter set. These include bat auditory cortex responses to echolocating calls, and spatial information processing of sounds in the midbrain of the owl. In these preparations, the choice of stimulus has been influenced by the behavioral repertoire of the animal. In less specialized animals, the choice of stimulus is more difficult. In such cases, no generalized schema exists for choosing the stimulus parameter set. It is likely that the inability to produce any desired sound has inhibited the development of a logical approach. Investigators using complex sounds have been limited by the particular library of sounds they have prerecorded. In view of this, I have developed a very flexible sound production system, consisting of a set of computer programs that control associated analog hardware. The programs define a language which enables the user to describe sounds (via numbers or drawings) in any domain he wishes. Typically, the frequency vs. time domain is chosen, this being a most convenient way to think of sounds. The computer then outputs appropriate control voltages to the hardware. The use of external hardware to generate the actual audio signal eliminates the computationally large task of calculating a signal that is modulated at the audio frequency; rather, the signal need only be modified at the rate of change of the audio frequency. The primary advantages of this approach are: (1) each new sound is defined and generated on-line, essentially instantaneously; and (2) the computer has enough time left over to carry out other tasks--specifically on-line data collection and data analysis. It will be interesting to see if this tool will be of significant advantage in developing a systematic approach to the study of the auditory system.

Reference:

Hubel, D. H. and Wiesel, T. N. (1962) J. Physiol. **160:** 106-154.

186. EAR OCCLUSION CAUSES SYSTEMATIC SHIFTS IN THE RECEPTIVE FIELDS OF AUDITORY UNITS

Investigators: **Eric I. Knudsen, Masakazu Konishi**

The ear openings of the barn owl (Tyto alba) are asymmetrically located on the head: the left ear is higher than the right. A consequence of this asymmetry is that the left ear is more sensitive to areas of space below the horizontal plane and the right ear is more sensitive above. Behavioral experiments show that ear occlusion causes systematic errors in the owl's ability to localize sounds: when the left ear is plugged, the owl localizes above and to the right of the sound target; when the right ear is plugged, it localizes below and to the left of the target.

A specialized region in the midbrain (MLD) of the owl has been implicated in spatial analysis of sound. Units in this region only respond to sounds from restricted areas of space (receptive fields) and are inhibited by sounds originating outside these areas. Furthermore, the units are arranged within this brain region according to the locations of their receptive fields so that they form a physiological map of auditory space.

We used a movable sound source to map the receptive fields of these units before and after plugging one ear. All units were affected in the same way. Plugging the left ear caused receptive fields to move down and to the left. Plugging the right ear caused fields to move up and to the right.

If the activity of a unit coded the location of a sound source in space, then an induced receptive field shift down and to the left, for example, would cause the owl to make a localization error above and to the right of the sound source--the effect that was, in fact, observed. These results support the contention that neurons in this specialized region of MLD are involved with spatial analysis of sounds.

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Summary: This past year we have continued our studies of the neural and neurochemical basis of motivation and learning. These studies have their origin in the 1954 discovery (Olds and Milner, 1954) that highly motivated approach behavior can be induced and maintained, to the exclusion of other behaviors motivated by naturally occurring stimuli, by direct electrical stimulation of the brain. These studies also showed that escape behavior from an aversive brain shock can be produced. The significance of these findings was twofold: first, they suggested the existence of neurons functionally organized to mediate motivation; and, second, they provided physiological psychology with an animal model for studying relationships between brain activity at particular sites and motivated behavior. Subsequent developments in this laboratory of methods for recording single and multiple unit activity in the rat during extended periods, while working for an electrical brain stimulus or while learning a task rewarded with food, made possible the initiation of studies aimed at understanding the neural circuitry underlying the motivational and cognitive components of behavior. The efforts of this past year in these two areas are summarized below.

I. Neural and Neurochemical Studies of Motivated Behavior. Previous studies in this and other laboratories have produced maps of the sites in the central nervous system of various species where electrical stimulation has rewarding or punishing effects. These maps did not succeed in identifying a classical anatomical pathway, or one of the more recently discovered anatomical systems which underlie the rewarding and punishing effects of electrical stimulation. These maps did, however, show a close overlap between the distribution of rewarding sites and the catecholamine systems, and between the punishing sites and the serotonergic systems. More recently, it has been shown that a similarly close overlap exists between rewarding sites and endorphin-containing neurons in the diencephalon of the rat, and between punishing sites and endorphin-containing neurons in the brain stem. It has also been shown that opiates have facilitatory effects on behavior maintained by brain-rewarding stimulation, and, at different doses, have analgesic effects on escape behavior from brain stimulation. These findings suggest that the catecholamine and the endorphin systems exert, at the very least, a modulation of the rewarding and punishing effects of brain stimulation. Our studies this past year have investigated the nature of this modulation. We have done this with methods which involved the permanent reduction of neural activity in the catecholamine systems through treatment of neonates with neurotoxins specific to the catecholamines and to serotonin; with methods for the detection of neurons whose output is directly a function of rewarding or punishing stimulation; and with methods which permitted the local application of putative transmitters at chronically denervated rewarding sites. We have also taken advantage of the availability of compounds with reinforcing properties, for example morphine, to test for reinforcing effects of local application at reinforcing sites. Another area of interest has been the relationship between reinforcing and stimulant effects.

Our findings this year show (1) that reinforcing effects can be produced by local infusion of morphine and the enkephalins at sites that are electrically reinforcing; (2) that the neural activity correlated with positive electrical or chemical reinforcement tends to be inhibitory; and (3) that the local application of compounds known to possess reinforcing properties in animals replicates the effect of the electrical rewarding stimulus on unit activity linked to the rewarding stimulus. In the past few months a method has been developed for recording single unit activity with a movable probe-cannula combination that permits the injection of minute substances in the vicinity of a reward-linked neuron and records its output during self-administration or self-stimulation.

II. Studies of Associative Changes in Auditory System. We have previously shown that multiple unit activity recorded from neurons in medial geniculate (MG) of the behaving rat exhibits associative changes in response to auditory stimuli that are made behaviorally significant or nonsignificant by conditioning procedures. It has been our goal to trace and identify the neural circuitry involved in these changes. Although this task has been complicated by the finding that only a small proportion of units recorded throughout MG show associative changes, the evidence now indicates that such

"conditionable" units may form a distinct subset of the population. Thus it may be that the conditionable units that make up only a small proportion of all MG units make up a large proportion of an identifiable subset of neurons. If it were possible to determine whether a neuron belonged to this subset prior to performing a conditioning experiment, the task of identifying the neural circuitry would be greatly facilitated and simplified. One of our primary research efforts during this past year has been toward this goal of identifying anatomical and physiological characteristics which differentiate units that exhibit associative change from those that do not. To this end we have recently completed a study in which multiple neurons were sampled systematically from the various subdivisions of MG during differential conditioning and reversal. This study showed that units in the medial and posterior portions of MG were more likely to show associative change than were those in other divisions, and that associative change was much more likely in units with a sustained response than in those with a transient response. We are following up these findings in three ways. First, we have developed techniques for single-neuron recording in behaving rats which allow us to describe more adequately the response properties of neurons in medial MG. Second, we are investigating whether these associative changes result from changes at the level of the MG or whether they are secondary to changes that occur earlier in the afferent pathway. This is done by recording from those parts of inferior colliculus which, according to the anatomical literature, project differentially to medial and posterior MG. Third, we are considering the possibility that these associative changes in MG may be brought about by changes in tonic efferent flow to MG. In this study we compare the changes that occur during conditioning with those that occur when tonal stimulation is preceded by electrical stimulation of paths efferent to MG.

Reference:

Olds, J. and Milner, P. (1954) J. Comp. Physiol. Psychol. **47**: 419-427.

187. SELF-ADMINISTRATION FOR INTRAHYPOTHALAMIC APPLICATION OF MORPHINE, LEVORPHANOL AND DALA

Investigators: **Marianne E. Olds, Kendrick N. Williams**

Adult male rats implanted chronically with a cannula guide and two electrodes glued to its shaft were tested for self-stimulation before and after self-administration tests for morphine, levorphanol, dextrophan, and a synthetic enkephalin, DALA.

For direct injection into the hypothalamus at sites yielding low to moderate rates of self-stimulation, rats learned to discriminate between two pedals placed at opposite ends of a Plexiglas test chamber, one that delivered 20 nl of morphine, 5 μg/μl for each depression of the lever, and the other that yielded nothing. The rates on the active lever were higher for morphine 5 μg/μl concentration than for the vehicle, which was artificial cerebrospinal fluid, and were higher for morphine 10 μg/μl than 5 μg/μl.

High-rate hypothalamic self-stimulators learned to reverse repeatedly when the active lever was changed from side to side in successive sessions and delivered 20 nl of 10 μg/μl morphine/press. Responding was reduced on both levers and discrimination between active and inactive levers was reduced when naloxone (1-2 μg/μl) was mixed with morphine, or when it was given systemically (1 mg/kg). Extinction after morphine was slow and often produced higher rates of responding than during the acquisition session.

High-rate hypothalamic self-stimulators self-administered DALA (0.1 and 1 μg/μl concentration) but rates were generally lower than for morphine and reversal was more difficult to achieve. Responding was attenuated for a mixture of DALA and naloxone injected into the hypothalamus. Levorphanol was approximately as effective as morphine but dextrophan yielded lower self-administration response rates.

Moderate-rate nucleus accumbens self-stimulators self-administered morphine and levorphanol. Non-self-stimulators with probe and cannula in the caudate nucleus did not self-administer morphine or did so at very low rates.

Post-drug self-stimulation tests yielded rates of responding for the electrical reward comparable to those seen in pre-drug tests, a result indicating minimal functional damage to the elements responsible for self-stimulation behavior even after repeated self-administration tests.

The data are interpreted to indicate the need of both opiate receptors and high rates of self-stimulation behavior to obtain self-administration for intracerebral injection of opiates. This finding is viewed as supporting the notion that structures known to support self-stimulation behavior may mediate the reinforcing properties of morphine, levorphanol, and DALA.

188. A LONGITUDINAL STUDY OF MOTILITY AFTER NEONATAL CATECHOLAMINERGIC DEPLETION BY 6-OHDA

Investigators: **Marianne E. Olds, James L. Fobes**

During the present decade interest in neurotransmitters has included a rapid expansion of investigations featuring selective depletion of neonatal catecholaminergic systems by 6-hydroxydopamine (6-OHDA). Although the 6-OHDA is typically administered intraventricularly, it is occasionally injected locally, and in either case a favored behavioral measure of the ensuing disruption in habituation is that of activity. This lesioning technique has provided considerable information on the role of the noradrenergic and dopaminergic systems, either of which may be selectively depleted by appropriate pretreatment that modifies the site of action of the 6-OHDA. One of the culminations of this line of inquiry has been the formation of a model of minimal brain dysfunction in humans.

Although intraventricular 6-OHDA injections at a very early age have usually resulted in subjects evidencing elevated activity levels compared with those for saline controls, the previous investigations have not examined a relatively wide age range for an extended test period. In addition, these earlier studies featured activity estimations by visual time span scans or with apparatus designed to record only two-dimensional movement.

In order more accurately to determine the effect of 6-OHDA on habituation, a parametric study was conducted that measured activity for 1 hr at 15, 25, 35, and 45 days of age. The activity of both 6-OHDA and saline controls was quantified by photocell activation due to displacements of the test cage's floor. Thus activity, which did not include movement along anterior-posterior or medial-lateral axes, could be included--for example, that arising from grooming or rearing.

The activity data counts, taken for each consecutive 10 min segment of the 60 min test sessions, were examined as a function of drug manipulation. Activity as a function of age at testing showed an inverted U-shaped function; activity increased between day 15 and day 25, remained the same through day 35, and decreased to its lowest level by day 45. When examined as a function of test segment, activity was found to decrease across time, and this reduction was more pronounced for saline controls than for the 6-OHDA group, whose activity rates were greater than those of the controls during most test segments.

189. UNIT ACTIVITY CORRELATED WITH TOPICAL OPIATE ACTION DURING SELF-ADMINISTRATION

Investigators: **Marianne E. Olds, Kendrick N. Williams, Dorwin L. Birt**

In a previous study we have shown that injection of morphine and a synthetic enkephalin, DALA, directly into the lateral hypothalamus leads to self-administration of the compounds, a behavioral measure indicative of reinforcing properties of the substances in the region where they are injected. Present knowledge of the neural action of these substances derives from iontophoretic injections in regions rich in opiate receptors or terminals that contain substances showing opiate-like properties. Under those conditions, morphine and DALA have mostly depressant effects. Exceptions are reported for the spinal cord and, in animals that are addicted, for other regions, especially the cortex. Under these conditions excitation is produced. Whether the findings are obtained in the immobilized animal with iontophoresis or in the awake animal with the systemic administration of the substances, they do not shed light on the neural action that underlies the behavioral effects of these substances. The two principal behavioral effects are analgesia, believed to be mediated at the level of the central gray, and euphoria, believed to be mediated in more rostral regions containing opiate receptors. By showing that rats will self-administer morphine and DALA administered directly in the hypothalamus, we have obtained a preparation in which the substance tested is self-applied repeatedly, yet preserves its positive reinforcing properties. Such a preparation offers the opportunity to investigate whether the neural activity related to positive reinforcement is a depression of the spontaneous or evoked rate of firing or is a gradual induction of excitation. We have initiated studies in which a rat implanted either with a combination cannula-single movable recording unit probe or with multiple fixed chronic electrodes implanted in the hypothalamus is trained to self-administer morphine. Simultaneously, information is obtained about the local neural activity before and immediately after each behavioral response.

These studies are in the preliminary stage and so far have revealed an initial depressant action of morphine that is blocked with repeated responses and may eventually be reversed to produce an excitatory effect. Controlled administration of the vehicle produced either a minor increase in the rate of firing or no effect. Our goal

is to confirm these preliminary findings at the single-unit level with the movable probe.

190. THE EFFECT OF [D-Ala^2]ENKEPHALIN AND OPIATES ON NEONATAL MOTILITY

Investigators: **James L. Fobes, Marianne E. Olds**

Although the analgesic and euphoric effects of opiates have received considerable attention in adult subjects, opiate influences on the behavior of neonates has not been examined. Consequently, the present pilot study began to examine this area by measuring the effect of morphine (MSO_4) and [D-Ala^2]enkephalin (Try-D-Ala-Gly-Phe-Met-NH_2) on movement across the ontogeny of neonatal rats. Each group of five subjects received subcutaneous injections of either MSO_4, [D-Ala^2]enkephalin, or normal saline 15 min prior to each test session. Test sessions were 24 min in length and took place on every fourth day from 4-32 days of age. Activity was determined on the basis of photocell activation due to cage floor displacement. This method of measurement is quite sensitive in that it provides quantification of three-dimensional activity rather than the two-dimensional movement along anterior-posterior and medial-lateral axes that is frequently reported. Thus, the present technique is also sensitive to such activities as rearing or grooming during which the animals' relative position in two-dimensional space frequently remains unchanged.

The saline control and [D-Ala^2]enkephalin groups did not differ significantly in their average activity, which ranged from 151 counts on day 4 to 12,656 counts on day 32, increasing linearly. In contrast, MSO_4 treatment resulted in considerable suppression of activity; the activity of neonates receiving morphine became increasingly less than that of the two other groups, with average activity ranging from 0 on day 4 to 3960 on day 32.

This preliminary examination justifies more extensive study that will feature replication of the three conditions mentioned above and the addition of three more groups. Groups to be added will comprise those receiving (1) naloxone; (2) morphine 15 min after naloxone pretreatment; or (3) [D-Ala^2]enkephalin 15 min after naloxone pretreatment. These groups will provide further control groups, in addition to the saline group, since naloxone can be expected to mask the effects of morphine and [D-Ala^2]enkephalin.

191. CORRELATION OF BRAIN STIMULATION REWARD AND CATECHOLAMINES IN DEVELOPING RATS

Investigators: **James L. Fobes, Marianne E. Olds**

The central catecholamine systems in the mammalian brain have been implicated in the mediation of positive reinforcement produced by brain stimulation on the basis of anatomical, physiological, and histochemical evidence. Permanent depletion of central catecholamine levels in adult rats by 6-hydroxydopamine (6-OHDA), a neurotoxin specific to the catecholamines, was found to affect differentially rates of bar pressing to obtain the brain stimulation reward applied in the hypothalamus, substantia nigra, raphe nuclei, and the locus coeruleus. Baseline patterns of responding differed in these regions with respect to the duration of the stimulus trains selected by the animal. After intraventricular treatment with 6-OHDA, the selection of duration for some regions was considerably altered. Responding for stimulation of the pontine area, and of periaqueductal midbrain region, was initially characterized by only short stimulation durations; this response pattern was not significantly altered by treatment with 6-OHDA. However, response patterns in hypothalamus and substantia nigra shifted from moderate to long duration of pulses.

Differential response reinstatement was evidenced subsequent to neurotransmitter replacement therapy in these various regions. Injections of norepinephrine (NE) or serotonin reinstated self-stimulation reinforced responding for stimulation in the hypothalamus but not in the other areas where responding was depressed by 6-OHDA. Dopamine (DA), though it did not effect a response change in the above areas, was accompanied by response enhancement for periaqueductal stimulation.

The sensitivity of these regions to 6-OHDA overlaps with neural distributions of NE and DA. However, the ontogeny of the relative contributions of each of these catecholaminergic systems that support motivational properties of self-stimulation has yet to be examined in neonates. In order to study developmental aspects of the neurochemical basis of the motivating effects of self-stimulation, chemical manipulations will be made in infant rats. These interventions will consist of selective disruption of NE and DA systems by local injections at various ages. At adulthood, these subjects will be tested to determine the consequences of the various neurochemical manipulations made in infancy by examination of the motivational properties of self-stimulation delivered

to hypothalamic and limbic regions. The ontogeny of the motivational aspects of self-stimulation itself will proceed in another group by testing for rewarding effects of brain stimulation delivered to hypothalamic and limbic regions from day 5 on.

192. DEPRESSANT EFFECTS OF TOPICAL MORPHINE ON SELF-STIMULATION-RELATED UNITS IN HYPOTHALAMUS

Investigators: **Marianne E. Olds, Robert Nienhuis**

This study investigated the effects of locally injected morphine on hypothalamic unit responses correlated with self-stimulation behavior. Adult male rats were implanted with probes for self-stimulation in the posterior lateral hypothalamus and in the substantia nigra. Each rat was also implanted chronically with a combination cannula-recording unit assembly in the lateral hypothalamus at the level of the ventromedial nucleus. Animals were selected for tests if they self-stimulated for shock applied either in the hypothalamus or in the substantia nigra and if the spontaneous rate of firing of units anterior to the hypothalamic reward site was reduced or inhibited during self-stimulation. In a test, three needles connected to polyethylene tubing filled with test solutions and individually controlled were inserted into the cannula guide. Two of the needles were used to inject morphine at two different concentrations (5 μg and 10 μg/μl artificial cerebrospinal fluid) and the third to inject naloxone (1 or 2 μg/μl).

Morphine at the 5 μg dose had small and variable effects. Repeated applications of this low dose tended to increase the number of units that became excited. Morphine at the 20 μg dose consistently depressed the rate of firing of the neurons. Repeated injections potentiated the depression. Simultaneous application of morphine and naloxone blocked or attenuated the depressant action of morphine at the 10 μg dose and sometimes even produced excitation. Naloxone by itself also produced excitation in a number of units. These results are taken to indicate a direct and specific action of morphine on neural activity in the hypothalamus related to a central positive-reinforcing mechanism. The action of morphine at this level may be part of a process that mediates the reinforcing effects of opiates on behavior.

193. UNIT ACTIVITY IN THE THALAMUS OF THE RAT RELATED TO A CONDITIONED EMOTIONAL RESPONSE

Investigators: **Marianne E. Olds, Kendrick N. Williams, James L. Fobes**

Evidence from lesion and stimulation studies suggests a role for the medial and reticular nuclei of the thalamus in the processing of information related to emotionality. Evidence for such a role at the neuronal level is still lacking, however. For this reason, unit activity studies of these thalamic nuclei were undertaken using a behavioral paradigm that resulted in the establishment of a conditioned emotional reaction.

Experiments were carried out in rats implanted with unit recording electrodes aimed at medial thalamic nuclei. Analysis of the results was performed on two groups of probes, those that fell in the above thalamic nuclei and those that fell outside these regions. In addition, each pattern of unit firing in response to the two auditory stimuli that were, respectively, 1 and 10 kHz tones, was characterized in terms of its strong, moderate, or no linkage to the auditory stimuli. In the medial nuclei of the thalamus, the distribution of responses indicated that a preponderance of units fitted the "moderate" or nonresponsive patterns of firing; in regions outside these structures, about a third of the probes yielded patterns of firing that fitted the category of "strong auditory" linkage, a third that of moderate auditory linkage, and the remainder that of nonresponsive.

Each animal was given three tests that yielded information about its behavioral response to the presentation of stimuli and about the pattern of firing of the units immediately prior to the presentation and immediately following it. In the first test, pseudoconditioning, a 1 kHz and a 10 kHz tone as well as a food pellet were each presented 180 times in random order. The consumption of two-thirds of the pellets delivered served as a marker that the animal was food-magazine trained. In the second test, conditioning, the 1 kHz tone always preceded by 1 sec the food reward, whereas the 10 kHz tone did not. In this test, the movement of the animal to the food-magazine during the presentation of the 1 kHz tone (conditioned stimulus) served as the marker that the animal had learned the association between one of the tones and the food reward. In the third test, the paradigm was essentially the same as in the second test, but the act of retrieving the food pellet from the magazine yielded not

only the food reward but also, and at the same time, a footshock. The unit data in the third test were analyzed in terms of two behavioral consequences, the 100% suppression of the learned behavior (movement of the animal to the food-magazine and retrieval of the food reward) and the 50% suppression of this behavior.

For each test, two questions were asked: (1) Do changes in absolute magnitude of the rate of firing favor one of the auditory stimuli? and (2) What was the direction of the change for each stimulus over the course of the trials?

The results of the pseudoconditioning test indicate that absolute magnitude changes favored the 10 kHz tone that was the higher frequency of the two stimuli. The direction of change showed increases over the course of the trial series for probes outside the medial and intralaminar nuclei, and no trend in either direction in the medial nuclei.

The results of the conditioning test showed a shift in the responsivity of the neurons from what they showed when the tones had no motivational meaning. In terms of absolute magnitude, the 10 KHz tone was no longer favored, and changes were balanced for both stimuli even though one was higher in frequency. The excitatory trend in the auditory and marginal unit patterns over the course of the trials was blocked, and in some cases replaced, by a minor shift in the negative direction.

The results of the conflict test (conditioned emotional behavior) showed a balance between the two stimuli in terms of the absolute magnitude of the changes, and the direction of the changes pointed to a marked decrease in the rate of firing to the presentation of both stimuli.

In the group of animals showing 50% behavioral suppression the changes in pattern of firing between test I, where responsivity was principally to the physical characteristics of the two auditory signals, and test III, where responsivity was determined by both the physical and the motivational properties, were more pronounced than in the group of animals showing 100% suppression.

In the thalamus, the findings show that the nonresponsive neurons, which were distributed more frequently in the medial nuclei than outside them, showed more changes than did the strongly sensory-linked neurons, which were found to be more widely distributed outside the medial nuclei. Under strong motivational conditions, the responsiveness of these neurons need not necessarily show an increase in the rate of firing but instead can show enhanced responsivity in relation to the "background" rate. The specific pattern of cellular discharge in the thalamus was not so much an absolute increase in the response following the stimulus presentation as a decrease in the background activity (noise of the system). The correlation between the significance of the conditioned stimulus and the pattern of unit activity was not specific in that it was not restricted to the CS+.

194. CHARACTERISTICS OF RAT MEDIAL GENICULATE NEURONS DURING DIFFERENTIAL CONDITIONING AND REVERSAL

Investigators: **Dorwin L. Birt, Marianne E. Olds**

We recently reported that, in a conditioning paradigm which eliminated a number of previously demonstrated nonassociative factors, a proportion of neurons in medial geniculate of rat showed short latency associative change. The present experiment was designed to determine the distribution of such units throughout the subdivisions of the medial geniculate and to identify response characteristics that might differentiate these units from units not showing associative change.

The multiple unit responses on 117 probes distributed throughout MG were recorded during differential appetitive conditioning, extinction, and successive reversals. Of these, 73 met criteria for responsiveness and stability of recording and were further analyzed. Eleven units that showed enhanced response to the positive stimulus relative to the response to the negative stimulus throughout four or more consecutive reversals were considered to be associative. These units were largely localized in the far posterior portion of MG where 25% (9 of 36) units were associative. Only 5% (2 of 37) units in the remainder of MG were associative. Associative units were further differentiated from nonassociative units by differences in the temporal pattern of their initial response prior to conditioning. Units with a more sustained response were more likely to show associative change than were those with a very transient response, and the magnitude of change was positively related to the initial duration of response. Of the units that initially were responsive for more than 80 msec, 71% (5 of 7) showed large magnitude associative change during conditioning. Only 9% (6 of 66) of the units that were initially responsive for 80 msec or less showed associative changes and these were of small magnitude.

These associative neural changes were shown not to

result from behavioral feedback, since their latency (as early as 16 msec after stimulus onset) preceded behavioral response and since they were not directly correlated with behavioral change. They appear to be more directly related to processing of the significance of stimulus information.

The facts that these changes are localized to a discrete portion of MG with known differential afferents and efferents, and that associative units have different temporal response patterns which are easily identified, lead to the potential for identifying the neural circuitry involved using single-unit analysis techniques.

195. UNIT STUDIES OF ASSOCIATIVE CHANGES IN THE INFERIOR COLLICULUS OF THE RAT

Investigators: **Kendrick N. Williams, Marianne E. Olds**

Early experiments in this laboratory aimed to identify sites in the mammalian CNS where unit activity might reflect cognitive changes related to the acquisition of a differential conditioning task signaled by auditory stimuli and rewarded with food. Using a paradigm which did not take fully into account the temporal relation between the food reward and the presentation of the conditioned and control stimuli and its possible effect on the state of arousal of the subject, some changes in the responsiveness of interior colliculus neurons at short latencies were considered associational changes. In subsequent experiments, where sources of nonassociational changes were minimized by a counterbalanced design, the evidence indicated that the changes reported earlier for this nucleus of the auditory pathway were of a nonassociative nature. Additional tests with criteria for associational changes that made use of multiple reversals further indicated an absence of changes that could be construed as of associational origin. However, histological verification of probe placement in these studies showed location of the sites of recording to be in the central nucleus of the inferior colliculus. Since the inferior colliculus includes several subdivisions with differential input and projections, it seemed of interest to investigate whether associational changes might be found in these subdivisions. With this goal in mind we are presently investigating the external nucleus and the dorsomedial region. The findings to date have not as yet shown that associational changes in auditory stimuli-evoked unit responses are reflected in these subdivisions; however, there may be changes, and these may be of a subtle nature requiring other criteria for identification.

196. RESPONSE PROPERTIES OF SINGLE NEURONS IN THE MEDIAL DIVISION OF MEDIAL GENICULATE IN THE BEHAVING RAT

Investigator: **Dorwin L. Birt**

Experiments using multiple unit techniques have indicated that neurons which exhibit associative changes during conditioning may be highly localized and have distinguishable response properties. We have felt that this possibility could be more rigorously studied using single-unit recording methods. For the past two years we have been developing a system that allows us to acquire data on a number of physiological characteristics of single neurons of auditory system of behaving rat. This system utilizes a miniature microdrive which can be attached to a platform on the rat's head and which permits movement along all three axes. Single-neuron potentials can be isolated and held for sufficient time to conduct a variety of tests. The first phase of these experiments has been aimed toward classifying response properties of neurons in the medial and posterior portions of MG.

The following properties are being routinely studied, using a combination of computer-controlled and manual stimulation and recording techniques: (1) mean spontaneous rate and temporal patterning of spontaneous activity; (2) latency and temporal pattern of response to tonal stimuli; (3) determination of the range of tonal frequencies to which the unit is responsive; (4) test of response to somatosensory stimuli; and (5) test of response to gross visual stimulation.

The neurons that have been studied in this region can be grouped in terms of their response to tonal stimulation into the following categories: (1) onset units that give a single spike or a short burst of spikes 10 to 20 msec after the onset of a stimulus; (2) sustained units that begin to fire about 10 to 20 msec after stimulus onset and continue to fire throughout the duration of the stimulus; and (3) long latency units that give a burst of spikes beginning at 40 msec or more following onset. Units that are inhibited by tone presentation have not been observed. Only a very few responses following stimulus offset have been seen. Although most units have responded more strongly to some frequencies of tonal stimuli than to others, the responses generally have tended to be very broad-band. Nearly all units that respond to tonal stimulation respond also to somatosensory stimulation. Units located anteriorly in medial MG have fairly restricted somatosensory receptive fields on the contralateral nose and face. Units located

posteriorly have broad somatosensory receptive fields that often cover the entire contralateral side of the body.

The next phase of these experiments will use conditioning procedures to alter the behavioral significance of auditory stimuli while keeping their physical characteristics unchanged. With the availability of detailed data concerning the response properties of these units we should be able to define much more adequately the properties that distinguish associative from nonassociative units.

197. MODULATORY EFFECTS OF THALAMIC RETICULAR FORMATION ON TONE-EVOKED RESPONSES OF MEDIAL GENICULATE OF RAT

Investigator: **Dorwin L. Birt**

One goal in studies of the neural circuitry of associative changes during learning is to determine the relative contribution of different components of the circuit to the observed changes. Some of the associative changes observed in medial geniculate are of such short latency that the neural events that follow the onset of a particular stimulus must involve a relatively circumscribed circuit. It seems quite conceivable, however, that in a sequence of conditioning trials changes involving very large neural circuits might occur in the intervals between trials and would alter or bias the way neurons respond to subsequent stimulus presentations. It is well established that activity in medial geniculate nucleus is modulated by activity in a variety of structures, including the mesencephalic reticular formation and the sensory projection areas of cortex, and by a system called the mediothalamic-fronto-cortical system. The final common pathway of several of these systems is through a thalamic nucleus called the thalamic reticular formation (TRF). This nucleus projects to, and has been shown to modulate, medial geniculate activity. We have chosen to study this nucleus as a first step in determining whether descending tonic modulatory effects might account for short latency associative changes in medial geniculate.

This study involves three principal procedures: (1) recording, during conditioning, the simultaneous activity of medial geniculate neurons and neurons in TRF to see whether systematic changes in the activity of TRF units in the intervals between trials are related to changes in the response of MG neurons to positive and negative conditioned stimuli; (2) assessment of the effect of changes in TRF activity on tone-evoked responses in MG by comparing tone-evoked MG responses in which the tone presentation has been preceded by electrical stimulation of TRF with those in which it has not; (3) assessment of the response of TRF units to the auditory stimuli used as positive and negative conditioned stimuli to determine whether any specificity exists in the modulatory effect of TRF on MG, and if so whether this specificity is related to the response properties of the TRF units.

This study is now at an early stage. Present results show that in some cases stimulation of TRF has a powerfully inhibitory effect on tone-evoked responses in MG. We have not yet seen evidence of the specificity that would be required for this modulation to account for associative changes in a differential conditioning paradigm, since the inhibition seems equally powerful on both the positive and negative stimuli, and since stimulation between a pair of electrodes in TRF seems to affect responses either on all the recording probes in MG or on none of them.

198. LOCUS COERULEUS AXONS EXHIBIT VARIABILITY IN IMPULSE CONDUCTION LATENCY

Investigators: **Gary Aston-Jones*, Menahem Segal**, Floyd E. Bloom***

It is generally conceived that axons carry impulses with constant velocity and act as simple transmission lines between soma and synapse. Recent studies have suggested, however, that certain axons can modulate their own impulse flow; indeed, we now report that this ability is especially pronounced in the axons of noradrenaline-containing locus coeruleus (LC) neurons.

Chloral hydrate-anesthetized rats were implanted with stimulating electrodes in anterior cingulate cortex and olfactory bulb. Glass micropipettes recorded isolated impulses from single LC neurons, subsequently identified histologically. While studying antidromic (AD) conduction properties of these neurons, we observed striking alterations in AD latency not previously reported. Each of 12 antidromically activated LC neurons tested in 8 rats exhibited a pronounced gradual and continuous increase in latency (in some cases >20 msec) during 2 to 10 Hz stimulation, and a gradual and continuous recovery to basal AD latency (mean = 55.3 $\pm$ 4.2 msec) over 1 to 2 min when stimulus frequency was reduced. During trains of constant frequency stimulation, the AD latency increased somewhat asymptotically to a new, fairly stable value after 50 to 400 stimuli. In addition to these AD latency

increases, closer examination revealed that the second and closely succeeding stimuli in a 10 Hz train yielded decreased latencies, followed by the larger increases in AD latency during subsequent stimuli in the train. In neurons tested with pulse pairs, a decrease in AD latency of 0.5 to 3.5 msec for the second stimulus was consistently observed. The magnitude of variation in AD latency was positively correlated with three factors: (1) the initial AD latency of the neuron, (2) the frequency of stimulation, and (3) the number of pulses in a train of stimuli. Preliminary experiments have revealed marked AD latency alterations in the raphe and substantia nigra systems as well.

Data indicate similar conduction time variability as a consequence of nonstimulated activity and suggest that changes in conduction velocity along the axons themselves underlie these AD latency fluctuations. These fluctuations may allow long, unmyelinated axon systems to transfer information most effectively with short bursts of activity, and suggest that large axon diameters and myelination may be necessary for high fidelity as well as for high velocity of impulse flow in nervous tissue.

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199. MONOAMINERGIC RECEPTORS IN VISUAL CORTICAL PLASTICITY

Investigator: **Takuji Kasamatsu**

This study aims to elucidate the type of monoamine receptors which are involved in synaptic plasticity in the developing kitten visual cortex. The basic strategy for answering this question is available (Kasamatsu and Pettigrew, 1979; Kasamatsu et al., 1979). A preliminary study showed blockade of the usual shift of ocular dominance following monocular lid-suture, if the visual cortex had been concurrently perfused with a β-adrenergic receptor antagonist, propranolol (Kasamatsu, 1979).

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200. INTRACORTICAL SPREAD OF NOREPINEPHRINE AND 6-HYDROXYDOPAMINE

Investigators: **Takuji Kasamatsu, Toru Itakura, Gösta Jonsson*, Jeremy P. Brockes**

We have been studying the extent of intracortical diffusion of norepinephrine (NE) and 6-hydroxydopamine (6-OHDA) delivered through the continuous perfusion technique (Kasamatsu et al., 1979). Four methods have been used.

Catecholamine Fluorescence Histochemistry. The kitten visual cortex was perfused locally with 4 mM 6-OHDA for a week, and then prepared for catecholamine histofluorescence using a modified glyoxylic acid-cryostat method. We found an area close to the perfusion site in which no greenish fluorescent fibers and terminals remained. The radius of this primary lesion area was 4 to 5 mm from the center of perfusion.

Endogenous Catecholamines. An electrochemical assay was performed for NE and dopamine (DA) (Keller et al., 1976) in kitten visual cortex perfused with 4 mM 6-OHDA for a week. Endogenous NE was less than 10% of the control 3 to 4 mm away from the perfusion site. It increased to 80 to 90% of the control at 7 to 8 mm. The DA content behaved similarly but was less affected by 6-OHDA treatment.

Intracortical Distribution of Tritiated NE and Tritiated 6-OHDA. The spatial distribution of tritium in the neocortex was studied following 1, 3, or 7 days of continuous perfusion with 50 μM nonradioactive NE mixed with a tracer quantity of tritiated NE. Radioactivity was maximal at the site of perfusion and decreased exponentially in the neighboring area. This distribution pattern was attained as early as one day after the start of perfusion. Although the radioactivity at a given site in the cortex increased with time, its maximum detectable extent was about the same (10 mm) for the three different perfusion terms. A similar study has been performed after perfusion with tritiated 6-OHDA to obtain the same range of intracortical spread.

Electrophoresis. The above study concerns the total radioactivity which is due to both unmetabolized NE and metabolites of NE. Therefore, we are going to examine the proportion of radioactivity which comes from unmetabolized NE, using electrophoresis to separate unmetabolized NE from all other catecholamines and their metabolites.

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201. MORPHOLOGY OF CATECHOLAMINE-CONTAINING TERMINALS IN KITTEN VISUAL CORTEX

Investigators: **Toru Itakura, Takuji Kasamatsu, John D. Pettigrew**

The intracortical distribution of catecholaminergic terminals has been studied with a modified glyoxylic acid histofluorescence method (Itakura et al., 1978). The ultrastructure of monoaminergic (MA) terminals has been also studied, using electron microscopy, in materials fixed with glyoxylic acid perfusion followed by immersion in potassium permanganate (Itakura et al., 1979).

Intensely fluorescent stem fibers entered layer VI from the white matter after changing their course vertically toward the cortical surface. Many collaterals were seen in various directions in the middle layers. In the superficial layer, fine terminal fibers with varicosities run parallel to the surface. The dense plexus formation was found in layers II and III.

MA (probably norepinephrine)-containing terminal boutons were identified as bulgings which contained small- and large-cored vesicles. Although most of these axon bulgings did not show the typical synaptic membrane specialization, about 10% of them showed membrane thickening as well as a widened synaptic cleft. We are particularly interested in clarifying the likely neural elements with which individual MA boutons maintain intimate contact. The most common targets of MA terminals were dendrites and dendritic spines throughout the six cortical layers. Certain unique features in particular layers were noted, however. For example, axo-somatic contacts were primarily found in lower layers and axo-spiny contacts in upper layers.

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202. BINOCULAR COMPETITION: ITS ENHANCEMENT BY NOREPINEPHRINE

Investigators: **Baruch Kuppermann, Takuji Kasamatsu**

It has previously been proposed that norepinephrine (NE) plays a role in synaptic plasticity in cat visual cortex (Kasamatsu and Pettigrew, 1976; Pettigrew and Kasamatsu, 1978). In the present study, cats given continuous local perfusions of 50 μM NE for seven days with a binocular environment were found to have a conspicuous lack of cells dominated by the ipsilateral eye, even though the ratio of binocularly/monocularly driven cells remains unchanged. This effect was not seen for periods of NE perfusion of less than three days. A slight innate contralateral bias in ocular dominance distribution has long been observed (Hubel and Wiesel, 1962). Therefore, NE is involved in exaggerating a natural bias in ocular dominance distribution. Several normal kittens were subjected to the same procedure, continuous local perfusion of 50 μM NE for seven days, but were simultaneously kept in the dark. The resulting ocular dominance histograms were quite normal, with no exaggerated contralateral skewedness observed. This suggests that the tendency for NE to induce a contralateral bias is activity-dependent (Kuppermann and Kasamatsu, 1979). In current experiments, spontaneous tonic retinal activity (Kasamatsu, 1976) is being directly manipulated to test this hypothesis further.

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203. EFFECTS OF EXOGENOUS NOREPINEPHRINE IN CORTICAL PLASTICITY: IONTOPHORETIC ADMINISTRATION

Investigators: **Paul Heggelund, Takuji Kasamatsu**

Previous studies suggested strongly that norepinephrine (NE) and NE-containing nerve terminals are necessary to maintain synaptic plasticity in kitten and cat visual cortex (Pettigrew and Kasamatsu, 1978; Kasamatsu et al., 1979). How quickly do the changes in synaptic plasticity take place in NE-manipulated visual cortex? We will study the effects of iontophoretically-injected NE and related substances on the identified visual cortical cells. Particular attention will be paid to the following points: (1) Are there differential effects of NE on different types of cortical cells? (2) Can one modify the cell's preference of ocularity under continuous administration of NE? and (3) Can electrical stimulation of the locus coeruleus mimic the changes induced by iontophoretically-injected NE in the same visual cells?

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204. AN HRP STUDY OF NEURONS PROJECTING TO TECTUM AND GENICULATE IN OWLS

Investigators: **Hermes Bravo, John D. Pettigrew**

Pressure injections of HRP were made into the optic tectum or the lateral geniculate nucleus of burrowing owls, in which the lateral surface of the diencephalon and/or mesencephalon had been directly visualized after dissection of the overlying bone. After 2 to 3 days' survival the animals were dark-adapted for 4 to 6 hr at night and perfused with formalin. The retinas were dissected off and postfixed for 5 min in 2.5% glutaraldehyde. Retinal and brain sections were reacted with benzidine to demonstrate HRP.

Patterns of labeling from each site were strikingly different. After tectal injections, two classes of retinal ganglion cell were labeled contralaterally, one small (5 to 9 μ) and the other large (15 to 23 μ), with a widespread retinal distribution and a marked concentration along the horizontal streak. In contrast, lateral geniculate injections labeled a single morphologically distinct class of retinal ganglion cells (7 to 9 μ), with a distribution largely restricted to temporal retina and a marked concentration in the fovea. No ipsilaterally labeled cells were observed after injections in tectum or lateral geniculate. In the visual Wulst, tectal injections led to labeling in a distinct class of large multipolar neurons located within 1 mm of the pial surface, toward which many of them could be seen to send an axon. Wulst neurons labeled after geniculate injections were very sparse, of small size, and also confined to the superficial layers.

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Graduate Students: Karen E. Gaston, Larry E. Johnson, Betty A. Vermeire
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Summary: Our work continues to be focused on problems in the following areas: (1) hemispheric specialization and functional organization in human commissurotomy, hemispherectomy and other neurosurgical patients with occasional study of normal subjects and children; (2) mechanisms and pathways for visual processing and interhemispheric transfer and recall of visual information in cats and monkeys and the problem of hemispheric functional asymmetry in monkeys; (3) factors underlying learning and interocular transfer of conditioned food aversion in newly hatched chicks; (4) neural plasticity as evidenced in the growth and developmental organization of the retinotectal pathways.

205. ANATOMICAL BASIS OF INTEROCULAR EQUIVALENCE AND FUNCTIONAL HIERARCHIES

Investigators: **Charles R. Hamilton, Suzannah B. Tieman*, Ronald L. Meyer**

The location in the brain of components of visual perception and their hierarchical relationship to each other may be studied by testing for interocular equiva-

lence in partially-split-brain monkeys with different regions of their interhemispheric commissures left intact (Biology 1978, No. 207). Lack of interocular equivalence implies that the mechanism in question lies before the levels left interconnected. By this test various sensory, perceptual, mnemonic, and coordinational processes have been sequentially ordered and located (Hamilton, 1980). More precise localization of function to different cortical areas appears feasible as judged from our anatomical studies of the localization of interhemispheric connections from different visual areas. Figure 1 illustrates some of

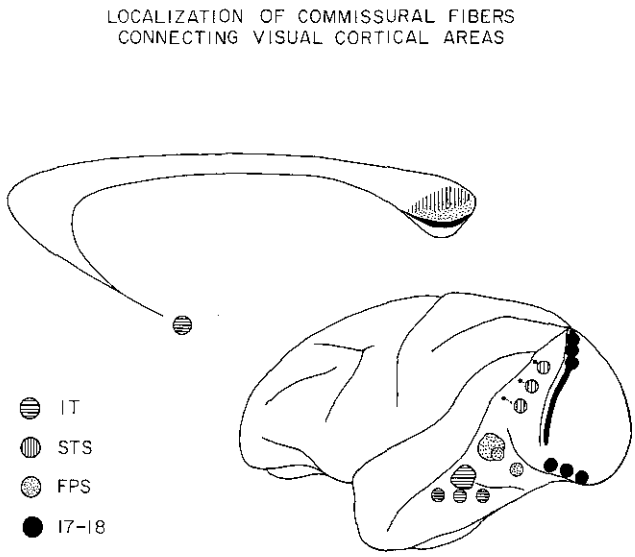

Figure 1. *The location of fibers in the corpus callosum and anterior commissure is shown based either on injections of tritiated proline or on lesions placed in the positions indicated on the diagram of the cortex. Circles represent injections and irregular shapes represent lesions. All are near the surface of the brain except the injections into the STS which entered at the dots and ended deep in the sulcus. IT = inferotemporal cortex; STS = posterior bank and depths of the superior temporal sulcus; FPS = foveal prestriate cortex; 17-18 = the vertical meridian of areas 17 and 18.*

these connections; additional brains being processed confirm and extend these results. The next three abstracts discuss experiments utilizing this approach to study visual behavior mediated at different hierarchical levels.

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206. LOCALIZATION OF SOME PERCEPTUAL PROCESSES IN MONKEYS

Investigators: **Charles R. Hamilton, Bonnie Blamick*, Anne L. Erdmann***

Psychophysical and physiological data suggest that perceptual mechanisms for certain visual aftereffects, such as tilt and motion, and for stereopsis are located early in the visual hierarchy. If so, they should show interocular equivalence in partially-split-brain monkeys with interhemispheric connections between early areas left intact, but not in monkeys with only deeper levels left interconnected (Biology 1978, No. 207). We have built an apparatus for adapting and testing partially-split-brain monkeys on the tilt aftereffect. Eight normal people have shown good aftereffects and interocular equivalence with this apparatus. Five partially-split-brain monkeys are now being trained on the basic tilt discrimination in preparation for tests of interocular equivalence of the aftereffects.

Tests of stereopsis and across-the-midline integration of information from the peripheral visual field are also being run with these animals. Since last year (Biology 1978, No. 207) we have found that either the splenium or the anterior commissure permits transfer of visual information from at least 45° off the midline. This seems to exceed estimates from anatomical and physiological studies of the extent of commissural connections around the vertical meridian of the visual field.

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207. LOCALIZATION OF DISCRIMINATION LEARNING

Investigator: **Charles R. Hamilton**

Animals without binocular cells in visual cortex lack stereopsis and do not show interocular equivalence for tilt and motion aftereffects yet do show interocular equivalence for learned discriminations. Partially-split-brain monkeys with the anterior commissure left intact also interocularly transfer visual discriminations despite a lack of all binocular cells in occipital cortex. Completely-split-brain monkeys, of course, show no interocular equivalence on learned discriminations. These results seem to place critical components of discrimination learning after occipital visual areas but before subcortical regions, possibly in the temporal cortex. More discrete partial disconnection of the cerebral hemispheres should show which segments of the commissures allow interocular equivalence and thereby indicate which cortical areas are

critically needed for discrimination learning. Furthermore, the critical levels may vary as a function of stimulus characteristics, such as movement, color, or the degree of stimulus categorization. Several monkeys have now learned visual discriminations based on movement in two types of apparatus and are being tested for interocular transfer through different commissural segments. Split-brain monkeys with unilateral lesions in specific visual areas are also being tested with these discriminations.

208. LOCALIZATION OF HIGHER-ORDER FUNCTIONS

Investigators: **Charles R. Hamilton, Betty A. Vermeire**

Most visual tasks depend on the splenium of the corpus callosum or the anterior commissure for interocular equivalence in monkeys with section of the optic chiasm. However, we have found that the development of visual learning sets, i.e., the increasing ease with which successive discriminations are solved as the animal learns the strategy or concept, can transfer through the frontal parts of the corpus callosum. This is now known to be true for object, reversal, and matching-to-sample learning sets. Because specific sensory information does not transfer through the frontal callosum these data cannot be explained by parallel development of learning sets in the two hemispheres but rather seem to require an interhemispheric transfer of the strategy. Several questions about this unique type of transfer are being investigated. Are duplicate memories formed for learning sets or are unilateral ones tapped by callosal connections? Do tactual learning sets show similar transfer? And most importantly, will a learning set for one pattern generalize to another, or from one sensory modality to another? These latter questions are difficult to answer in normal animals because of interference from the specific previously-learned discriminations, but may be more easily tested in partially-split-brain monkeys where the untrained hemisphere knows only the strategy or rule for solving problems and not the specific discrimination.

209. SELECTIVE ATTENTION IN MIRROR-IMAGE DISCRIMINATION BY MONKEYS

Investigators: **Betty A. Vermeire, Charles R. Hamilton**

Animals often find left-right mirror images (LR) harder to discriminate than up-down ones (UD). The finding of paradoxical interocular transfer of LR, that is, preference for the previously unrewarded stimulus when the untrained eye is tested, in several preparations has led to explanations for the confusion that are based on commissural inversion of topographic representations in the cortex. These explanations have been shown to be inadequate (Biology 1972, No. 106) and explanations based on selective attention to stimulus cues, especially because of a blind hemifield, have been suggested instead (Biology 1972, Nos. 107 and 108).

We are testing to see if other forms of selective attention more applicable to normal animals operate in producing mirror-image confusion. For example, both normal and chiasm-sectioned monkeys may code orientation relative to asymmetries in the environment or attend to cues near their place of response. Chiasm-sectioned monkeys are being trained to discriminate UD and LR with the place of response restricted to a peg on one side of the discriminanda. With the peg aligned with the axis of symmetry of the stimuli, discriminations are easier than with an orthogonal placement, as predicted by selective attention. In addition, because of the split chiasm, LR are learned more easily than UD, as previously found. In the aligned peg condition, interocular transfer tested with the peg position changed 180° leads to reversed transfer of both UD and LR. Testing LR shows, in addition, increased magnitude of reversed transfer associated with masking by the retinal scotoma. Reversal associated with manipulating the place of response is half the magnitude of that associated with masking. A similar experiment with normal monkeys shows that they too reverse their performance on UD and LR when the place of response is reversed. Results of these experiments together with those obtained previously (Biology 1975, No. 137; Biology 1976, Nos. 152 and 153) indicate that the concept of selective attention can explain mirror-image confusion by normal as well as split-chiasm animals.

210. HEMISPHERIC SPECIALIZATION IN MONKEYS

Investigators: **Betty A. Vermeire, Charles R. Hamilton**

Tests for hemispheric specialization in split-brain rhesus monkeys have shown no differences between the hemispheres in learning rates for visual discriminations employing types of stimuli that demonstrate lateralized processes in humans (Biology 1976, No. 154). Likewise, there are no differences between the two hemispheres in their ability to perform tests of conditional relationships between visual stimuli involving delays of up to 20 sec (Biology 1977, No. 208).

Current investigation of hemispheric specialization in monkeys concentrates on two areas.

(1) The two hemispheres of split-brain monkeys are being compared for their ability to learn auditory-visual conditional relationships and to subsequently perform over delays imposed between the auditory and visual elements. Similar tests have shown differential deficits in monkeys after unilateral cortical lesions. To date, several monkeys have learned this difficult task and are now being tested with increasing delays.

(2) Tests of visual preferences (Biology 1977, No. 209; Biology 1978, No. 205) have suggested that the left hemisphere is more inquisitive than the right. In situations that required operant responding it spent more time looking, although it did not respond differentially as a function of stimulus content. We are now using tests in which the monkeys view different stimuli and their social and/or agonistic behavioral responses to them are observed. Tests of each hemisphere alone could reveal differences in their abilities to identify and analyze socially relevant stimuli and direct appropriate behaviors toward them.

211. THE COMPREHENSION OF GRAMMAR BY THE DISCONNECTED RIGHT HEMISPHERE

Investigator: **Eran Zaidel**

As part of a systematic investigation of the comprehension of grammatical structures by the right hemisphere of commissurotomy patients, I administered several tests of pluralization. The tests were adapted from a battery developed by Susan Curtiss and Jeni Yamada of the Department of Linguistics at UCLA, and are used among others to check the hypothesis that a recently discovered isolated child who was not exposed to and did not develop language until puberty is now using her right hemisphere to learn and use language.

Pairs of colored pictures were presented to each hemisphere of two selected patients fitted with contact lenses for free ocular scanning in one visual half-field at a time. The patient had to point to the correct picture in response to the linguistic stimulus spoken aloud by the examiner.

In a noun pluralization test ("point to the box," "point to the hats," etc.) both hemispheres of both patients performed errorlessly. In a verb pluralization test for the use of the auxiliary ("the fish is eating" vs. "the fish are eating") both hemispheres performed equally well and competently. By contrast, the same verb pluralization test for the use of inflections ("the fish eats" vs. "the fish eat") again showed good performance by the left hemispheres but poor performance by the right hemispheres.

The disconnected right hemisphere seems to comprehend grammatical distinctions when they are signaled lexically (by whole words) but not when they are signaled by suffixes (parts of words), or by long phrases, as shown previously.

212. THE EFFECT OF ATTENTION ON THE RIGHT EAR ADVANTAGE IN DICHOTIC LISTENING

Investigators: **Eran Zaidel, Bennett Kashdan***

When simultaneous competing auditory stimuli reach the two ears the ipsilateral ear → hemisphere channels are suppressed and the subject perceives more accurately the stimuli reaching the ear contralateral to the hemisphere specialized to process them. The split-brain preparation eliminates cross-callosal interactions and permits a direct assessment of ipsilateral suppression and hemispheric specialization.

A dichotic tape containing pairs of syllables from Bee, Dee, Gee, Pee, Tee, Kee, was presented to commissurotomy patients and normal controls. Dichotic pairs were followed by pictures of letters (B, D, G, P, T, or K) flashed briefly to the right or left of fixation. The subject had to indicate by pointing whether or not the letter matched the sound in either ear. A significant right ear advantage (REA) signaling left hemisphere (LH) specialization was observed for normal right-handed male subjects and a massive REA was recorded for the LH of commissurotomy patients. How is the REA affected by attention to one ear or the other?

Subjects were instructed to (1) attend to both ears in the usual manner, (2) attend to one ear for the whole test, and (3) attend to the random ear receiving a beep 1 sec before the dichotic pair. The REA for normal subjects in either visual half-field was not affected by attention instructions. Thus the test seems an unusually robust and reliable index of LH specialization.

In both disconnected hemispheres attention to the contralateral ear had little influence on the laterality effect, whereas attention to the ipsilateral ear resulted in a substantial change. This change was especially strong and unpredictable in the RH. Again, the RH is seen to be more cognitively labile than the LH. It follows that

attention, like stimulus familiarity (Biology 1978, No. 203), can change the degree of ipsilateral ear suppression in dichotic listening. The effect of attention on the ear advantage in the split but not in the intact brain shows that cross-callosal competition is a more important factor than ipsilateral suppression in producing the REA.

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213. THE EFFECT OF SHORT-TERM MEMORY ON EAR ADVANTAGE IN DICHOTIC LISTENING

Investigators: **Eran Zaidel, Bennett Kashdan***

Using the same cross-modal paradigm as in the previous experiment, we varied the delay between a dichotic pair of monosyllables and a subsequent tachistoscopic flash of a comparison letter to one visual half-field, from 0 through 250, 500, and 1000 msec.

Delay had relatively little effect on the right ear advantage (REA) in the disconnected left hemisphere (LH). The laterality effect [$f = (R_c - L_c)/R_e + L_e$) where $R_c(R_e)$ = percentage of correct (erroneous) right ear responses] was largest ($f = 1$) for 500 msec and smallest ($f = 0.81$) for 1000 msec but high in all conditions. In the disconnected RH, however, only 500 msec delay resulted in a LEA ($f = -0.21$); 0 and 1000 msec delays resulted in LH control and no LEA. Thus the RH seems to need enough time to process the auditory information without interference but cannot store the results very long before decay sets in. The different susceptibility of the two hemispheres to delay suggests that they process and store the information differently.

When the visual probe following the dichotic pair was changed to a line drawing instead of a letter (a flying "bee," a "pea" pod, etc.) the effects of delay changed again, showing that the sounds were now interpreted and processed differently. Again, delay had little affect on the REA in the LH, although the laterality effect was maximal for 500 msec ($f = 0.97$) and smallest for 0 msec ($f = 0.67$). By contrast with letter probes, the picture probes in the RH showed a similar and consistent LEA for all delays, also maximal for 1000 msec ($f = -0.67$) and smallest for 0 delay ($f = -0.29$). Clearly, the same hemisphere, especially the right, interpreted and processed the sounds differently depending on the subsequent probe and comparison. The information stored by the RH for comparison with picture probes was more resistant to decay than the information stored for letter comparisons. Again the representation of information is seen to be more labile in the RH than in the LH.

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214. IDENTIFICATION AND CATEGORIZATION OF UNILATERAL VISUAL STIMULI IN SPLIT-BRAIN MAN

Investigator: **Larry E. Johnson**

Humans who have undergone forebrain commissurotomy for severe epilepsy have been found to use two different, but not completely autonomous, mechanisms to analyze visual stimuli projected to their left visual field (LVF). These two mechanisms are apparently dependent upon whether the visuolinguistic task requires the stimulus to be classified or categorized dichotomously (like a choice discrimination), or be verbally identified or named. Four split-brain patients, one partially-split-brain patient (splenium intact), and several normal subjects were tested in the following experiments. When required to name a number or letter flashed to their LVF or RVF, split-brain patients took significantly longer to respond, correctly or incorrectly, to LVF stimuli than to RVF stimuli. This visual field-reaction time difference was not seen when the same stimuli were named by normal or partially-split-brain humans. However, when the split-brain subjects were required to categorize manually or verbally a stimulus as odd or even, $\geq$ or <5, number or non-number, or letter or non-letter, the LVF/RVF reaction time ratio was now close to 1.0 and no longer distinguished the completely-split-brain subjects from the partially-split-brain or normal subjects. These differences in results between the two kinds of discrimination tasks cannot be easily explained by simple cross-cuing, behavioral strategies, or right hemispheric speech. In addition, when categorization and identification responses were combined, interference was observed between the two mechanisms as indicated by a marked change in accuracy or a change in the expected LVF/RVF response time ratio.

Therefore, split-brain human subjects seem to use different neural mechanisms when LVF stimuli are to be categorized and when they are to be named. The two mechanisms are not completely independent, however, and can interact to cause a change in expected accuracy or reaction speed. It is also concluded that verbal responses to LVF stimuli are secondary to information transfer from the right to the left cerebral hemisphere in spite of the surgical section of the forebrain commissures.

215. CROSS-INTEGRATION OF BILATERAL VISUAL STIMULI IN SPLIT-BRAIN MAN

Investigator: **Larry E. Johnson**

When two stimuli are presented simultaneously to the left and right visual fields (LVF and RVF) of split-brain patients, only one of four subjects (NG) was able to consistently categorize the two stimuli (patterns, numbers, or letters) as same or different, using either manual or verbal responses. When the task was changed to that of stimulus identification, again only one subject, this time LB, was able to correctly name the two stimuli presented. It would thus appear that categorization and identification of bilateral stimuli are distinct processes (compare with the preceding abstract): some split-brain subjects can perform one discrimination but not the other, while other subjects can do neither. However, in the two subjects tested further (NG and LB), when the two tasks (categorization and identification) were combined in a single experiment they seem to interfere strongly with one another causing a marked decrease in accuracy in the discrimination that had been easily performed alone.

These results show, therefore, that some split-brain subjects can indeed cross-compare, or identify, bilateral visual stimuli. In addition, while the ability to categorize accurately or name the two stimuli differs between patients for unknown reasons, performing the two tasks in tandem seems to cause a conflict which results in poor accuracy of both discriminations. Because the forebrain commissures have presumably been cut in these patients, any categorization or identification which occurs between stimuli in opposing visual fields most likely takes place via the midbrain-pretectum region that continues to unite the two halves of the brain following commissurotomy.

216. RIGHT HEMISPHERE LANGUAGE AFTER MASSIVE LEFT CEREBRAL STROKE

Investigators: **R. W. Sperry, Pamela J. Cox**

In studies based on the effects of left hemisphere lesions, the right hemisphere has been inferred to be typically incapable of speech, writing, and mathematical calculation, and also to be word-blind and word-deaf, as well as apraxic, and correspondingly deficient in higher cognitive function generally associated with language and mathematical logic. These latter impairments are not similarly apparent in the disconnected right hemisphere of commissurotomy subjects. The disconnected right hemisphere following commissurotomy appears to be neither word-deaf nor word-blind, nor apraxic nor lacking in higher intellectual ability as in the classical view drawn from asymmetric brain damage. The problem of this disparity, which still remains a source of confusion and controversy, is being investigated in a study of the effects of left hemisphere lesions with application of lateralized test procedures and task material similar to those applied to commissurotomy subjects, in order to make possible more direct comparisons in respect to the symptom disparities.

A patient is under study in which a complete occlusion of the left internal carotid artery resulted in a massive left hemisphere stroke with severe right hemiplegia and global aphasia. Recovery of language abilities and of some related praxic and gnostic functions was followed over a 9-month period of combined therapy and testing by which time the functional condition had largely stabilized. Improvements occurred particularly in the early months and advanced most markedly in the comprehensional aspects of language. Analysis of the individual task capacities and of the overall profile of the recovery pattern showed a close correspondence in most respects with the picture of right hemisphere capacities as derived from commissurotomy studies. The results are taken to support our earlier conclusion that the symptoms of lateral focal lesions are misleading in the presence of the commissures because latent functional abilities present in the undamaged hemisphere fail to gain expression over the defective function of the system as a whole.

217. LATERALIZED DIVISION OF ATTENTION IN THE COMMISSUROTOMIZED AND INTACT BRAIN

Investigators: **Leah Ellenberg, R. W. Sperry**

Previous studies with complete commissurotomy patients have shown that in the absence of the corpus callosum it is possible for separate information processing to occur simultaneously in the two hemispheres. Normal subjects have been reported to demonstrate some capacity for dual processing if simultaneous tasks involve different sense modalities or are simple to perform. The extent to which double performance in normals may be enhanced by allocation to separate hemispheres remains to be clarified.

In the present study, the degree to which independent attentional systems for simultaneous left and right performances can be maintained voluntarily for prolonged periods was assessed in five complete commis-

surotomy patients (A.A., L.B., N.G., N.W., and R.Y.), two partial commissurotomy patients (N.F. and D.M.) and eight normal subjects. Subjects performed bimanual sorting tasks requiring (1) the same, (2) different, or (3) opposite simultaneous decisions by left and right hemispheres. Normals were also given additional practice sessions with the same tasks in an effort to determine limitations in the capacity for dual processing.

The results suggest that the cerebral commissures force the two hemispheres to work together and maintain attentional unity in the intact brain. Complete commissurotomy patients can maintain independent attentional mechanisms in the two hemispheres with each isolated hemisphere demonstrating competent task performance. Normals and partial commissurotomy patients, however, have great difficulty making different decisions for left and right hands operating concurrently. Practice increases the capacity for simultaneous processing in normals, but this enhancement appears to occur via automation of performance decreasing the need for attentional supervision, rather than through a division of the normally unitary attentional system.

218. DIFFERENTIAL LEARNING BY THE TWO HALF-BRAINS OF MONOCULARLY TRAINED CHICKS

Investigator: **Karen E. Gaston**

Previous studies have shown that young chicks avoid drinking a novel liquid (green sucrose solution) which has been paired once with delayed LiCl-induced illness, and that this conditioned aversion is mediated by the visual (color) cue and not by taste (Gaston, 1977). Tests with monocularly trained chicks demonstrated good interocular transfer of the aversion learning (Gaston, 1978). To examine the possibility that novel taste might play some role in acquisition and/or interocular transfer of the visual aversion, the present experiment evaluated the effects of monocular training with novel color in combination with either novel or familiar sucrose taste.

128 ten-day-old chicks with one eye occluded were made sick with LiCl after drinking green sucrose solution. Half the animals had been preadapted to the sucrose taste. The chicks were tested the following day, with the trained or the untrained eye open, for learned aversions to green or uncolored sucrose. Chicks tested with the trained eye displayed a marked aversion to the colored sucrose, but not to uncolored sucrose, regardless of the taste variable. This result demonstrated that taste adaptation did not interfere with monocular learning and that novel taste was not required for acquisition of the visual aversion. In contrast, animals tested with the untrained eye showed a significant aversion to either colored or uncolored sucrose, but in each case only if the taste was novel during training. The avoidance of uncolored sucrose clearly represents a taste aversion which was acquired only by the hemisphere deprived of direct visual information during training.

The present results, in combination with those of the earlier studies, suggest that the seeing whole or half-brain utilized only novel visual information (color) for acquisition and expression of an illness-induced aversion. However, when one eye was occluded and vision through that eye thus eliminated, the nonseeing half-brain was able to use novel taste information in forming an aversion. These findings indicate that, in monocularly trained chicks, each half-brain can independently acquire an illness-induced food aversion, with learning in the seeing and nonseeing hemispheres mediated by novel color and taste cues, respectively.

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219. LACK OF INTEROCULAR TRANSFER OF A PATTERN DISCRIMINATION IN CHICKS

Investigator: **Karen E. Gaston**

Since complete optic decussation in birds restricts direct visual input from each eye to the contralateral half-brain (Cowan et al., 1961), interocular transfer of visual learning depends on interhemispheric connections. Lee-Teng and Butler (1971) showed that very young domestic chicks could learn a simultaneous pattern discrimination in a situation which entailed key-pecking for heat reward. The present experiment was designed to assess interocular transfer of this pattern discrimination learning in chicks pecking for heat. A second purpose was to determine whether the extent of transfer might vary with age, possibly as a result of progressive maturation of participating interhemispheric fiber systems. Preliminary results were reported last year (Biology 1978, No. 211).

Three groups of 24 chicks were trained at 3-6, 7-10, and 11-16 days of age, respectively. With one eye occluded, a chick was required to discriminate between two simultaneously presented patterns (+, Δ) projected on stimulus keys. A peck at the correct pattern produced

5 sec heat reward; a peck at the incorrect pattern produced 5 sec nonreward. Discrimination training continued to a criterion of 36/40 correct responses. 30 min after the end of the monocular training session, each chick was retrained to criterion on the pattern discrimination with either the same eye (monocular retention test) or the other eye (interocular transfer test).

The results demonstrated that pattern discrimination learning failed to transfer from trained to untrained eye in chicks pecking for heat. Subjects tested with the trained eye had excellent retention of the discrimination task, as shown by large percentage savings scores for initial performance and for total trials- and errors-to-criterion at all three age levels. In marked contrast, animals tested for interocular transfer had no significant savings with the second eye by any measure. Further, there was no evidence that transfer was developing in progressively older chicks up to 16 days post-hatch.

These findings indicate that pattern discrimination learning in one side of the chick brain was not available to the other hemisphere. Similar lack of interocular transfer in normal chicks has been reported for monocular adaptation to a visual cliff (Zeier, 1970) and for monocular extinction of a passive avoidance habit (Benowitz, 1974). Thus, in the intact avian brain as in the surgically bisected mammalian brain, restricting visual input to one eye may result in learning which remains confined to one hemisphere. Such findings show the extent to which the two sides of the avian brain are separately organized and able to function independently under certain conditions. The present results also suggest that monocular visual learning can be stored in the form of a unilateral engram which is not retrievable through the untrained hemisphere.

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220. LEFT-RIGHT NEUROSPECIFICITY: EVIDENCE FROM UNCROSSING OPTIC FIBERS IN GOLDFISH

Investigator: **Ronald L. Meyer**

In goldfish, fibers from each eye normally connect only with the contralateral side of the brain, principally with the optic tectum. However, a limited ipsilateral projection can be produced surgically by excising a group of optic fibers from one tectum and inserting them into the opposite tectum. If the optic fibers normally innervating this recipient tectum are also severed, then both the deflected and the cut normal (undeflected) fibers will both regrow into the same tectum. Previously it was found that the two groups of fibers eventually come to innervate separate regions of tectum in a mutually exclusive fashion. The present experiment is an autoradiographic study of the development of this innervation from two weeks to three months after surgery.

At up to four weeks deflected and undeflected fibers appeared to overlap completely. At six weeks there was some hint of segregation and by eight weeks a clear but still incomplete segregation was evident. At three months the segregation was nearly complete. This sequence was the same regardless of whether fish were maintained in diurnal illumination or in complete darkness.

In another group of fish at four to five months after surgery the pathways of optic fibers were analyzed by allowing a sufficient period after labeling (ten days) so that the shafts of the fibers became heavily labeled. This showed that the pathways taken by the two groups of fibers also overlapped and indicated that the deflected fibers followed diverse paths to their site of innervation.

These results together with other considerations strongly suggest that an intrinsic difference between fibers from left and right eyes underlies the development of this segregation.

221. A RETINAL PROJECTION IN GOLDFISH TO AN INAPPROPRIATE REGION OF TECTUM WITH A REVERSAL IN POLARITY

Investigator: **Ronald L. Meyer**

In adult goldfish optic fibers will regrow to their original target sites when severed and so will reestablish the original retinotopic projection onto the midbrain tectum, its major projection site. Normally this projection is strictly contralateral. However, in this experiment a select fraction of optic fibers, those normally innervating posterior (contralateral) tectum, were deflected into the anterior end of the opposite (ipsilateral) tectum. The deflection technique involved teasing free and cutting optic radiation fibers from one tectum, directing them across the midline, and inserting them into the other tectum. At the same time, the normal anterior innervation of the recipient (ipsilateral) tectum was permanently eliminated by electrocoagulation of temporal retina of its

corresponding (contralateral) eye. Thus deflected fibers were made to grow into an inappropriate but denervated region of a tectum in which the appropriate target region for the deflected fibers was already occupied.

Electrophysiological recording and autoradiographic tracing showed that the deflected fibers were confined to the inappropriate anterior region of tectum and the undeflected (normal) fibers remained in their appropriate posterior region. This is perhaps the first evidence in this system that time and position variables can permanently alter the position of regenerated fibers. In addition, the organization of deflected fibers, while retinotopic, was reversed with respect to tectum along its anterior-posterior axis. This latter result is thought to be the most direct evidence to date that optic fibers interact selectively and directly with each other in forming a retinotopic projection.

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Summary: Our research on the primate visual system concerns the organization and function of visual areas in the cerebral cortex of macaque monkeys. One of our major goals has been to define the total number of distinct visual areas and their precise locations. During the past year we have determined the complete boundaries of two of the six areas in the occipital lobe known from previous studies, namely V2 and MT. In addition we have established the presence of two additional visual areas, for a total of at least eight visual cortical areas in the macaque occipital lobe.

Our experimental approach generally involves a combination of physiological and anatomical techniques. Physiological recordings with microelectrodes are used to study the topographic organization of individual visual areas, i.e., the way in which the visual field is represented within the cortex, and also to determine the functional properties characteristic of neurons in each area (e.g., selectivity for stimulus orientation, direction, or color). Our main anatomical approaches include the analysis of cortical myeloarchitecture, which allows the recognition of the boundaries of certain visual areas, and several procedures for tracing pathways within the CNS.

A separate facet of the research in this laboratory concerns the process of synapse elimination during development of the neuromuscular junction. We are currently studying this process in skeletal muscles from the rabbit. The large size of neonatal rabbits facilitates various types of surgical intervention designed to explore the mechanisms underlying synapse elimination. It is known that the process involves competitive interactions between terminals innervating the same muscle fiber; our goal is to determine the rules governing this competition.

222. TWO-DIMENSIONAL MAPS OF THE CEREBRAL CORTEX

Investigators: **David C. Van Essen, John H. R. Maunsell**

In order to study the organization of visual areas lying within the rather deep sulci of the macaque cerebral cortex, we have found it very convenient to work with two-dimensional, unfolded representations of the cortical surface. Our procedures for constructing such maps have recently been refined to the extent that the entire cerebral cortex can be represented accurately in a single map. The outlines of histological sections through the hemisphere are used to generate cortical maps whose contour lines are analogous to those of geographical "topo" maps.

The accuracy with which the cortical surface is represented in our maps has been tested by comparing them to three-dimensional representations of the cortex generated with a computerized reconstruction technique. The surface areas of specific cortical regions calculated with these two independent techniques generally agree to within 10 to 20%. This degree of accuracy is comparable to the inherent variability in linear measurements within the cortex introduced during histological processing.

Cortical maps are useful for displaying a wide variety of information ranging from basic anatomical subdivisions of the cortex to markings of individual electrode recording sites. The fidelity of the representation greatly facilitates the attainment of quantitative information such as the exact size of individual cortical areas and their precise internal organization. For example, it can be readily determined that more than half of the cerebral cortex is concerned with processing visual information, and that the largest single cortical area, the striate cortex, occupies one-sixth of the entire hemisphere.

223. ORGANIZATION OF THE MIDDLE TEMPORAL AREA IN THE MACAQUE

Investigators: **John H. R. Maunsell, David C. Van Essen, John L. Bixby**

Last year (Biology 1978, No. 214) we reported that the middle temporal area (MT), a specific region in the superior temporal sulcus receiving direct inputs from the striate cortex, could be recognized in myelin-stained sections of the cortex. We have now characterized this area further with respect to its size, location, function, and topographic organization.

MT is relatively small in size, averaging about 35 mm^2 in surface area, which is only 3% of the extent of striate cortex. It occupies a characteristic position deep within the superior temporal sulcus and is nowhere exposed to view on the surface of the hemisphere. Electrophysiological recordings from the superior tempo-

ral sulcus have confirmed that about 90% of the cells in MT are directionally selective, i.e., they respond well to stimuli moving in one direction but weakly or not at all to oppositely directed stimuli. MT is surrounded by two distinct cortical regions, which differ markedly in their functional characteristics. Lateral and dorsal to MT is a region, possibly part of V4, in which very few cells are directionally selective; many are instead selective for stimuli of particular colors. The transition in functional properties occurs very abruptly (within 0.25 mm) right at the architectonic border of MT. Medial to MT is a separate region in which a high percentage of cells are directionally selective, just as in MT; they differ mainly in that receptive fields are much larger, often covering one or more quadrants of the visual field.

We have also examined the way in which the visual field is represented within MT and found unexpected complexities in its topographic organization. At a relatively crude level there is a systematic representation of the visual field in MT, with the long axis of MT representing a progression from central to peripheral receptive fields, and the short axis representing a progression from inferior to superior fields. At a finer level, however, there are significant deviations in this progression, and the detailed organization differs among individuals.

This initial characterization of the major organizational features of MT will provide a sound basis for more detailed functional studies now in progress. These in turn should permit a clearer understanding of the role of MT in the detection and analysis of moving stimuli.

224. THE ORGANIZATION OF VISUAL AREA V2 IN THE MACAQUE

Investigators: **David C. Van Essen, John H. R. Maunsell, John L. Bixby**

The striate cortex (V1) in the macaque projects directly to three cortical areas, V2, V3, and MT. V2 lies immediately adjacent to V1 and is the largest of its target areas. We have determined the full extent of V2 by examining its myeloarchitecture and its pattern of inputs from V1. Although the myeloarchitecture of V2 is far from homogeneous, there are certain characteristic features (pertaining to the myelination of cortical layers IV and V) that distinguish V2 from most surrounding regions of cortex. Although we have been unable to determine all of the borders of V2 solely on the basis of myeloarchitecture, the remaining gaps (e.g., the V2/V3 border) have been filled in by analyzing the topographic organization of projections from striate cortex.

V2 is fairly constant in width (10-15 mm) along much of its extent, but near the foveal representation on the lateral surface of the hemisphere it narrows to less than 2 mm; in some cases there may even be a complete gap between dorsal and ventral portions of V2. An interesting feature of the projections from V1 to V2 is that single loci in V1 in some cases project to multiple patches in nearby parts of V2. In future experiments we hope to explore the reasons for this occurrence in some hemispheres but not in others and to consider how the patchiness relates to the function of cells in V2 (e.g., as a possible anatomical substrate for binocular disparity processing).

225. A DORSOVENTRAL ASYMMETRY IN THE ORGANIZATION OF EXTRASTRIATE VISUAL AREAS

Investigators: **David C. Van Essen, John L. Bixby, John H. R. Maunsell**

Both V1 and V2 in primates are organized in a symmetrical fashion along the dorsoventral axis, with the superior half of the visual fields represented dorsally and inferior fields represented ventrally in the cortex. A similar organization has been proposed for V3 in the macaque, but our results suggest that this may not be the case. First, the projections of striate cortex are asymmetric, in that dorsal striate cortex projects to three areas, V2, V3, and MT, whereas ventral striate cortex projects only to V2 and MT. Thus V3 may contain an incomplete representation of the visual field and be restricted to dorsal extrastriate cortex. If, alternatively, V3 extends further ventrally, it must have an asymmetric pattern of inputs.

A separate line of evidence for dorsoventral asymmetry concerns the organization of interhemispheric connections within the occipital lobe, as revealed by the distribution of degenerating terminals following transection of the corpus callosum. Previous studies have shown a remarkably complex pattern of degeneration in dorsal extrastriate cortex; we have now found that this pattern is much simpler in the ventral part of the occipital lobe. One important feature of this pattern is a strip of cortex receiving interhemispheric inputs that run parallel to and several millimeters anterior to the border

of V2, all the way from the region of foveal representation laterally to the far peripheral representation medially. This pattern is quite similar to what has been described for the bush baby and owl monkey (Newsome, 1979). Current studies on the physiological organization and connections of this region in the macaque should clarify whether these regions are genuinely homologous in different primates.

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226. INNERVATION RATIOS AND THEIR RELATIONSHIP TO NEUROMUSCULAR MATURATION

Investigators: **John L. Bixby, John H. R. Maunsell, David C. Van Essen**

We reported last year (Biology 1978, No. 218) that the time course of synapse elimination at the neuromuscular junction differs considerably among various body muscles in the rabbit. Among these differences were significant variations in the peak level of multiple innervation (assessed in terms of synapses per muscle fiber) and in the maximal rate of loss of synapses (assessed as synapses per day per muscle fiber). An interesting possibility is that one or both of these parameters might display more uniformity among muscles if they were scaled with respect to motor neurons rather than muscle fibers. In other words, is the peak number of synapses per motor neuron and/or the maximal rate of loss of synapses per motor neuron the same for all muscles? In order to answer these questions it is necessary to know the innervation ratio for different muscles, i.e., the total number of motor neurons divided by the total number of muscle fibers. We therefore determined the innervation ratio for three muscles by making direct counts of both muscle fibers and motor neurons in adult rabbits. From these data and our previous results on immature muscles, we calculate that the peak number of peripheral synapses per motor neuron during development is relatively similar in all three muscles (507, 561, and 694 synapses per motor neuron), suggesting that similar factors may limit the total number of synapses motor neurons are able to establish. In contrast, there were large differences in the peak rate of loss of synapses per motor neuron (15, 24, and 42 synapses per motor neuron per day) suggesting significant variations among muscles in the factors influencing the rate of loss of synapses.

227. FINE STRUCTURAL OBSERVATIONS ON DEVELOPING NEUROMUSCULAR SYNAPSES

Investigator: **John L. Bixby**

The innervation pattern of neonatal rabbit diaphragm muscles is being examined in serial thin sections with the electron microscope. The major goals are to characterize the detailed morphology of terminals during and after the period of synapse elimination and to search for anatomical clues to the factors determining which synapses are to persist and which are to disappear. Of particular interest is a possible role of axonal myelination in deciding the fate of synapses, since myelination of peripheral nerves is known to occur during the early postnatal period.

The results to date suggest that Schwann cells do not play a central role in synapse elimination. Myelin, which develops first near the ventral roots and proceeds gradually towards the terminals, has apparently not reached many intramuscular branches at a time when decisions relating to synapse elimination are being made. Indeed, the axons near the end plates seem to bear the same relationship to the Schwann cells regardless of whether they are forming synapses on singly- or multiply-innervated fibers. Obviously, the possibility remains of axon-glia interactions which are simply not apparent in thin sections of fixed material.

In addition, serial reconstructions have been made of developing end plates. In contrast to the findings of some other investigators, no degenerating terminals have been seen during the period when synapse elimination is in progress. The relationship between different terminals coexisting on an end plate of the same muscle fiber has been analyzed. In the cases examined so far there is no extensive intermingling of the terminal processes from separate axons. Some synapses have prominent, elongated terminal branches which leave the fiber surface to end blindly several microns above the fiber. Such profiles are not seen in adult muscle, and their presence even on synapses of singly-innervated fibers in these immature muscles suggests that substantial modifications of synaptic terminals can occur subsequent to the synapse elimination process.

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NEUROGENETICS

Seymour Benzer

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Professor: Seymour Benzer
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Summary: Our activities are directed at the mechanisms underlying behavior, making use of genetic methods and the fruit fly, Drosophila. Work is in progress on two main levels--basic neurophysiology and the higher order problem of memory formation.

Drosophila turns out to be excellent for neurophysiology, since it contains large muscle fibers that are easily impaled with microelectrodes. The properties of the larval neuromuscular junction have been studied in detail, and certain mutants, detected by behavioral abnormalities in the adult form, have physiological abnormalities demonstrable in this larval system. Some show temperature-reversible blocks in axonal impulse propagation. Others show abnormal transmission or facilitation at the neuromuscular junction. This larval system thus makes it possible to use behavioral mutants to dissect physiological mechanisms and work toward the identification of the molecular components of neural structures. In addition, the adult fly contains "giant" axons that permit intracellular recordings of action potentials, and defects in mutants can be demonstrated.

Normal flies can learn to avoid selectively a specific odor that has been associated with electric shock. A mutant, dunce, does not learn this task, in spite of having essentially normal ability to sense the odorants and the shock. The defect is due to change of a single gene, and the mutant lacks one of the forms of cyclic AMP phosphodiesterase, which may implicate that enzyme in the process of memory formation.

228. FURTHER STUDIES ON THE PHYSIOLOGY OF THE nap^{ts} MUTANT

Investigators: **Chun-Fang Wu, Barry S. Ganetzky**

We have reported that the nap^{ts} (no action potential, temperature sensitive) mutation blocks the sodium action potential in nerves at high temperatures but does not block the calcium action potential of muscle (Wu et al., 1978). Thus, nap^{ts} appears to affect a component of axonal membranes. Using the Drosophila larval preparation we have examined the tetrodotoxin (TTX) sensitivity of the nerve action potential in nap^{ts} and in normal individuals. The excitatory junction potential (ejp) was recorded intracellularly from larval muscle at different doses of TTX as a means of monitoring the activity of the motor axon at permissive temperatures for nap^{ts} (20° to 23°C). Conduction in motor axons appears to be blocked by a 3- to 5-fold lower concentration of TTX than for wild-type controls. The concentration of TTX required to block conduction ranges from 5 to 15 nM (9.8 $\pm$ 4.4 nM; $\bar{X} \pm$ S.D.; n=11) in nap^{ts} and from 20 to 65 nM (40.3 $\pm$ 11.1 nM; n=9) in normal larvae. This difference in TTX sensitivity is not restricted to the motor axon because similar results were obtained by monitoring the compound action potentials of larval segmental nerves.

We have also examined the refractory period of the compound action potential in mutant and normal larvae. Following a single nerve stimulation, a second stimulation (even several times above the initial threshold) is ineffective in eliciting another response during the absolute refractory period. As the interval between the two stimuli increases, the second response gradually recovers to the amplitude of the first response. The interval necessary for recovery to 50% amplitude is 3 msec for wild-type larvae at 20° to 23°C. This interval is between 7 to 10 msec for nap^{ts} larvae under the same conditions.

Since the refractory period is a function involving the kinetics of Na and K channel activation and Na channel inactivation, it seems that nap^{ts} alters an active property of the electrical excitability of the axonal membrane. These results together with the TTX sensitivity of nap^{ts} and the altered TTX binding of membrane preparations from nap^{ts} brain homogenates (see following abstract) points toward a defect that involves the Na channel.

Reference:

Wu, C.-F., Ganetzky, B., Jan, L. Y., Jan, Y.-N. and Benzer, S. (1978) Proc. Nat. Acad. Sci. USA **75**: 4047-4051.

229. TETRODOTOXIN BINDING TO HOMOGENATES OF NORMAL AND nap^{ts} FLIES

Investigator: **Lawrence M. Kauvar**

Electrophysiological studies of nap^{ts} mutant larvae suggest that this gene may code for a component of the voltage-sensitive sodium channel in nerve membranes. Investigation of this possibility at the level of biochemical analysis is made possible by the availability of high specific activity [^{3}H]tetrodotoxin (TTX). This toxin, whose high specificity for the sodium channel has been established in other organisms, has standard pharmacological effects on Drosophila nerves.

In collaboration with M. Raftery's group in the Division of Chemistry and Chemical Engineering, we have obtained competible, saturable binding of labeled TTX to a crude particulate fraction prepared from a homogenate of fly heads. For normal flies, the dissociation constant (K_d) is 11 nM; the maximal binding (B_{max}) is 7 fmoles/mg protein in the total head homogenate. Examination of the nap^{ts} mutant with this technique has shown abnormalities in both parameters. For nap^{ts} flies, the K_d is 4.5 nM and the B_{max} is 4.5 fmoles/mg protein. These differences support, but do not yet prove, the hypothesis that nap^{ts} codes for a channel component.

Further studies of the TTX binding characteristics of nap^{ts}, particularly concerning stability at elevated temperatures, are in progress. Analysis of other temperature-sensitive paralytic mutants is also under way.

230. PHYSIOLOGICAL AND GENETIC ANALYSIS OF THE SHAKER (Sh) LOCUS

Investigators: **Alberto Ferrus, Mark E. Tanouye**

Shaker (Sh 1-57.7) is a dominant mutation that produces vigorous leg shaking in Drosophila adults when they are lightly anesthetized. Jan et al. (1977), working with the larval neuromuscular preparation, proposed that the Sh mutation may affect neuronal K^+ channel activity. We are analyzing these mutants by intracellular recordings of the giant axons in the cervical connective of the adult fly.

In normal flies, after a single stimulation to the brain, the giant fiber typically responds with a characteristic waveform containing a single action potential (Tanouye, 1978). Shaker flies, however, produce several spikes. Each allele shows characteristic abnormalities in waveform, time course, and number of spikes (see inside back cover).

Genetic analysis of the Shaker locus is being done to obtain information on the nature of the gene product. Using the proximal element of the translocation $T(1;4)B^S$, it is possible to construct flies with different doses of Sh and Sh^+ genes. It is found that Sh/+ males or Sh/+/+ females, despite having normal dosages of the normal gene, continue to show some leg-shaking activity. This result suggests that the Sh locus produces an abnormal molecule capable of interfering with normal function. If this interpretation is correct, heterozygous deficiencies for the Sh^+ locus should not show the Shaker phenotype. The generation of such chromosomal rearrangements is in progress.

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Tanouye, M. A. (1978) Ph.D. Thesis, Yale University, New Haven, Connecticut.

231. MECHANISM OF REPETITIVE FIRING IN Sh MUTANTS AND ITS SUPPRESSION BY nap^{ts}

Investigators: **Barry S. Ganetzky, Chun-Fang Wu**

Last year (Biology 1978, No. 221) we reported that the temperature-sensitive paralytic mutant, nap^{ts} (no action potential, temperature sensitive) suppresses the leg-shaking behavior of Sh (Shaker) mutants. Preliminary experiments suggested that this suppression is at the neurophysiological level.

Jan et al. (1977) found that Sh mutants have an abnormally prolonged excitatory junctional potential (ejp), as recorded intracellularly from larval muscles. L. Y. Jan and Y. N. Jan also demonstrated that this prolonged ejp is associated with repetitive firing of the motor axon (unpublished). We have found that in nap^{ts} Sh double mutants the ejp has a nearly normal time course and the repetitive firing of the motor axon is decreased or absent. This has led us to seek a better understanding of the physiological basis of repetitive firing in Sh mutants and its suppression by nap^{ts}.

Initially, it might seem that Sh simply increases nerve excitability leading to repetitive firing of the motor axon which in turn triggers a prolonged ejp. Several observations support this view. First, both the multiple firing and the prolonged ejp can be entirely blocked by TTX ($\geq$50 nM), a Na^+ action potential blocker. Second, at subcritical doses of TTX (15-25 nM) the number of nerve spikes and the duration of the ejp are coordinately reduced. Third, the multiple firing and prolonged ejp are

suppressed by nap^{ts}, a mutant with known defects in nerve excitability.

However, there is evidence that the axon terminal is also involved, since recording from the nerve after cutting it near the terminal shows no repetitive firing. When the terminal Ca^{++} current is blocked with 0 $[Ca^{++}]$ Ringer's solution or with 5 mM Co^{++} (which blocks Ca channels) the repetitive firing of the axon is reversibly blocked. Furthermore, in the presence of TTX (1 μg/ml), electrotonic depolarization of the Sh nerve terminal by a stimulus of sufficiently high intensity leads to prolonged release of neurotransmitter (Llinás et al., 1976).

We propose the following explanation: the Sh nerve terminal is abnormally excitable and capable of developing a presynaptic Ca^{++} action potential which can be prolonged (Katz and Miledi, 1969; Llinás et al., 1976; Jan et al., 1977). This Ca^{++} current serves as a source of stimulation to trigger additional Na^{+} action potentials in the axon. These Na^{+} spikes in turn further depolarize the terminal or help maintain the depolarization. In other words, there is a positive feedback cycle between the axon and the terminal which generates both the prolonged ejp and the repetitive nerve spikes.

This model predicts that the repetitive nerve spikes should travel antidromically from the terminal. This has been confirmed by recording the nerve spikes at two different points along the nerve. The model also predicts that interrupting the feedback cycle at either the terminal or the axon would inhibit the generation of repetitive nerve firing and prolonged ejps. This explains the blockage of the repetitive firing by TTX which acts on the axon and by 0 $[Ca^{++}]$ or 5 μM Co^{++} which act at the terminal. The nap^{ts} mutant appears to interrupt the feedback cycle in a manner similar to that of subthreshold doses of TTX. This is consistent with the known effects of nap^{ts} on axonal conduction.

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232. cAMP-PHOSPHODIESTERASE IN NORMAL AND DUNCE FLIES

Investigators: **Duncan Byers, Lawrence M. Kauvar, Sandra L. Shotwell**

Normal flies can be trained to avoid an odorant previously associated with electric shock. Flies carrying the dunce mutation fail to learn in this paradigm, despite their ability to sense the odorants and perform normal locomotor behavior.

In the past year, genetic mapping has shown that the two dunce alleles previously isolated in this laboratory belong to the same complementation group as two other point mutants isolated by D. Mohler at the University of Iowa. Furthermore, J. Kiger and R. Davis at the University of California at Davis have mapped these point mutants to region 3D3-4 on the X chromosome by use of overlapping duplications and deficiencies. Mohler's mutants were originally isolated as female steriles (Mohler, 1977). Region 3D3-4 was originally identified as influencing levels of cAMP-phosphodiesterase (cAMPase) in whole flies (Kiger and Golanty, 1977).

The fact that dunce is in this same complementation group suggests a role for cAMP in Drosophila learning. Before drawing this conclusion, however, it is necessary to show that the cAMPase enzyme deficiency is the primary defect, since a regulatory component might also disrupt other processes which affect learning ability. For understanding the connection between the biochemical and behavioral phenotypes, it will be important to determine the tissue distribution and developmental history of the defective enzyme.

Kiger's group has published a limited purification by means of sucrose gradient velocity sedimentation which resolves two peaks of phosphodiesterase activity (Kiger and Golanty, 1979). One peak, designated form II, is greatly reduced or absent in dunce flies. By the use of Sephadex, DEAE, and affinity chromatography we have prepared a fraction which co-purifies with the form II enzyme.

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Summary: The Drosophila brain, like vertebrate brains, is bilaterally symmetrical. Thus a neural subsystem, such as a circadian oscillator, should be represented on both sides of the brain. Using genetic techniques, we have constructed mosaic flies whose brains have an oscillator with a 19 hr periodicity on one side and a 22 hr periodicity on the other. Some of these flies show activity rhythms having both 19 hr and 22 hr components, indicating that there are indeed two oscillators which can function independently. A candidate cell group for the oscillator is a neurosecretory cell group in the posterior part of the brain whose morphology is affected by clock mutations in two Drosophila species. Transplantation studies have already suggested that activity rhythms are controlled by a humoral factor.

While typical circadian oscillators operate in the range of 18 to 30 hr, Randall Smith has discovered that a genetic translocation can produce very long rhythms of up to 35 hr. He has also shown that extra doses of a wild-type gene specifying period length shorten the period of the eclosion rhythm, suggesting that increased gene product activity may be the basis for the 19 hr short-period mutation. Dominic Orr has discovered that clock mutations affect the response of the oscillator to constant dim light. These mutations therefore alter several basic properties of the oscillator, including periodicity and responses to light and temperature. Alfred Handler has continued studies on the morphological and biochemical integrity of transplanted brains, showing that protein synthesis occurs at approximately normal levels in transplanted brains, though a few bands appear to show altered rates of synthesis. Steven Green has been studying neural pathways in mutants which transform or eliminate body structures. He has obtained evidence that, just as in certain vertebrate systems, nerve axons seem to be able to find their targets in the fly brain even when forced to take an abnormal path.

233. MOSAIC ANALYSIS OF A DROSOPHILA CLOCK MUTANT

Investigators: **Ronald J. Konopka, Steven M. Wells**

Analysis of locomotor activity rhythms of gynandromorphs mosaic for a circadian clock mutation, per^s, as well as surface markers yellow (y), singed (sn^3), and miniature (m) indicates that the rhythm focus is about 10 sturts from the anterior orbital bristle, the outer verticle bristle, and the vibrissae, and about 50 sturts from the humeral bristles, posterior supraalar bristle, wing, posterior notopleural bristle, and leg III. These results are consistent with a brain location for the rhythm focus. Further studies using acid phosphatase histochemistry of mosaic brain tissue and a mutation (so) lacking parts of the visual system suggest that the rhythm focus is not in the lamina or ventral subesophageal ganglion. Brain mosaics showing some degree of bilaterality exhibited locomotor activity patterns that were arrhythmic, or that showed both male and female components in periodograms.

234. GENETIC COMPLEXITY AT THE PER LOCUS

Investigators: **Randall F. Smith, Ronald J. Konopka**

With very few exceptions, chromosome rearrangements in Drosophila obey "Lefevre's Rule," i.e., rearrangements with a breakpoint at the same locus have an identical phenotype and this phenotype is equivalent to that of the null mutational allele for that locus (Lefevre, 1973). Exceptions to Lefevre's Rule occur when a locus contains more than one genetic element, as in the bithorax gene complex. In our analysis of the circadian rhythm phenotypes of six chromosomal rearrangements affecting the per clock mutant locus, we have identified one rearrangement which does not obey Lefevre's Rule. We have determined through deficiency analysis that the null functional state of the per locus results in an arrhythmic clock phenotype. However, individuals hemizygous for the per locus breakpoint of the translocation T(1;4)JC43 have been observed to express up to six cycles of erratic rhythmicity with cycles that are very long (approximately 35 hr) and quite variable in duration from cycle to cycle. Since the phenotype of this rearrangement differs from the null mutational phenotype of this locus,

per could be operationally characterized as a complex locus. If on the other hand the per locus contains a single genetic element, then (1) the breakpoint of T(1;4)JC43 must alter, rather than eliminate, per expression, and (2) a new class of exceptions to Lefevre's Rule must be recognized.

Reference:

Lefevre, G. (1973) Cold Spring Harbor Symp. Quant. Biol. **38:** 591-599.

235. MUTANT ACTION OF THE PER ALLELES

Investigator: **Randall F. Smith**

Three mutants (arrhythmic, long-period, and short-period) have been previously isolated which drastically alter the normal 24 hr period of circadian rhythmicity of Drosophila. Genetic mapping and complementation analysis have shown that all three mutants affect the same gene (called per) on the distal X chromosome (Konopka and Benzer, 1971). We are currently using a variety of genetic techniques to analyze the mode of action of these mutations on the expression of the per gene. Our analysis of the clock phenotypes of chromosomal deficiencies of the per region indicates that the arrhythmic mutant phenotype results from the total absence of per function. Two very interesting genetic studies suggest that the short-period mutant phenotype results from increased per activity levels. First, the eclosion rhythm period of short-period (per^S) homozygotes (approximately 20.5 hr) is significantly shorter (i.e., more extreme in phenotype) than the period of per^S/deficiency individuals (approximately 22 hr). In contrast, for the vast majority of all known mutants in Drosophila, mutant homozygotes have either an identical or a less extreme phenotype than when the mutation is over a deficiency, indicating that these mutations either eliminate or decrease gene function. Second, the addition of an extra dose of per^+ to a wild-type per^+/per^+ female shortens the normal 24 hr period about 1/2 hr while the deletion of one dose lengthens the period by about the same amount. Although the addition of two extra doses of per^+ shortens the period by about 1 hr, the addition of more than two extra doses does not cause a further reduction of period length. Therefore, the per^S mutation may shorten period length by increasing the activity of the per product, rather than increasing the levels of per^+ product. The per dosage studies also suggest that the long-period allele (and possibly the translocation T(1;4)JC43; see preceding abstract) lengthen period by reducing but not entirely eliminating per function.

Reference:

Konopka, R. J. and Benzer, S. (1971) Proc. Nat. Acad. Sci. USA **68:** 2112-2116.

236. GENE DOSAGE EFFECTS ON ACTIVITY RHYTHMS

Investigator: **Alfred M. Handler**

Screening mutagenized Drosophila for aberrant circadian eclosion and activity rhythms has thus far revealed four circadian rhythm mutants which all map to the X chromosome. We have recently begun to screen regions of the Drosophila genome for dosage effects on circadian rhythmicity. For this study we have monitored the locomotor activity of heterozygous-deficient females for regions covering 75% of the X chromosome, and flies duplicated for regions covering 40% of the autosomes.

While most of these aneuploid animals showed slight aberrations in their entrained or free-running activity, the periodicities of the rhythms were normal. However, two overlapping deficiencies in region 5 on the X chromosome had striking effects on the activity pattern. Animals carrying either of these deficiencies showed high levels of constant activity under constant conditions. Some animals showed similar levels of constant activity during entrainment as well. By testing smaller deficiencies and duplications in this region, and especially those in the overlapping region, we hope to locate more precisely the dose-dependent locus.

237. EFFECT OF LIGHT ON PERIODS OF WILD-TYPE AND MUTANT CLOCKS

Investigator: **Dominic Orr**

One approach to determining how mutations which alter the period of a circadian oscillator are affecting its mechanism is to study the response of mutant oscillators to natural entraining agents, such as light and temperature. In this study, we observe changes in the circadian period of the Drosophila locomotor activity rhythm effected by tonic application of dim light.

Wild-type and clock-mutant flies are reared in constant light of about 150 lux. Individual young flies are then put into constant darkness and their locomotor rhythms monitored by infrared light. Such a transition from constant light into darkness initiates a rhythm in locomotor activity with a period sharply centered at 24 hr

for wild-type flies, sharply centered at 19 hr for the short-period flies, and more broadly centered at 29 hr (range: 28-31 hr) for the long-period mutant.

After the period of a fly is established during the initial dark free run, a dim light of various intensities (0.001 to 6 lux) is turned on. This generally results in a lengthening of the period of wild-type, short-period, and long-period strains during the next 10 days. However the conditions under which lengthening occurs differ greatly among these three strains. Thus, for the wild type, marginal lengthening of half to one hour is observed at intensities as low as 0.002 to 0.004 lux, while a maximum lengthening of 11 hr is observed at 6 lux; with intermediate values in between. For the short-period mutant, lengthening of 1 to 6 hr in period is observed between light intensity of 0.04 to 0.6 lux. However, no significant lengthening can be induced in the short-period mutant flies at light intensities lower than 0.04 lux, and, at intensities higher than 1 lux, their activity tends to become arrhythmic. For the long-period mutant, period lengthening of 2 to 7 hr has been observed in the light intensity range of 0.001 to 0.2 lux. However, arrhythmicity in activity has been observed in this mutant at a light intensity level as low as 0.002 lux. We conclude that the light intensity required for a given amount of period lengthening is increased in the short-period mutant and lowered in the long-period mutant, and that both mutants can be made arrhythmic within the range of light intensities studied while the wild type cannot.

238. A MORPHOLOGICAL CORRELATE OF ARRHYTHMIC CLOCK MUTATIONS

Investigators: **Ronald J. Konopka, Steven M. Wells**

Gene mutations which produce arrhythmic eclosion and adult locomotor activity rhythms have been isolated in Drosophila melanogaster and Drosophila pseudoobscura. In D. pseudoobscura, five mutations are known which comprise two complementation groups. The single D. melanogaster mutation and two pseudoobscura mutations, one from each complementation group, have similar effects on a neurosecretory cell group located in the brain. Normally, these cells are found at the rear of the brain in a cluster of four cells per side, about halfway between the dorsal and ventral edges of the brain. In the arrhythmic mutants, as compared with the wild-type strain from which they were derived, a significantly greater number of cells are displaced toward the top edge of the brain. In addition, the percentage of brains in a population with three or more cells located abnormally near the top edge of the brain is greater in the mutants than in wild type. In D. melanogaster, the effect is similar in males and females, while in D. pseudoobscura, the effect is significantly greater in females than in males.

239. NEUROANATOMY OF FLIES WITH MISPLACED ANTENNAE

Investigator: **Steven H. Green**

I initiated studies on the mutant "antennaless" originally in order to determine the fate of the antennal lobes in the brain when they are deprived of inputs. Preliminary investigation showed that all areas of the neuropil which project to or are the target of the antenna appear normal, using the volume of neuropil and number of cell bodies in the rind as the criteria. The mutation removes one or both antennae and in all affected flies examined there was no antennal nerve if the corresponding antenna was absent.

Further investigation has shown that the "missing" antenna is actually present, metamorphosed inside and out in the head cavity, often in the optic lobe. Thus, the fly is not actually "antennaless" but the antennal disc fails to evert during metamorphosis. It seems likely that the targets of the antennal nerve receive their normal inputs albeit by a highly abnormal pathway. Close investigation of silver-stained brains does indeed show fibers meandering from the antenna through the cell body layer and into the antennal mechanosensory area in the neuropil. These fibers reach their targets after following extremely abnormal routes suggesting cues other than strict mechanical guidance are available to the developing axon.

The mutation antennaless seems to map close to lobe on the second chromosome (one recombinant in 262 flies). The penetrance is low but can be increased by lower temperature or uncrowded bottles. I interpret this as meaning that affected flies have reduced viability (not surprising in view of the fact that they have a full size antenna stuck in their brain). The combination ant;ss^{a} is affected in the same way as ant so the effect is not specific to antennal tissue.

240. NEUROANATOMY OF FLIES WITH ABNORMAL APPENDAGES

Investigator: **Steven H. Green**

Work in progress is aimed at trying to understand some of the principles used by the central nervous system to organize its complex structure during development. Our method is to compare the neural ultrastructure, using a variety of techniques, of normal flies and flies with mutations which one may expect to cause changes in their innervation patterns.

The specific mutations used are spineless aristapedia (ss^a) and Antennapedia (Antp); these are homeotic mutations, which partially transform antenna into leg. These mutants have been characterized behaviorally and morphologically using light and scanning electron microscopy. Wild-type flies, ss^a and Antp, have the same general sorts of sensory receptors but differ in number and location of these receptors. In addition ss^a flies consistently have taste receptors which are behaviorally and morphologically similar to those on the normal tarsus (foot).

Duplicated antennae are obtained from eyeless (ey) flies. Duplicated legs or antennae can be found after heat-pulse treatment of the temperature-sensitive lethal l(1)726 provided by Dr. Jack Girton.

Tarsi that are behaviorally or morphologically abnormal exist in a number of stocks including ss^a and Antp.

Preliminary studies of silver-stained brains of some of these mutants did not show significant differences from wild type. However, further studies with an improved silver stain and cobalt-backfilled preparations with silver-sulfide intensification have shown unusual neuronal pathways in the transformed portions of the homeotic antennae. Work is being done presently to determine the functional significance of these altered projections. In addition, a modified cobalt procedure allowing transsynaptic fills as well as more conventional silver and Golgi methods are being used to look at changes in morphology of central neurons in response to the aberrant inputs.

241. IN VIVO CULTURE OF DROSOPHILA BRAINS

Investigators: **Alfred M. Handler, Steven M. Wells**

Brains dissected out of rhythmic donor Drosophila can be transplanted into the abdomens of arrhythmic host flies. Some of the arrhythmic hosts subsequently express activity rhythms representative of the donor animals. The relatively small number of host animals which become rhythmic has been attributed to degeneration of the donor brain after transplantation. We have begun to analyze the viability of transplanted brains, both histologically and biochemically, after varying time periods in in vivo culture.

Histological examination of donor brains has included toluidine blue and silver staining of brains within the host abdomens, as well as silver staining of donor and host brains retrieved from the host animals. Examination of brains kept in vivo for times ranging from several days to five weeks showed, in general, increased cell death and neuropil degeneration with time. While cell death was usually greatest in between the optic lobes and protocerebrum, neuropil degeneration showed no consistent pattern. Some dissected host brains also showed cell death which is attributed in some cases to age, and stress placed on the tissue during dissection.

The biochemical integrity of the donor brains was determined by assaying de novo protein synthesis. Host flies ranging in age from 1 to 5 weeks were injected with [^{35}S]methionine. Equal numbers of host or donor brains (1 to 5 brains per sample) were run on SDS polyacrylamide slab gels which were then autoradiographed. In general, the amount of protein synthesis was proportional to the number of brains in the sample, with only small differences in total protein synthesis between host and donor brains. Also, the amount of time donor brains were kept under in vivo culture conditions did not affect total protein synthesis. One protein species of about 45,000 daltons, which co-migrated with muscle actin, was synthesized at significantly higher levels in donor brains than in host brains. This protein's relationship to Drosophila actin is presently being investigated.

Thus far it is apparent that isolated brains kept under in vivo culture conditions do degenerate morphologically, which is due at least in part to the dissection and transplantation procedure. The nervous tissue which does remain is apparently viable, but may produce an altered pattern of protein synthesis. We have yet to resolve whether cell death in specific regions of the brain, or altered patterns of protein synthesis, prohibits donor brains from producing activity rhythms in the host.

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DEVELOPMENTAL GENETICS AND IMMUNOGENETICS

Edward B. Lewis

Professor: Edward B. Lewis
Sherman Fairchild Distinguished Scholar: Matthew S. Meselson
Visiting Associate: Danielle Thierry-Mieg
Research Fellows: Loring G. Craymer III, Ian W. Duncan
Research Staff: John Wen-Kiang Chu, Madeline A. Crosby, Nancy Hwang, Eileen P. L. Roy, Ker-hwa Susan Tung, Gudrun Vener
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Summary: The segmentation pattern of many higher animals constitutes a well-defined set of endpoints or levels of development for analyzing the genetic control of development. In Drosophila we have found that much of the basic body segmentation of the thoracic and abdominal (AB) segments is under the control of a giant cluster of genes known as the bithorax gene complex (BX-C). This complex comprises a minimum of nine genes which appear to code for substances which control specific body segment transformations. Genes in the distal portion of BX-C "abdominalize" segments AB2 to AB8, inclusive, while genes of the proximal portion abdominalize AB1 and modify the third thoracic segment so that it bears halteres instead of wings. For four of the genes of the complex, cis-dominant regulatory-like elements have been identified. The entire complex acts as if it is under negative control mediated by a repressor coded for by another gene, Polycomb (Pc^+). Three major rules govern the behavior of the bithorax genes during early development. (1) The more posterior the segment the more genes of the complex become derepressed; (2) genes once derepressed in a given segment tend to be derepressed in all segments posterior thereto; and (3) the more proximal a gene is in the complex the more likely (with one exception) it is to be derepressed. The way in which the complex controls normal development can perhaps best be understood if there are two gradients: a gradient along the embryo in a substance produced by Pc^+ and a gradient along the chromosome in the affinity of the cis-regulatory elements for that substance.

Dr. Craymer is applying some elegant new screening techniques to recover more deficiencies in the Pc and BX-C regions. The recovery of deficiencies in the Pc region should provide a more precise localization of the Pc^+ gene. The recovery of additional BX-C deficiencies should help us to recognize any additional genes in the complex.

242. MATERNAL EFFECTS OF THE POLYCOMB GENE ON THE SEGMENTATION PATTERN OF DROSOPHILA

Investigators: **Edward B. Lewis, Gudrun Vener, Nancy Hwang, Ker-hwa Susan Tung, John Wen-Kiang Chu**

The bithorax gene complex acts as if it is under negative control mediated by another gene Polycomb (Pc) that lies outside the complex. Thus, flies carrying one dose of an extreme Polycomb mutant, Pc^3, and one dose of the normal or Pc^+ gene have weak phenotypic effects resembling those produced by dominant cis-regulatory mutants of the bithorax complex (see Biology 1979, Nos. 243 and 244). Furthermore, Pc^3 when combined with such mutants strongly enhances the cis-dominant phenotypes. It is possible with the aid of insertional translocations to construct individuals which are hemizygous for Pc^3, or $Pc^3/0$; that is, they have one dose of Pc^3 and lack entirely a Pc^+ gene. Such individuals die late in embryonic development and show a pronounced tendency for the first seven abdominal segments to be transformed towards the eighth abdominal segment, as if there is extensive derepression of all of the genes of the complex in those segments. The degree of abdominalization of the thoracic segments of $Pc^3/0$ animals has been discovered to depend very strongly upon whether the mother carries two doses of Pc^+ or one dose. In the former case there is poor transformation of the embryonic thoracic segments towards the eighth abdominal state, whereas in the latter case all three of the embryonic thoracic segments come to resemble closely the embryonic eighth abdominal segment in several respects. Evidently the Pc^+ substance is incorporated into the egg before fertilization and the amount incorporated is directly related to the number of doses of Pc^+ in the mother.

243. POLYCOMB TRANSFORMATIONS THAT MIMIC THOSE OF BITHORAX REGULATORY-LIKE MUTANTS

Investigator: **Ian W. Duncan**

Several cis-dominant regulatory-type mutants have been identified within the bithorax complex (BX-C). One possibility is that the genes of BX-C are under negative control and that these mutants act to reduce the affinity for a repressor of certain regulatory elements within

BX-C. Under this model, it should be possible to mimic the effects of these mutants by lowering the amount of repressor present. In fact, $Pc^3/+$ flies occasionally have patches of epidermal tissue that show transformations associated with either of two of the regulatory mutants within BX-C. Sectors of tissue normally found only in the haltere (identified by characteristic bristles and small, densely-packed trichomes) are found in the posterior part of the wing of $Pc^3/+$ animals, a transformation also shown by the regulatory mutant Cbx. In addition, patches of epidermis characteristic of the fifth abdominal segment have been found on the fourth abdominal segment, a transformation shown by the regulatory mutant Mcp. These patches are identified in the male tergite by the darker pigmentation and lower trichome density of the cuticle from the fifth as compared to the fourth abdominal segment. For the latter transformation it has been shown by examination of X-ray-induced clones homozygous for the cell marker, pawn, that the cells within a transformed patch are clonally related and that the decision to express the fifth abdominal level of development must have been made in these cells by the late larval-early pupal stage. These observations support the model that BX-C is under negative control by the Pc^+ gene.

244. ABDOMINAL SEGMENT TRANSFORMATIONS

Investigator: **Madeline A. Crosby**

We are continuing our investigation of genes within the bithorax complex which control the development of the fly's fourth and fifth abdominal segments. We have succeeded in isolating a mutant which impairs the function of infra-abdominal-5 (iab-5), a structural gene necessary for the normal development of the fifth and probably the sixth and seventh abdominal segments.

As previously reported, we have used a dominant cis-regulatory mutant, Miscadestral pigmentation (Mcp), in mutagenesis experiments to find new mutants within the distal portion of the bithorax complex. One of these mutants has now been separated from Mcp by recombination and has been designated iab-5^{C7}. The iab-5^{C7} mutation appears to damage a structural gene necessary for the normal development of the fifth abdominal segment of the fly. In animals which are iab-5^{C7} over a deficiency (Df-C4) this segment looks like the fourth abdominal segment. Not only is the fifth abdominal segment affected: the sixth and seventh abdominal segments also show characteristics of the fourth abdominal segment. It may be that iab-5^+ is necessary for the normal development of these segments as well. Another possibility is that iab-5^{C7} affects additional hypothetical structural genes within the complex.

Mcp causes the fourth abdominal segment to acquire characteristics of the fifth, the transformation opposite of that seen in iab-5 mutants. We postulate that Mcp is a mutation in a cis-regulatory element that turns on (or derepresses) iab-5^+ in the fourth abdominal segment. One way to examine this relationship is the cis-trans test. In the trans configuration (Mcp +/+ iab-5^{C7}) the phenotype of the fly is identical to that of Mcp over wild type. In the cis configuration (Mcp iab-5^{C7}/++) the Mcp phenotype is weak, indicating that to be expressed Mcp requires iab-5^+ on the same chromosome. We are now testing whether this effect is enhanced when Mcp iab-5^{C7} are over a chromosome with a rearrangement in the region of the bithorax complex.

245. TEMPERATURE-SENSITIVE SUPPRESSION IN DROSOPHILA

Investigator: **Madeline A. Crosby**

There exist in Drosophila several mutations which cause the suppression of certain other mutations. At least one of these, suppressor of Hairy-wing, su(Hw), is a general suppressor which is allele specific but not gene specific. We have been testing for suppression new spontaneous alleles of rudimentary, a gene for which the protein product is known and partially characterized. We have found several alleles which are suppressible, but only at certain temperatures. When combined with su(Hw) the rudimentary phenotype of these alleles is suppressed completely at low temperatures (18°C) but suppressed incompletely or not at all at higher temperatures (25°C). We have not yet been able to determine whether there are alleles of other genes which also exhibit temperature-sensitive suppression.

246. THE USE OF DUPLICATIONS IN SCREENING FOR DEFICIENCIES

Investigator: **Loring G. Craymer III**

As a consequence of techniques which I have developed for the genetic manipulation of chromosomal rearrangements, a methodology has developed which should have great power in screening for deficiencies (and for tandem duplications) to be used in cytogenetic analyses. This methodology utilizes the lethality of

grossly aneuploid genotypes to recover selectively deletional events in screening for deficiencies. In the simpler form of this type of screen, a duplication of the form 1 2 3 4/8 7/2 3 4 5 . 6 7 8 9, which can be synthesized as a recombinant between two overlapping inversions, is used; the particular duplication used is chosen so that the hyperploidy for regions 2 3 4 and 7 8 results in lethality, and the duplication is kept balanced over a chromosome which carries the complementary deficiencies. If males carrying this duplication are mutagenized and mated to structurally normal females, the few surviving progeny will include individuals which carry deficiencies in the 2 3 4 region as a result of events which delete the duplication. The alternative form of the screen is to use smaller duplications, duplications which are viable in heterozygotes with a normal chromosome but not in homozygotes, and to use a criss-cross lethal system to recover selectively possible deletion bearing progeny: by crossing females of genotype Dp $1_1/1_2\ 1_3$ to irradiated Dp $1_2/1_1\ 1_3$ males, the only possible survivors are of genotype Dp 1_1/Dp* 1_2. The first of these methods is being used to carry out a cytogenetic study of the mediodistal (61A to 73A) region of the left arm of chromosome three of Drosophila melanogaster; the second method is being employed towards cytogenetic analyses of the Pc and BX-C regions.

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THE FOLLOWING REPORTS ARE BY GRADUATE STUDENTS IN THE DIVISION OF BIOLOGY WHO ELECTED TO DO THEIR THESIS WORK IN THE DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING.

247. PROTEASE EFFECTS ON THE STRUCTURE OF AN ACETYLCHOLINE RECEPTOR

Investigators: **Michael W. Klymkowsky, John E. Heuser*, Robert M. Stroud****

Support: **National Institutes of Health, USPHS National Science Foundation**

Protease digestion of acetylcholine receptor-rich membranes derived from Torpedo californica electroplaques by homogenization and isopycnic centrifugation results in dramatic changes in the morphology of the normally 0.5 to 2 μ diameter spherical vesicles when observed by either negative-stain or rapid-freeze deep-etch electron microscopy (Figure 1). Removal of peripheral, apparently nonreceptor polypeptides by alkali stripping (Neubig et al., 1979) results in increased sensitivity of the acetylcholine receptor membranes to the protease trypsin as indicated by SDS gel electrophoretic patterns and the extent of morphologic change observed in vesicle structure. Trypsin digestion of alkali-stripped receptor membranes results in a complete degradation of all four presumed receptor subunits without appreciably changing either the appearance of the receptor particles in negative-stain electron micrographs or affecting their ability to bind α-bungarotoxin specifically. Trypsin digestion does not alter the sedimentation value of detergent-solubilized receptor. The protease-induced morphological transformation of receptor membranes is unaffected whether the receptor exists as a monomer or as a disulfide-bridged dimer.

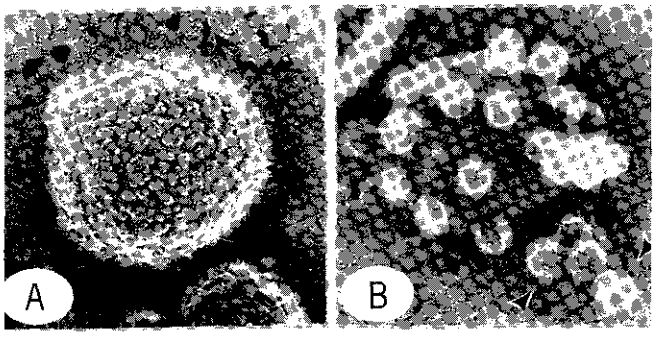

Figure 1. *Rapid-freeze, deep-etched and rotary-shadowed acetylcholine receptor membranes before (A) and after (B) trypsin digestion. All of the receptor is found in the small budding minivesicles. Arrows in (B) point out released minivesicles.*

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248. LOW RESOLUTION STRUCTURE OF A MEMBRANE-BOUND ACETYLCHOLINE RECEPTOR

Investigators: **Michael W. Klymkowsky, Robert M. Stroud***

Support: **National Institutes of Health, USPHS National Science Foundation**

Electron microscopic analysis of acetylcholine receptor-rich membranes derived from Torpedo californica indicates that the molecules extend by no more than 1 nm on the intracellular surface. Image reconstruction of small crystalline patches of dimeric receptor, from which peripheral proteins have been removed, shows that the receptor is asymmetric at approximately 3 nm resolution (Figure 2). The molecule has a large central channel, accessible to both phosphotungstate and uranyl ions, descending down to the membrane surface. The molecules pack in a unit cell with dimensions a = 8.8 ± 0.6, b = 8.8 ± 0.3 nm, and γ = 118° ± 2°. The 11 nm thick unit cell is

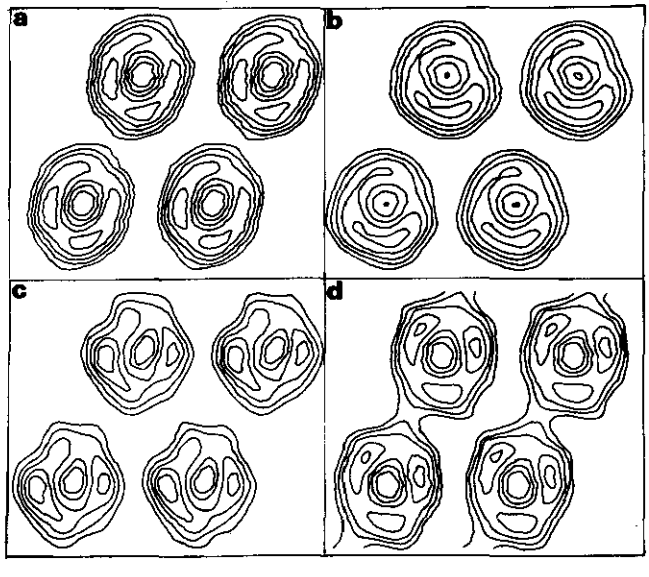

Figure 2. *Image reconstructions of four ordered receptor areas which reveal common features that reflect the true structure of the molecule.*

approximately 60% lipid and protein, with a calculated lipid/protein ratio of 1:3. The molecule has a calculated radius of gyration of 4.2 nm.

A second set of rarely occurring crystalline membranes has also been identified with a significantly larger unit cell ($a = 9.7 \pm 0.4$, $b = 9.5 \pm 0.5$ nm, $\gamma = 121° \pm 3°$). The repeating unit in these areas is a featureless ball. By immunochemical and biochemical techniques these areas have been demonstrated to be different from the receptor.

Thus by utilizing both X-ray diffraction and electron microscopic analysis (Ross et al., 1977; Klymkowsky and Stroud, 1979a,b) the low resolution structure of an integral membrane neurotransmitter receptor protein has been determined. Our efforts are now aimed at resolving higher resolution features such as the ligand binding site and structural changes occurring during channel activation.

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249. RECONSTITUTION OF PURIFIED NICOTINIC ACETYLCHOLINE RECEPTOR

Investigators: **Wilson C.-S. Wu, Michael A. Raftery***

Support: **National Institutes of Health, USPHS**

Biochemical characterization of the membrane-bound nicotinic acetylcholine receptor (AcChR) has provided considerable information on its structure and composition as well as its interaction with cholinergic ligands, local anesthetics, and other agents such as histrionicotoxin, a putative ligand for the receptor-associated ionophore. However, basic questions regarding the polypeptide composition of the AcChR still remain. In fact, suggestions have been made to implicate the involvement of a component (of molecular weight 43×10^3 daltons) other than those principal polypeptides believed to be characteristic of the AcChR in the formation of the receptor ionophore.

In continuing attempts to define the molecular species necessary for agonist binding and consequent cation translocation, we are studying agonist-induced cation flux across membranes reconstituted from purified protein components and phospholipids. Previous attempts to reconstitute purified AcChR have either been unsuccessful or lacked reproducibility. One of the major problems associated with the previous procedures lies in the method by which AcChR was purified, namely, affinity chromatography of Triton-extracted membranes. These procedures probably irreversibly altered the properties of the protein.

Recently, advances have been made which allow purification of membranes that contain essentially only those principal polypeptides believed to comprise the acetylcholine receptor (40, 50, 60, and 65×10^3 daltons). These membranes are shown to exhibit pharmacological properties expected for an in vitro model of a nicotinic cholinergic synapse (Hsu-Moore et al., 1979).

In preliminary experiments (Wu and Raftery, 1979), we dissolved these purified AcChR membranes with 2% (w/v) sodium cholate and then removed all insoluble matter. The solubilized purified receptor preparation was reassociated with phospholipids when the detergent was removed by dialysis. Such a reconstituted receptor preparation, lacking the 43×10^3 dalton polypeptide, was shown to retain $^{22}Na^+$ with a relatively slow leak rate and is capable of translocating the cation in response to carbamylcholine binding in a highly reproducible manner. The dose response curve for this effect is similar to that observed for the original electroplax membrane preparation and the carbamylcholine-induced signal is completely blocked by the antagonist α-bungarotoxin.

Experiments are now under way to further characterize these reconstituted membranes with respect to their pharmacology and morphology.

References:
Hsu-Moore, H.-P., Hartig, P. R., Wu, W. C.-S. and Raftery, M. A. (1979) Biochem. Biophys. Res. Commun. **88:** 735-743.
Wu, W. C.-S. and Raftery, M. A. (1979) Biochem. Biophys. Res. Commun. In press.

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

VISITING LECTURERS

GRADUATES

FINANCIAL SUPPORT

VISITING LECTURERS

Brian Roberts, Brandeis University: Cell-free translation and its application to gene mapping.

Tom Kaufmann, University of Indiana, Bloomington: Cytogenetic analysis of a homeotic gene complex in Drosophila.

Janet Boyles, University of California Medical Center, San Francisco: Neutrophil adherence: The changing patterns of plasma-membrane-associated filaments in the small amoeboid cell.

Derek Fisher, University of London: Chemically-induced cell fusion.

I. J. Russell and P. M. Sellick, University of Sussex: Intracellular studies from hair cells of the guinea pig cochlea.

Piotr Slonimski, Centre de Genetique Moleculaire du CNRS, France: Mosaic organization and expression of the yeast mitochondrial DNA region coding for cytochrome c reductase and oxidase.

Mark Ellisman, University of California, San Diego: Unexpected glial ionic concentrations and axonal relations at nodes of Ranvier: Cytoskeletons in the closet.

Gerald Rubin, Harvard University Medical School: Disbursed repeated genes in Drosophila.

William Wickner, University of California, Los Angeles: Assembly of proteins into biological membranes: The membrane trigger hypothesis.

Ron Evans, The Salk Institute: The structure of the promoter and cap site of a late adenovirus mRNA.

Wah Chiu, University of California, Berkeley: Electron crystallography of proteins: DNA helix.

Mary-Dell Chilton, University of Washington School of Medicine: Molecular basis of crown gall tumorigenesis.

James Goding, Stanford University Medical School: Production and characterization of monoclonal antibodies.

Freda Newcombe, The Churchill Hospital, Oxford University: Brain and language: Some recent results.

Raymond Daynes, University of Utah School of Medicine: Regulation of tumor immunity.

C. C. Tan, Futan University, Shanghai: Biology in China.

P. M. Bhargava, Center for Cellular and Molecular Biology, Regional Research Laboratory, Hyderabad, India: Evidence for nutritional control of translational errors in E. coli.

Victor Vacquier, University of California, San Diego: Cell-surface macromolecules involved in sperm adhesion.

Harold Weintraub, University of Washington: Chromosome structure and replication.

Ernest Wright, University of California, Los Angeles: Biochemical dissection of intestinal epithelial cells.

Alan Grinnell, University of California, Los Angeles: Factors influencing synaptic efficacy at frog neuromuscular junctions.

Edward Kravitz, Harvard University Medical School: Amines and neuronal modulation in the lobster nervous system.

Anthony Bretscher, Max Planck Institut, Tübingen: Microfilament organization and membrane attachment in the brush border: Villin, a new microfilament-associated protein from the microvillus.

Robert Nowinski, University of Washington: Expression of mouse leukemia virus in lymphocytes.

Ellen Rothenberg, Sloan-Kettering Institute: Synthesis and processing of murine thymus leukemia antigen.

David Klein, National Institutes of Health: The molecular basis of circadian rhythms in the pineal gland.

Norman Klinman, Scripps Clinic and Research Foundation: The ontogeny of antibody diversity.

Michael Grunstein, University of California, Los Angeles: Histone gene activity in sea urchin development.

Fritz Melchers, Basel Institute, Switzerland: Differentiation of B cells.

Denis Baylor, Stanford University Medical School: The membrane current of single rod outer segments.

J. Michael Bishop, University of California Medical Center, San Francisco: The origins and possible functions of avian retrovirus transforming genes.

Sheryl A. Scott, Carnegie Institution: The Merkel cell: The target of sensory neurons in the salamander?

Laurinda Jaffe, Marine Biological Laboratory, Woods Hole: Fertilization potentials and electrical polyspermy blocks.

Leonard A. Herzenberg, Stanford University Medical School: The fluorescent-activated cell sorter and hybridoma cell clones: New tools for the study of cell populations.

James F. Crow, University of Wisconsin: Attempts to measure the impact of mutation on the population.

Jim Patrick, The Salk Institute: Acetylcholine receptors on nerve cells.

Paul Berg, Stanford University Medical School: Dissections and reconstructions of a viral chromosome.

Anthony Strelkauskas, Harvard University Medical School: Mapping the T lymphocyte population in man.

Vincent Dionne, University of California, San Diego: Synaptic responses in snake slow muscle fibers.

John Lis, Cornell University: Characterization of cloned heat shock genes of Drosophila.

Robert Roeder, Washington University, St. Louis: Transcription of specific eukaryotic genes in reconstituted systems.

Peter Strick, Veterans Administration Hospital, Syracuse: Multiple representations in the primate motor system.

Eric Shooter, Stanford University Medical School: Nerve growth factor and neuronal development.

David Housman, Massachusetts Institute of Technology: Commitment to erythroid differentiation of murine erythroleukemia cells.

Edward Ziff, Rockefeller University: Sites for RNA initiation, splicing and polyadenylation.

Peter Marler, Rockefeller University: Innate knowledge: Ethologist's view of perceptual development.

Ray Lund, University of Washington: Plasticity in the development of the visual system.

Dennis Bray, MRC Biophysics Unit, Kings College, London: Studies on the mechanism of neurite extension in tissue culture.

David Gilbert, Cell Biophysics Unit, Kings College, London: Giant axon architecture.

Lee Rubin, Harvard University Medical School: Regulation of acetylcholinesterase appearance at newly formed neuromuscular junctions

James Rothman, Stanford University Medical School: Mechanisms of membrane assembly.

Donna Arndt-Jovin, Max Planck Institut: Cell surface properties and functional prerequisites for commitment to differentiation determined by flow sorting.

Spyros Artavanis-Tsakonas, Stanford University Medical School: Heat-activated genes of Drosophila melanogaster.

Susan Brown, Stanford University Medical School: A close look at nonmuscle actin.

Eliott Meyerowitz, Stanford University Medical School: Isolation and study of developmentally regulated Drosophila genes: An example and a new technique.

Maureen Gilmore-Hebert, University of California School of Medicine, Los Angeles: The organization and expression of immunoglobulin genes.

Kathleen M. Karrer, Indiana University: Determination of the germ line in Drosophila.

Michael O'Shea, University of Southern California: The properties of identified monoaminergic neurons.

Nobuo Suga, Washington University, St. Louis: How biologically significant information is coded in the bat auditory cortex.

Nicole LeDouarin, Institute of Embryology, CNRS, France: Environmental influences on the development of the peripheral nervous system.

Adelaide Carpenter, University of California, San Diego: An intimate view of meiotic recombination.

Juan Korenbrot, University of California Medical Center, San Francisco: Light-dependent ion transport by rhodopsin: A model systems approach.

Richard Mains, University of Colorado Medical Center, Denver: ACTH, endorphin, and related molecules.

Melvin Simon, University of California, San Diego: The mechanism of phase variation in Salmonella.

Francisco Bozanilla, University of California, Los Angeles: Gating currents in squid giant axon.

Ray Guillery, University of Chicago: On the laminar arrangement of the visual relays.

Nicholas Proudfoot, MRC Laboratory of Molecular Biology, Cambridge: The structure of known globin genes.

Susumu Tonegawa, Basel Institute for Immunology, Switzerland: Immunoglobulin gene rearrangements.

David Botstein, Massachusetts Institute of Technology: Translocatable genetic elements in prokaryotes.

Andrew Harris, Albert Einstein College of Medicine: Regulation of gap junction conductance by pH and transjunctional voltage.

Max Oeschger, Georgetown University: Isolation of E. coli ts suppressor strains and their application to the study of RNA polymerase in vivo.

Mary Kennedy, Yale University School of Medicine: Calcium and cyclic AMP-dependent phosphorylation of a specific protein in nerve terminals.

Walter Heiligenberg, Scripps Institute of Oceanography, University of California, San Diego: Electrolocation and jamming avoidance in electric fish.

Bela Julesz, Bell Laboratories: Counter examples to a quasi-linear conjecture on texture perception yield the fundamental building blocks of form.

Elizabeth Bikoff, Sidney Farber Cancer Center, Boston: Regulation of lymphocytes.

Welcome Bender, Stanford University Medical School: Chromosome walking to bithorax.

Horace Barlow, University of Cambridge, England: The evolution of consciousness: A biologist's view.

Michael Botchan, Cold Spring Harbor Laboratory: The mechanism of excision of SV40 DNA from chromosomes.

John Palka, University of Washington: Neurobiology of homeotic mutants in Drosophila.

Lindesay Harkness, Harvard University: Depth perception in chameleons.

Louis Sokoloff, National Institute of Mental Health, National Institutes of Health: The [^{14}C]-deoxyglucose method for measurement of local cerebral glucose utilization: Its use in mapping local functional activity in CNS.

Malcolm Gefter, Massachusetts Institute of Technology: Enzymatic unwinding of DNA during DNA replication.

Klaus Eichmann, Deutsches Krebsforschungszentrum Institut für Immunologie and Genetik, Heidelberg: Antigen receptors on mouse lymphocytes: Their function as cell communication molecules.

Kevin Struhl, Stanford University Medical School: New methods in eukaryotic genetics: Expression of the yeast His III genes in yeast and in E. coli.

Albert Deisseroth, National Institutes of Health: The expression of human globin genes in human-mouse cell hybrids.

S. J. Singer, University of California, San Diego: Membrane dynamics in cell biology. I. The red blood cell and its mechanichemistry.

S. J. Singer, University of California, San Diego: Membrane dynamics in cell biology. II. Molecular interactions.

Susan Pierce, Northwestern University: Regulation of the humoral immune response at the single cell level.

Sidney Altman, Yale University: Ribonuclease P, an essential enzyme for tRNA biosynthesis which has an RNA component.

Lewis Tilney, University of Pennsylvania, Philadelphia: The role of actin in nonmuscle movement.

William Hahn, University of Colorado Medical Center, Denver: Complex populations of poly(A)+ and poly(A)- mRNA in brain: Are they regulated post-transcriptionally?

Barbara Wold, College of Physicians and Surgeons, Columbia University: Introduction and expression of a rabbit globin gene in mouse fibroblasts.

S. J. Singer, University of California, San Diego: Membrane dynamics in cell biology. III. On the role of products of the major histocompatibility complex in cellular immunology.

Kenneth Paigen, Roswell Park Institute: The molecular genetics of mammalian glucuronidase.

Tom Benjamin, Harvard University Medical School: Transforming genes and gene products of polyoma virus.

Richard J. Cogdell, University of Glasgow, Scotland: Carotenoids: Their involvement in bacterial photosynthetic energy transfer and the modulation of their synthesis by light.

Elizabeth Craig, University of California, San Francisco: Organization of the heat shock genes in Drosophila melanogaster.

Peter Dallos, Northwestern University: Cochlear physiology: Some current concepts.

Charles Straznicky, Flinders University of South Australia: The acquisition of tectal positional specification and its modifiability during development.

Gregory Brewer, University of Southern California School of Medicine: Membrane morphogenesis: Studies with bacteriophage PM2.

Leslie Wilson, University of California, Santa Barbara: "Microtubule treadmills": Dynamic cellular machines for structure and movement.

Bunsiti Simizu, National Institute of Health, Tokyo: Inhibition of host DNA synthesis by western equine encephalitis.

Lynna M. Hereford, Brandeis University: Isolation and characterization of yeast histone genes.

Barbara Myers, Harvard University: Control of lambda repressor synthesis.

Robert Nowinski, University of Washington: Monoclonal antibodies against mouse leukemia viruses and some thoughts about the future.

Erhard Geissler, Forschungszentrum for Molecular Biology and Medicine, Berlin: Mutagenic effects of SV40 virus on mammalian cells.

David Yaffe, Weizmann Institute, Rehovot, Israel: Gene expression during myogenesis.

GRADUATES

Thirty-five students in Biology were awarded the B.S., M.S. or Ph.D. degree in June, 1979. Names, degrees conferred, titles of doctoral theses (with plans of recipients for future work), are as follows:

Bachelor of Science

Clifford James Beall, B.S. with honor.

Philip Lee Chang, B.S.

Debra Anne Cisar, B.S.

Charles Joseph Curatalo, B.S. with honor.

Robert Garo Dergarabedian, B.S. with honor.

Steve T. Eckmann, B.S.

Leila Margarita Gonzalez, B.S. with honor.

Paul Andre Gutierrez, B.S.

Leonard Robert Levy, B.S. with honor.

James G. Llewellyn, B.S.

John Christopher Manley, B.S. with honor.

Martin Edward Mann, B.S. with honor.

Patty Pik-Ying Pang, B.S. with honor.

Theodore Warren Post, B.S. with honor.

Mark Ragins, B.S. with honor.

Tony Blake Ramey, B.S. with honor.

Joy Diane Shaffer, B.S. with honor.

Charles Alva Shaller, B.S.

David Andrew Wheeler, B.S.

Master of Science

Eileen Angelique Bagdonas, M.S.

Deanna Kae Ojala, M.S.

Doctor of Philosophy

David Lee Armstrong, Ph.D. Thesis: The kinetics of curare action and restricted diffusion within the synaptic cleft of motor nerve terminals on frog skeletal muscle. Postdoctoral research, University College, London.

Elizabeth Peters Blankenhorn, Ph.D. Thesis: Immunogenetic studies of the mouse and the rat. Postdoctoral research, Clinical Research Centre, Middlesex, England.

Anne Chomyn, Ph.D. Thesis: Studies on protein synthesis after heat shock in Drosophila melanogaster. Postdoctoral research, California Institute of Technology.

Antonia R. Claudio, Ph.D. Thesis: The acetylcholine receptor and its role in induction of experimental autoimmune myasthenia gravis. Postdoctoral research, California Institute of Technology.

Susan Ellen Conrad, Ph.D. Thesis: I. Sequence organization of Drosophila melanogaster 5S RNA and 4S RNA genes. II. In vitro studies on replications of plasmid DNAs. Postdoctoral research, University of California, Berkeley.

Michael Lee Cooper, Ph.D. Thesis: Studies in the visual system of the cat. I. The retinothalamic pathway in normal and Siamese cats. II. The vertical horopter. Postdoctoral research, Yale University.

A. J. Hill, Ph.D. Thesis: Investigations of "spatial" firing in dorsal hippocampus of the rat. Postdoctoral research, University of Virginia.

Bruce D. Hubbard, Ph.D. Thesis: Biochemical studies on the 10 nm filaments of avian muscle. Employed by TRW, Inc.

Elwyn Yuan Loh, Ph.D. Thesis: Amino acid sequence studies of immunoglobulins: Implications for the storage, processing, and expression of genetic information. Student, Stanford Medical School.

Charles Edward Novitski, Ph.D. Thesis: Aspects of regulation of mitochondrial DNA replication and transcription in mammalian cells. Postdoctoral research, City of Hope National Medical Center.

Richard Carl Parker, Ph.D. Thesis: I. Methods for restriction endonuclease studies of DNA structure. II. Restriction endonucleolytic characterization of animal mitochondrial DNAs and human globin genes. Postdoctoral research, University of California Medical School, San Francisco.

David Eugene Presti, Ph.D. Thesis: Studies of the blue light receptor in Phycomyces. Postdoctoral research, California Institute of Technology.

Mavis Shure, Ph.D. Thesis: I. Studies of closed circular DNA: Physical and biological implications of heterogeneity in the topological winding number, a. II. The structure of virion SV40 DNA in situ examined by chemical modification with dimethylsulfate. Postdoctoral research, California Institute of Technology.

William Edward Stumph, Ph.D. Thesis: Gene enrichment using antibodies to DNA/RNA hybrids: Mapping the ribosomal DNA of slime mold and rat. Postdoctoral research, Baylor College of Medicine.

FINANCIAL SUPPORT

The financial support available for the work of the Division of Biology comes from many sources: from general Institute endowment and from special endowment funds for broad areas of work; from grants or contracts with individuals, companies, foundations, and U.S. governmental agencies for specific projects; from unrestricted annual gifts; from fellowships for the support of individual scholars; and from contributions to general funds provided by Industrial Associates and Institute Associates, as follows:

Fund--Research Support	Purpose
The Ahmanson Foundation	Research in molecular biology
Rita Allen Foundation	Cancer research
American Cancer Society	Cancer research
Anonymous Gift Fund	Research in educational programs
John E. Barber Fund	Biological research, particulary as related to the brain
Louis D. Beaumont Foundation	Neuroscience research
Beckman Instruments, Inc.	Funds for equipment
Bing Endowment Fund, Inc.	Professorship in behavioral biology
Biology Research in Neuroscience	Neuroscience research
Biomedical Research Support (NIH)	Biomedical research
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The James G. Boswell Foundation	Virus research
Ethel Wilson Bowles and Robert Bowles	Professorship in chemical biology
Mrs. Leah Hills Bruce	Research in biology
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The Commonwealth Fund	General support
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Mrs. Mary Bruce Delbrück	Research in biology
Josephine V. Dumke Fund	Cancer research
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Leland Fikes Foundation, Inc.	Research in molecular biology
The Max C. Fleischmann Foundation	Research in molecular biology
Foundation for Cardiovascular Research	Dr. F. Noble lectureship
Gloria Gartz Fund	Research and education in biology
E. S. Gosney Fund	Research in genetics
The Hearst Foundation	Research in auditory anatomy and physiology

Frank P. Hixon Fund	Neurobiology, physiological psychology, and related research
Hoag Foundation	Medical research program
Irma Hoefly Fund	Cancer research
Hoffman-LaRoche, Inc.	Research on barbiturate and minor tranquilizers
Arnold R. Jones	Cancer research
The Kroc Foundation	Research in biology
Lasker Award Fund	Chemical genetics
Litton Bionetics, Inc.	Instrumentation grant
Louis B. Mayer Foundation	Medical research program
The John A. McCarthy Foundation	Research in molecular biology
Merck and Company, Inc.	Research in biology
The Merck Company Foundation	Faculty development
Muscular Dystrophy Associations of America, Inc.	Research in biology
National Aeronautics and Space Administration	Space research
The National Foundation - March of Dimes	Research in birth defects
National Institutes of Health, USPHS	Studies in basic experimental biology, animal physiology, biochemistry, biological systems analysis, biophysics, developmental biology, genetics, neurobiology, plant and cell biology, psychobiology, and virology; graduate research training, biomedical sciences support grant
National Science Foundation	Studies in animal physiology, biochemistry, biophysics, developmental biology, genetics, neurobiology, plant and cell biology; undergraduate research participation program
Northwest Area Foundation	Medical science program in biology
Dr. and Mrs. Raymond Pearson	Research in chemical biology
Ann Peppers Foundation	Research in auditory physiology and anatomy
Pew Memorial Trust	Neuroscience research program
The President's Fund	Research and development at JPL
President's Venture Fund	Medical science
Research Corporation	Genetic research
The Rockefeller Foundation	Chemical biology research
Albert Billings Ruddock Fund	Professorship in biology
Damon Runyon-Walter Winchell Cancer Fund	Cancer research
Edwin H. Schneider Fund	Study and research in genetics
Alfred P. Sloan Foundation	Neuroscience research
Alfred P. Sloan Fund for Basic Research	General support
The Spencer Foundation	Behavioral biology research

Richard Steele	Grace C. Steele professorship of immunology
The Stone Foundation	Psychobiology research
A. H. Sturtevant Memorial Fund	General support
Sundry donors	Chemical biology research
Albert Tyler Memorial Fund	Annual lectureship in biological research
Union Oil Company	Corona Del Mar Marine Station
Joseph G. Venable Fund	Arthritis research
The Del E. Webb Foundation	Neuroscience research program
Martin Webster Fund	Immunology and virology basic to the problems of multiple sclerosis
Jean Weigle Memorial Fund	General support
Whitehall Foundation	Neurobiology
Robert E. and May R. Wright Foundation	Medical science
The Zanetti Grant	Biochemistry, cancer research

Fund--Fellowship Support

American Cancer Society

Earle C. Anthony Fellowship

The James G. Boswell Foundation

California Foundation for Biochemical Research

California State Graduate Fellowship

Cancer Research Institute, Inc.

Carnegie Institution of Washington

Centre National de la Recherche Scientifique, France

Chinese Nationalist Embassy

Lucy Mason Clark Fund

The Jane Coffin Childs Memorial Fund for Medical Research

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European Molecular Biology Organization

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German National Cancer Center

E. S. Gosney Fund

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The John A. McCarthy Foundation

Medical Research Council, Canada

Muscular Dystrophy Associations of America, Inc.

National Institutes of Health, USPHS

National Science Foundation

Gordon Ross Medical Foundation

Rubber Research Institute of Malaysia

Damon Runyon-Walter Winchell Cancer Fund

Evelyn Sharp Fellowship

Smith, Kline and French Laboratories

The Spencer Foundation

Swiss National Agency

Walter and Sylvia Treadway Fund

Vern Underwood Undergraduate Scholarship

Veterans Administration

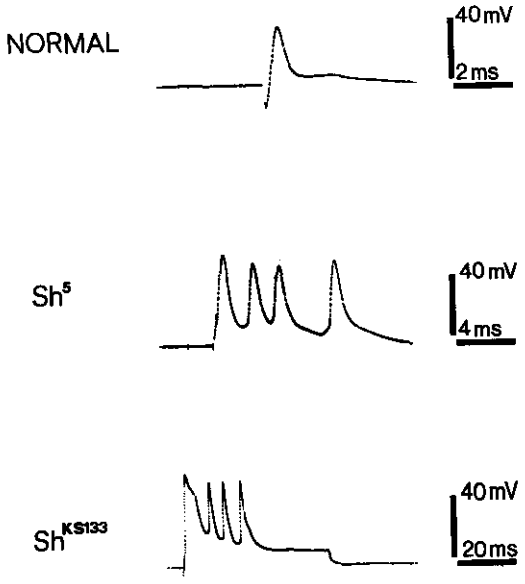
The Del E. Webb Foundation

Chaim Weizmann Foundation

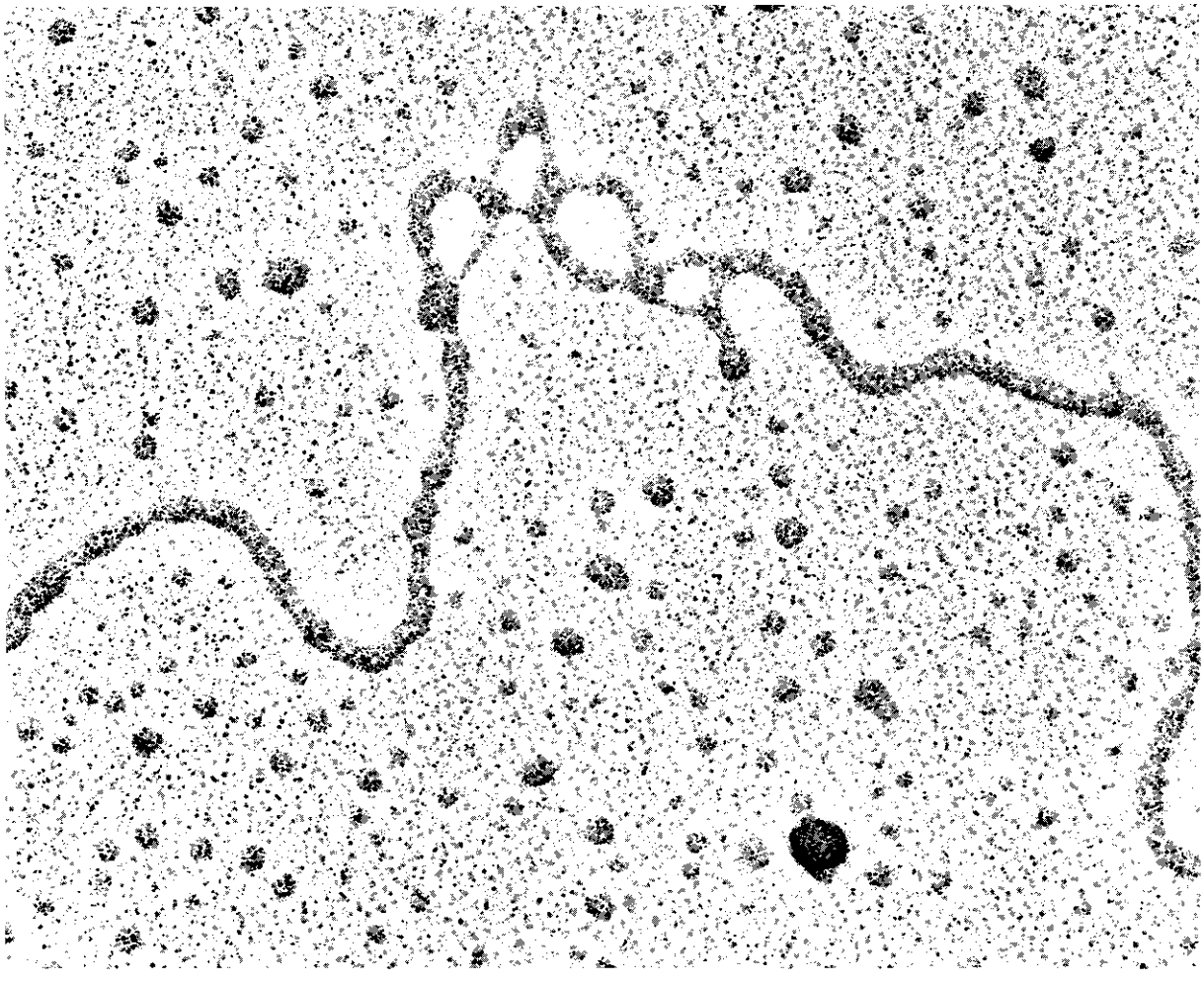
Helen Hay Whitney Foundation

AUTHOR INDEX (by page number)

FINANCIAL SUPPORT INDEX (by page number)



Action potentials from the giant axons of Drosophila. Intracellular recordings from normal giant axons show a single spike after a single, short duration (0.1 msec) electrical stimulus is delivered to the brain. The Sh mutation produces abnormal action potentials after such a stimulus. The Sh^5 allele shows multiple firing. The Sh^{KS133} allele has plateau potentials as well as multiple firing (M. Tanouye).



R-loop structure formed by hybridization between mouse DNA containing the gene for IgM heavy chain constant region (combined with a phage DNA cloning vector) and the mRNA for an immunoglobulin containing the mu constant region. The structure shows four coding regions interrupted by three small intervening sequences. The intervening sequences appear to separate segments coding for the four structural domains of the mu constant region (K. Calame and L. Hood).