A Report for the Year 1961-1962
on the Research and other Activities
of the
Division of Biology
at the
California Institute of Technology
Pasadena, California
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 publish papers bearing on the projects reported, but a complete list of publications by members of the Division is not given here. Such lists are published in the Institute's bulletin Publications of the Staff, copies of which may be obtained upon request.
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INTRODUCTION

Professor Alfred Henry Sturtevant reached retirement age this year, and was designated Thomas Hunt Morgan Professor Emeritus of Biology. His seventieth birthday was celebrated with the publication of Genetics and Evolution, a collection of some of his most significant papers. The book serves as a reminder of how great and how sustained over a long period have been Sturtevant's contributions to Biology.

Curriculum

In his final year of formal teaching responsibility, Sturtevant worked with Dr. Charles Brokaw to carry the Division's curriculum revision an important step farther. As in other sciences, the need in teaching biology has been to get on with the subject faster; to cover basic areas more efficiently so that time would remain to students for absorbing the recent and current knowledge that is increasing so rapidly these days. Brokaw's and Sturtevant's new encapsulation of Animal Biology in a one-quarter course, and last year's similar accomplishments in Plant Biology by Professor Lang and in Cell Biology by Professors Hodge, Sinsheimer, Bonner and others, superimposed upon Professor Bonner's highly successful one-quarter course in General Biology, now provide an efficient and intense sequence in the basic areas of our science, upon which specialization in diverse areas can well build. A curricular experiment next year will be a new two-quarter course in Biophysics of Macromolecules, to be taught by Professors Sinsheimer and Hodge of Biology, and offering an extensive laboratory in the complex modern instrumentation of this subject.

Students

The trend toward increasing interest in biology among Caltech undergraduates, noted last year, has continued. The Summer Apprenticeship Program this year fostered residence at the Institute of 48 students from Caltech and a number of other schools, most at the end of their junior year, each devoting a period of at least 10 weeks to gaining laboratory experience under the supervision of a member of our faculty. This year the wave of interest in careers in modern biology on the part of excellent students found marked expression also at the level of applications for admission to graduate work; from an unprecedented number of excellent applicants we admitted to graduate standing for 1962-63 as many as we felt we could provide with proper space, facilities, and supervision. The result is that graduate student enrollment in the Division will approximately double this coming year.
Research Activities: Virus DNA

The rather diverse and very extensive research activity in the Biology Division is summarized in this annual report. Only two categories of research activity will be selected for brief review here.

In Professor Sinsheimer's group, effort is directed mainly toward understanding the structure and mode of replication of two selected bacterial viruses: ΦX174, which contains a single-stranded DNA, and MS2, an RNA-containing bacteriophage. Only the work on ΦX174 will be mentioned here.

It is possible to obtain infection of bacterial protoplasts with the free nucleic acid of the virus. This result not only indicates that the nucleic acid component carries the essential genetic information, but also serves as a useful tool to follow the development of the viral nucleic acid during infection. When the single-strand viral DNA is introduced into the bacterial cell, it is rapidly converted into an altered form, called a "replicative form." Many properties of this replicative form--its density, chromatographic behavior, resistance to inactivation by ultraviolet radiation or by formaldehyde--suggest that it is two-stranded. During infection, this replicative form--which is also infective to protoplasts--is multiplied 30-50 fold. Then at a later stage in infection, the single-strand viral form reappears. It is never found free, however, but appears to be built into mature progeny virus particles as soon as it is formed.

During this year it was discovered that the infective viral form of ΦX DNA is not only single-stranded but is circular; it has no ends. There is one discontinuity of unknown nature in this nucleic acid ring, detectable by its resistance to enzymatic digestion. It may be a "coupling" between the ends of the nucleic acid chain. The significance of the ring structure is unknown; it may well have an important role in the biosynthesis of this unusual DNA.

Whether the "replicative form" is circular or not is unknown. Current research is attempting to isolate the replicative form in sufficient quantity to study its physico-chemical characteristics. Other research is attempting--by means of appropriate isotope experiments and by means of substitution of 5-bromouracil for the thymine of ΦX DNA--to ascertain the manner of replication of the replicative form and to decide whether the replicative form serves as a precursor of, or template for, the progeny viral DNA.

A ΦX DNA molecule contains 5500 nucleotides. Derived from a homogeneous viral preparation, a preparation of this DNA is presumably homogeneous. Hence it is feasible to attempt experiments to learn some of the details of the nucleotide sequence of the DNA. Current work is directed toward an understanding of the pyrimidine sequences of the DNA. By appropriate means the pyrimidine sequences can be isolated (from labeled viral DNA, using calf thymus DNA as a "carrier"). These can then be fractionated according to size, and then within a given size class according to composition. In this way it has been shown that the longest pyrimidine sequence in ΦX DNA is a stretch of 11 pyrimidines. There is one such sequence in the DNA. There are two sequences of 10 pyrimidines, which have been separated, and four sequences of 9 pyrimidines, of which two appear to be similar in composition. Detailed studies of the composition and sequence of these longer pyrimidine stretches are under way. Possibilities exist for carrying this type of research considerably further.
Having 5500 nucleotides, and assuming a coding ratio of 3 nucleotides per amino acid, ØX DNA could—if it is all used for this purpose—code for about 1800 amino acids or perhaps 10 proteins. Only one of these is known, the viral coat protein. To obtain some idea what the other proteins specified by the virus might do mutant forms of the ØX virus are being studied. Mutants can be obtained with extended host-range (presumably effected by a change in the coat protein). Temperature mutants—which will grow normally at reduced temperature but not at normal temperature—are also readily produced. These can be crossed and recombinants scored and a genetic map constructed. Complementation between mutants defective in different functions can also be observed; thus far two distinct complementing groups of mutants have been obtained. Studies have begun of the nature of the block to replication of these mutants to obtain clues as to the functions carried out by some of the proteins specified by the viral DNA.

Studies of the one protein we do know to be specified by the viral DNA, the coat protein, indicate that there is a protein subunit of the viral coat of molecular weight about 26,000. The coat might comprise 180 such units. The synthesis of the coat protein, detected by its capacity to combine with anti-virus serum, is also being examined during the process of infection. These studies have indicated that the synthesis of viral coat protein begins early in infection and precedes by several minutes the appearance of intact progeny particles. The physical form of the nascent coat protein is unknown, as is the manner of assembly of the virus.

Research Activities: Plant Growth

The study of how environment affects plant growth may seem very distant from the studies of nucleic acids mentioned above. In fact, however, the two areas are related, forming a kind of point and counterpoint. A complex organism like a higher plant is the result of a constant interplay between the genetic information contained in its DNA, and responsible for its specific structure, and the environment in which the organism develops and lives. In order to understand how the organism comes to be as we see it we must understand not only its genetic information but also the environmental factors that have affected its development.

Studies of environmental effects on plant growth are carried out in the Biology Division's plant research unit, which now includes the Earhart and Campbell laboratories (the former incorporating the old Clark greenhouse), the Dolk greenhouse, and adjacent small experimental garden plots. The Earhart and Campbell laboratories include about 30 separate rooms which are operated under different conditions of light and temperature. By shifting plants among these rooms the investigator can create a wide variety of environmental combinations ("climates") in a precise and reproducible way, and thus differentiate and integrate the effects of various climatic factors to learn both about the individual contributions of these factors and their complex interactions. This work is supplemented by organ-culture, microscopic, and biochemical studies carried out in the main laboratories of the Biology Division, resulting in a broad and multi-level attack on various problems of plant development.

Several kinds of environmental effects on plant growth can be distinguished. At the most direct level, the environment may exert its action immediately, and the action lasts only as long as the environment does not change. In less direct situations it may take some time before the effect of an environmental factor becomes evident; in fact, the effect may appear only after the environment has changed, but the effect may last for extended periods of time, sometimes for the remainder of the life of the plant. Such effects are called "inductive effects." Finally, work done in the Earhart and Campbell laboratories has revealed a startling possibility that the
effects of environment may be carried over to the offspring of a plant, for as many as two or three generations. Similar but even more persistent effects, responses to certain mineral nutrients regimes, have been noted by A. Durrant in England.

Present work is mainly directed at understanding how environment causes these different effects. In certain instances, the effect of abnormally low or high temperatures can be overcome by supplying the plants with certain chemicals, such as vitamins or nucleosides. This situation suggests that abnormal temperatures in these instances do not cause a general depression of the plant's functions but affect only particular processes which then become bottlenecks limiting growth. Other results, however, show that the relations of environment and growth are often more complex. Growth may depend not so much on the absolute level of particular environmental conditions as on cyclic changes. Tomato plants, for example, fail to grow on continuous light and constant temperature but can be made to grow either by light-dark cycles or by cycles of higher and lower temperatures. For optimal effects, these changes must be at or close to a 24-hour periodicity. However, inferior responses to other periodicities can at least in part be counteracted by changing the light:dark ratio. These responses are probably related to the existence, in plants and other organisms, of internal "clocks" operating on a diurnal periodicity; for optimal performance the internal and external rhythms must be closely coordinated. We are still quite ignorant of the mechanism of the clock, but by learning more about how the clock affects growth we may also obtain some ideas about the nature of the clock itself.

Inductive effects commonly involve some kind of amplifying mechanism. The energy which is put into the system from the environment is often much too small to account for the response directly; it evidently results in a re-channeling of other energy available to the systems. Experiments in the Earhart and Campbell laboratories have shown that some of the environmental responses are accompanied by striking changes in the levels of particular growth hormones, the gibberellins, and that similar responses may be evoked by application of these hormones to the plant, thus suggesting that gibberellins may act as part of the amplifying systems.

The nature of the possible carry-over effect between generations is still obscure. These effects are not due to the selection of preexisting, genetically different types. Neither are they direct, heritable changes in the Lamarckian sense, because the observed changes do not result in improved adaptation of the plant to the particular environment. They seem to be based on persistent modifications of gene function, under the influence of environmental factors, perhaps similar to those that have come to attention recently in certain microorganisms. Present work in the Institute's Plant Research Laboratories is directed at specifying the conditions which induce this kind of change. Dr. Durrant has joined the Division's plant biology group for a year in order to test the behavior of his plants under the controlled conditions of the Earhart and Campbell Laboratories. While his results must be checked in further generations, they indicate that the conditioning effect of mineral treatment is dependent on the temperature, and that other temperature regimes tend to reduce the differences between the induced types.

Much work remains to be done in this area, but our present knowledge already makes it clear that the plant as we see it is the product, on the one hand, of its "specific structure," and on the other hand of the environment to which it is exposed, the environment to which it has been exposed in the past, and probably sometimes the environment to which its progenitors were exposed.
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Intermediate in the terminal stages of hemoglobin synthesis have been found among the hemoglobin minors in rabbit reticulocytes. The experimental procedure was to incubate washed reticulocytes prepared from phenylhydrazine-treated rabbits in a reaction medium containing a labeled amino acid (leucine or valine). After incubation the cells were again washed and in a chase treatment were re-incubated in the same reaction medium in which the labeled amino acid was replaced by the unlabeled form. At the end of the incubation, whether submitted to the chase treatment or not, the cells were again washed, the red cells lysed leaving the white cells intact, the lysate centrifuged at 10,500 x g, the supernatant dialysed against the appropriate buffer and chromatographed on either DEAE-cellulose or Amberlite CG-50. The criterion for an intermediate was that it have a higher initial specific activity than the final product and lose specific activity in a chase treatment. A number of minors conforming to this criterion were isolated; three have been firmly established; there are probably several others. In a typical experiment the specific activity of the major increased linearly for 20 minutes during the incubation with labeled amino acid, increased further by 35 per cent at the beginning of the chase period and then leveled off. The specific activity of the fraction containing the minors also increased at nearly a linear rate during the incubation with labeled amino acid, attaining a level of 5 to 13 times that of the major, but during the chase treatment lost specific activity leveling off at about the specific activity of the major. Three minors, and there are probably several others, have been established as conforming to the above criterion for an intermediate in the synthetic...
process. All the labile minors behaved on chromatography and electrophoresis as if they were more acidic than the major.

A number of tests have rendered it unlikely that the labile counts, i.e. those lost in the chase treatment, are due to adsorbed free labeled amino acid. After adsorbed free leucine had been excluded, there were two obvious explanations for the loss in specific activity of the minors during chase treatment: 1) dilution of labeled by unlabeled material synthesized during the chase; 2) a simple precursor relationship in which a labeled precursor is replaced by an unlabeled one. Both these explanations appear to be excluded by the following considerations.

First, the minors comprise 11 per cent of the total hemoglobin. In order to reduce their specific activity to one half by dilution at least 11 per cent of the total hemoglobin would have to be synthesized during the chase treatment. In the time interval of the chase treatment there was only a 0.2 per cent increase in hemoglobin.

Secondly, a simple precursor relationship is excluded by the fact that a chase does not reduce the specific activity of any one labile fraction to zero.

The combined minor fractions accounted for 35 per cent of the total counts before the chase treatment; after the chase there was only 21 per cent of the total counts in the minors. Therefore, 14 per cent of the total counts were labile; this phenomenon clearly is metabolically significant.

The first of the labile minors to be obtained in sufficient quantity and purity for molecular weight determination was found to have, within experimental error, the same molecular weight as the major.

One possible explanation for the data we now have is that as hemoglobin comes off the template it becomes associated with low molecular weight acidic material, which is responsible for the chromatographic and electrophoretic difference of these hemoglobins from that of the final major or minor forms. This would account for the higher specific activity of the labile minor fractions. But in order to account for the decrease and final leveling off of their specific activity during the chase, it is necessary to invoke a rapid equilibration of the acidic material with the related pre-existing pool of major and minors; thus a steady state pool of the more acidic hemoglobins would exist, and the specific activity of the minor fractions would not drop below that of their ultimate forms--major or minor.

Our findings suggest that minors may be classified as either genetic or metabolic. This would help to explain the increasing number of minors which are being found in the fractionation of hemoglobins.

2. A COMPARISON OF THE PROCESS OF HEMOGLOBIN SYNTHESIS IN RABBIT ERYTHROID MARROW CELLS AND BLOOD RETICULOCYTES

Investigators: Henry Borsook, Jerry B. Lingrel, Robert L. Millette, José Scaro
Support: National Institutes of Health, U.S. Public Health Service
U.S. Atomic Energy Commission
National Council of Scientific Investigation of Argentina
American Cancer Society, Nutrition Foundation

In the current theory of protein synthesis the specific amino acid sequence is determined by the base sequence of a messenger RNA which is specific for the protein
and which is copied from the base sequence of the DNA on which it is synthesized. Thence it migrates to a ribosome on which it serves as the template for the amino acid sequence of the protein. The ribosome according to this theory is no more than a carrier, which is necessary but of low specificity for the messenger RNA.

The evidence comes largely from findings in cell-free extracts. In these systems synthesis is much slower than in intact cells, 1 per cent or less at its maximum, and comes to a stop in about 30 minutes, whereas in intact cells it proceeds, even in vitro for hours. We thought it might be instructive to compare erythroid marrow cells and circulating reticulocytes in the following respects: rate of hemoglobin synthesis, concentration, rate and degree of labeling of their ribosomes, and the changes in hemoglobin synthesis and ribosomes during the maturation of erythroid marrow cells to circulating erythrocytes.

The marrow cells and circulating reticulocytes were obtained from rabbits made anemic by phenylhydrazine, washed and then incubated in a reaction medium which contained a complete amino acid mixture, an iron, salt, citrate, bicarbonate, plasma and a labeled amino acid; this reaction medium supported more rapid hemoglobin synthesis than any yet reported. Differential lysis separated the cell contents of the erythrocytes from the white cells which were left unlysed. Red cell counts were made so that the analytical data could be expressed on the bases of concentrations and rates per cell.

Under these experimental conditions the rate of hemoglobin synthesis, expressed as µmols x 10^-14 per minute per cell, was on the average in the marrow erythroid cells, 3.1 in the first 10 minutes; it rose to 7.5 for the 60 minute interval and then fell to 5.3 for the 120 minute interval. In reticulocytes, on the other hand, the rate was constant, 3.4, over the whole 120 minute interval; the rate in reticulocytes was practically the same whether they were produced by phenylhydrazine or bleeding. Reticulocytes in normal blood were only about one quarter as active as those in anemic blood.

Marrow erythroid cells contain, on the average, about 6 times the ribosomes as reticulocytes. Average values, expressed as µmols x 10^-12 per cell, were 1.1 and 0.16 for marrow cells and reticulocytes respectively. These data indicate that in the process of maturation to reticulocytes marrow erythroid cells lose 80 to 90 per cent of their ribosomes. The ultraviolet absorption spectra of the ribosomes from the two groups of cells over the range 230 to 240 mµ are the same, which indicates that there is no change in the protein/RNA ratio as the marrow cells lose most of their ribosomes.

The ribosomes in the two groups of cells differ in their behavior. Reticulocyte ribosomes attained their maximum labeling in the first five minutes of incubation, and remained at that level up to two hours of incubation. When the labeled amino acid was replaced by the unlabeled form (chase treatment) the specific activity of the ribosomes fell quickly to 10 to 30 per cent of that before the chase. The "fixed" counts, i.e. those which remained after the chase, were incorporated at the same time as the labile counts and did not increase with prolonged incubation. The ribosomes in the marrow erythroid cells differed in three respects. They attained their maximum labeling in about 15 instead of 5 minutes; the maximum value of labile counts was not maintained, it fell after 60 minutes' incubation to less than a third of the maximum; there was a continuous increase of "fixed" counts throughout the incubation; after 30 minutes the "fixed" counts became greater than the labile counts. The relatively large and continued increase of "fixed" counts in the marrow cells is to be construed as synthesis of ribosomal protein in marrow cells, which is to be ascribed to the erythroblasts and normoblasts and is dependent (indirectly) on the DNA in their nuclei. In reticulocytes, which have no nuclei, synthesis of ribosomal protein is slight and stops in the first few minutes of incubation.
However, the synthesis of hemoglobin, which is the major protein in both groups of cells, may continue long after the disappearance of the nucleus, through to the terminal stage of the reticulocyte before it becomes an erythrocyte. The difference in the time course of hemoglobin synthesis in marrow cells and reticulocytes parallels the difference in incorporation of "labile" counts into their ribosomes. In marrow cells where the ribosomal labeling changed during the incubation there was a linear relation between rate of hemoglobin synthesis, ribosome activity and labile counts in the ribosomes; the straight line goes through the origin.

The activity per unit of ribosome was 2.5 times greater in reticulocytes than in marrow cells, but since the latter had 6 times the ribosomes per cell their maximum rate was 2.03 times greater. The falling off in hemoglobin synthesis with the prolongation of incubation in marrow cells but not in reticulocytes suggests that some ribosomes become inactive and it is these which are lost as the marrow cells mature.

The turnover of ribosomes, i.e. the rate at which their labile counts are converted into hemoglobin counts, was greater in reticulocytes than in marrow cells (compensated by the higher concentration of ribosomes in marrow cells). The turnover varied only from 9.2 to 13.4 per minute in batches of reticulocytes from six different animals and from 6.2 to 6.5 per minute in marrow cells from three animals.

Yet the steady state labeling in the reticulocyte ribosomes varied from 0.42 to 1.70 µmols of labeled amino acid per µmol of ribosome. This variation may represent different degrees of labeling in different batches of cells in which all the ribosomes are active, or variations in the fraction of the ribosomes which are active. In either case in reticulocytes the rate of hemoglobin synthesis is not related to the average degree of steady state labeling of the ribosomes. More likely it is the rate of release of finished globin peptide from the template which is the limiting factor, and the relative constancy of turnover of the ribosomes indicates the constancy of the release factor in different batches of cells prepared, as these were, under standardized conditions.

The time required to synthesize a hemoglobin molecule is given by the ratio 
\[(\mu\text{mols of a given amino acid per } \mu\text{mol of } a \text{ or } \beta \text{ peptides of hemoglobin}) \div (\mu\text{mols of ribosomes}) \div (\mu\text{mols of amino acid incorporated per minute into hemoglobin}).\]

On the assumption that the $a$ and $\beta$ peptides are made on different ribosomes and that two ribosomes, one of each kind, make a tetramer of hemoglobin, the time was 3.6 minutes in reticulocytes and 6.4 minutes in marrow cells. If four ribosomes, two of each kind, are necessary, i.e. one for each globin peptide, the time for synthesis of a tetramer would be 1.8 and 3.2 minutes respectively.

The picture of the maturation process outlined by the above findings is: Erythroid cells lose ribosomes as they mature; the rate of hemoglobin synthesis decreases mainly for this reason as erythroid cells mature; some ribosomes become inactive before they are lost to the cell; the rate of hemoglobin synthesis is proportional to the labile counts on the ribosome. This picture was confirmed and some further details supplied by radioautography on fractionated marrow erythroid cells and reticulocytes.

A. Miller (Biology Division, C.I.T.) had observed on radioautography of reticulocytes that had been incubated with tritiated leucine that only about 35 per cent had any grains; i.e. had been synthesizing hemoglobin; 65 per cent were inactive although in the preparation 85 per cent of the cells had the appearance of reticulocytes after appropriate staining. Miller noted also that there was not a Gaussian distribution of grains among the cells, indicating that in the cells which had no grains synthesis had been stopped relatively quickly.
These observations were extended to marrow cells and to reticulocytes that had been fractionated. In marrow cells the fractionation was obtained by differential staining after radioautography and counting the grains in each class of cells: basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts and reticulocytes. In all four classes there was a relatively high percentage of cells with no grains: 11.9, 37.5, 73.0 and 46 per cent in the above four classes respectively. Also the marrow reticulocytes as a group had more grains than the orthochromatic erythroblasts; an explanation for this finding may be that some basophilic erythroblasts skip one or more cell divisions in their maturation to reticulocytes; a less likely explanation is that some orthochromatic erythroblasts resume hemoglobin synthesis after they become reticulocytes. There was not in any group of cells a Gaussian distribution of grains. The outstanding observations were that even in some as immature as basophilic erythroblasts hemoglobin synthesis stops; and that, as maturation proceeds on the average, hemoglobin synthesis becomes slower. It was unlikely that cells in the early stages of maturation had lost all their ribosomes. The complete cessation of hemoglobin synthesis in even some of these is to be ascribed to inactivation of all their ribosomes by the appearance of an inhibitor. The inhibitor appeared at all stages in the maturation to stop hemoglobin synthesis completely in that cell regardless of its maturation stage.

The same picture was obtained when reticulocytes were fractionated in a bovine serum albumin gradient by the method of Vinograd and Leif (Chemistry Division, C.I.T.). Leif and Millette had found that when reticulocytes which had been incubated with labeled amino acid were fractionated by this method hemoglobin with the highest specific activity was found in the lightest cells (at the top); in the heaviest cells (in a pellet at the bottom) the specific activity was very low, and there was a gradient of specific activity in between. We extended these observations by a) determination per cell of the ribosome and hemoglobin concentrations and their specific respective specific activities and b) radioautography of the cells in different layers. It was observed that the most active cells, which were at the top, were also the largest, contained the most hemoglobin per cell and the most ribosomes. The least active cells were in the pellet at the bottom; they contained the least hemoglobin and the least amount of ribosomes per cell. The ribosomes in the top-most cells had the highest specific activity and the highest turnover; vice versa in the pellet. Between the top and bottom there were gradients in all these respects. The pellet cells contained only 13 per cent reticulocytes; in all the other layers the cells appeared all to be reticulocytes.

On radioautography, in the top-most layer, which was the most active and had the highest concentration of ribosomes per cell, there were 40 per cent with no grains. It is a reasonable surmise that the inactive cells still had ribosomes, but they had been rendered inactive. As in the marrow erythroid cells there was not a Gaussian distribution of the grains. In the layers a short distance below the top the proportion of cells with no grains increased rapidly, more rapidly than the decrease in average ribosome concentration. In the pellet cells it was uncertain whether there were any grains above background.

The outstanding observation on the fractionated reticulocytes was that the most active cells also had the most hemoglobin per cell; the least active cells the least amount of hemoglobin per cell. It followed from this observation that one should find among normal erythrocytes (fully mature cells) varying amounts of hemoglobin per cell. Vinograd, et al., had already found this to be the case in human erythrocytes. We have now observed it in rabbit erythrocytes.

The picture that comes from the foregoing observations is that hemoglobin synthesis may be stopped in erythroid cells at any stage in their maturation. The longer hemoglobin synthesis persists through the several maturation stages of an erythroid cell the more hemoglobin it will contain in its final erythrocyte state; e.g.
where hemoglobin synthesis was stopped in a basophilic erythroblast the erythrocyte derived from it contained less hemoglobin than the cell in which synthesis had persisted through to the terminal reticulocyte stage. At all stages synthesis depends on the presence of active ribosomes. When they become inactive they are lost from the cell, with the result that although hemoglobin synthesis slows down in the course of maturation, the activity per unit of ribosome which remains decreases more slowly.

Publications:


3. ERYTHROPOIETIN

Investigators: Geoffrey Keighley, Peter Lowy

Support: American Cancer Society

Erythropoietin is found in anemic animals. As its concentration increases with the severity of the anemia, and as the polycythemia which it produces when injected into normal animals is proportional to the dose it appears to be a hormone which regulates the size of the red cell mass. The previously described 60 to 80% ethanol fraction "LD" from rabbit plasma has been further purified by a four-stage ethanol fractionation. Over 50% of the activity is recovered in a 65 to 80% ethanol precipitate with a 7- to 10-fold increase in specific activity. The enrichment based on plasma protein is 5,000- to 10,000-fold. The material has the electrophoretic behavior of an $a_2$-glycoprotein.

Normal inactive plasma filtrate carried to the same state of fractionation yields inactive material of the same character.

In parallel investigations with human urines successive ethanol fractionations in the presence of phenol gave consistently good recoveries of activity.

Differences in ethanol solubility and electrophoretic behavior are found in the most purified fractions from pooled rabbit plasma and in urines from different individuals. It is assumed at present that the differences are due to attachment of the active moiety to different inert carriers.

Erythropoietic activity cannot be found in the plasma of normal animals. Even in anemic animals there is little or none unless the anemia is severe. This situation certainly reflects the sensitivity of the assays, and in the absence of pure samples it is impossible to relate the assays to absolute amounts of the hormone. In this
situation it would be useful to know something about the biological half-life. Soluble erythropoietin concentrate is quite stable. When incubated in vitro with blood, plasma or bone marrow at 37°C under 95% O₂ 5% CO₂, from 50% to 90% of the activity can be recovered even after several hours. When the same preparations are injected intravenously into rats or mice the activity disappears from the plasma with a half time of about one hour. Pretreatment of rats with X-irradiation, CCl₄, splenectomy, bilateral nephrectomy, or removal of the abdominal digestive tract with no circulation in the liver had no appreciable effect on the rate of disappearance. Investigation of the situation in anemic rabbits showed that the lymph contained activity, which was related quantitatively to the activity in the plasma. Therefore, lymph is part of the fluid space for erythropoietin and in the experiments with mice and rats the loss from plasma was largely controlled by the rate at which it entered the lymph.

As in the past, work is being continued with Dr. Denman Hammond of the Children’s Hospital of Los Angeles, particularly on anemic patients who excrete erythropoietin in their urine. Collaboration is being continued with Dr. Elizabeth S. Russell of the Jackson Memorial Laboratory, Bar Harbor, Maine, on the red cell regulation in normal and genetically anemic mice.

These subjects were discussed at the Conference on Erythropoietin and Regulation of Erythropoiesis in San Francisco, held in January, 1962 under the sponsorship of the Public Health Service and the University of Chicago. Three papers will appear when the Conference collection is published. A paper, "Response of Normal and Genetically Anemic Mice to Erythropoietic Stimuli," by Geoffrey Keighley, Elizabeth S. Russell and Peter Lowy has been accepted for publication by the British Journal of Haematology.

Publication:


4. SPECIES DIFFERENCES IN ERYTHROPOIETINS

Investigators: José Scaro, Geoffrey Keighley, Peter Lowy
Support: American Cancer Society

Erythropoietin from rabbits, rats, sheep, humans, is effective in rats and mice but not in guinea pigs. It was not known whether this was a species difference in erythropoietins or whether guinea pigs did not produce and use erythropoietin. Guinea pigs have been made anemic with phenylhydrazine and plasma from them has been tested by injecting it into other guinea pigs which had been sensitized by exposure to low concentrations of O₂ for two weeks or by fasting. The responses of the recipients were measured by the uptake of Fe⁵⁹ into the red cells. In both cases the anemic guinea pig plasma stimulated erythropoiesis in the assay guinea pigs. No clear evidence of erythropoietic activity was found when the same anemic guinea pig plasma was tested in sensitized mice and rats.

5. FURTHER STUDIES ON RETICULOcyTE RIBOSOMES

Investigator: Ellen R. Glowacki
Support: National Institutes of Health, U.S. Public Health Service

In an effort to produce a specific anti-ribosome antibody, as had been reported by Dr. Barbu, who injected rabbits with E. coli ribosomes, chickens were injected with rabbit reticulocyte ribosomes. In chicken serum one encounters a great deal of non-specific precipitation with ribonucleoprotein, but this difficulty was
finally eliminated by a clarification spin at 105,000 g for three hours and subsequent fractionation of the serum for γ-globulin with ammonium sulfate. Preliminary tests indicate that a true antibody to ribosomes was obtained, but whether this is an antibody to the protein or to the RNA is still in question.

Recently experiments on the extraction of ribosomal RNA with phenol and SDS were carried out and it is hoped that in the near future these RNA preparations can be tested to determine their effect upon the cell-free incorporating system.

6. STRUCTURAL STUDY OF RABBIT RETICULOCYTE RIBOSOMES

Investigator: Hildegard Lamfrom
Support: National Institutes of Health, U.S. Public Health Service

Rabbit reticulocyte ribosomes were examined in the electron microscope using the phosphotungstic acid (PTA) negative staining method. Particles homogeneous in size but irregular in shape were observed in 70S preparations. When the experimental conditions of Huxley and Zubay were employed, the structure of rabbit reticulocyte ribosomes was found to differ from that reported for E. coli. There was no obvious cleft in the 70S particles examined, indicating a more intimate association of 50S and 30S components. Moreover, the diameter of the 70S particles was considerably greater (250 x 340 Å).

Treatment with PTA alone, without formaldehyde fixation, led to breakdown of the particles, yielding a uniform structure 170 x 210 Å with a sedimentation value that did not correspond to any component commonly seen in ribosomes prepared in the absence of PTA. Parallel experiments using the analytical ultracentrifuge and the electron microscope revealed that individual particles may vary greatly in morphological appearance, although there may be a homogeneous sedimentation boundary in the ultracentrifuge.

Particles treated with the low pyrophosphate concentration which gave 70% dissociation in the ultracentrifuge still appeared to have a fairly uniform size distribution in the electron microscope. However, at the higher pyrophosphate concentration required for 99% dissociation, the products were very heterogeneous in structure, ranging from small spheres to threads. Reassociation of these 30S and 50S subunits yielded particles which were more heterogeneous in size and shape than the original 70S ribosomes, and these reassociated particles showed an irregular internal structure. These observations suggest that loss of protein synthesis activity in these particles may be due to structural alteration.

This work was done in collaboration with Frederick A. Eiserling, Departments of Zoology and Bacteriology, University of California, Los Angeles.

Reference:

7. PURINE OXIDATIVE ENZYMES IN FROG ONTOGENY

Investigator: Dennis Barrett
Support: National Institutes of Health, U.S. Public Health Service

A new assay for uricase, mentioned in Biology 1961, has been developed to a reliable and reproducible stage. Uricase decarboxylates uric acid-6-C14 at the 6C position, releasing C14O2 which can be trapped and counted in a liquid scintillation counter.
Once the difficulties of a high spontaneous uric acid degradation blank were overcome, the method proved a sensitive assay for uricase, and showed the error of previous results which suggested that uricase is absent until its synthesis is "turned on" at a specific phase in tadpole ontogeny. Instead uricase can now be demonstrated in the egg before fertilization, with an initial activity per egg of $5 \times 10^{-12}$ moles of substrate oxidized per minute, which figure corresponds to $4 \times 10^8$ molecules per egg (on the basis of the turnover number which Mahler has determined for swine uricase).

Xanthine oxidase, the enzyme preceding uricase in the purine degradative pathway, was similarly investigated, using a high excess of added uricase, with xanthine-6-C$^{14}$ as substrate. The oxidase defied detection in crude extracts of early embryos until nicotinamide-adenine-dinucleotide (DPN) was added, indicating that the enzyme is predominantly a dehydrogenase resembling the xanthine dehydrogenase reported for chicken liver rather than the well-known true xanthine oxidase found in milk. Frog liver xanthine dehydrogenase favors nicotinamide-adenine-dinucleotide more than tenfold over nicotinamide-adenine-dinucleotide-phosphate (TPN) as an acceptor, and is inhibited by 3-pyridinaldehyde-DPN.

Attempts to demonstrate a true oxidase activity (DPN-independent) have not yet been successful and it seems probable that only the dehydrogenase is present in the embryos and adult organs so far studied. The dehydrogenase has an activity per egg of $3 \times 10^{-15}$ moles of substrate oxidized per minute, corresponding to $1.4 \times 10^6$ molecules of enzyme per egg (calculating with the chicken liver enzyme turnover number). While these order-of-magnitude figures seem large, sensitive enzymatic assays have rarely been used on developmental materials, and no report of a lower enzymatic activity is known to be extant in the embryological literature.

Assays of a complete developmental series to give an indication of how much of the enzyme is maternal and when embryonic synthesis begins must precede attempts to analyze the regulatory mechanisms at work.

8. INHIBITION OF GROWTH AND SPORULATION OF BACILLUS MEGATHERIUM BY O-FLUOROPHENYLALANINE

Investigators: R. L. Heath, H. K. Mitchell
Support: National Institutes of Health, U.S. Public Health Service
Arthur McCallum Fund

In the course of studies on protein synthesis during spore formation in B. megatherium ATCC 8245, it was found that o-fluorophenylalanine completely inhibits sporulation in a synthetic medium (sucrose-glutamic acid-salts) at a concentration of $5 \times 10^{-5}$ M when the inhibitor is added either (a) to fresh medium together with an inoculum of growing cells, or (b) to a fully grown culture just prior to sporulation. The inhibitor does not, however, prevent the pH changes (a drop to about pH 5.5, followed by a rise to pH 9) which normally accompany sporulation, and which are prevented by some other inhibitors of sporulation such as a-picolinic acid (Nakata and Halvorson, 1960).

Further investigation showed that the inhibitor, while not preventing growth when added with the inoculum, causes a lag before growth can begin, amounting to 4 hours ($3 \times 10^{-6}$ M) to 48 hours ($10^{-3}$ M); very slow linear growth occurs during this period, at the end of which exponential growth at the normal rate suddenly sets in. The final cell population in each case is the same as in the absence of inhibitor. The effects on both growth and sporulation are completely reversed by yeast extract or phenylalanine.
Studies are currently under way to measure incorporation of o-fluorophenylalanine into protein during growth and spore synthesis, and its release from cell proteins prior to spore synthesis, using a colorimetric assay procedure for fluoride ions after fusion with sodium peroxide.

References:


9. TYROSINE METABOLISM IN DROSOPHILA PUPAE

Support: National Science Foundation
National Institutes of Health, U.S. Public Health Service

Cell-free extracts of Drosophila pupae, just after their formation, show a net increase in total protein, determined by the biuret reaction on the material precipitated by hot trichloroacetic acid, of 10 to 15% in 60 minutes at 30°C. It was observed that at the same time tyrosine is incorporated into the protein fraction over 1,000 times faster than 8 other amino acids tested. This was found to be true also in vivo by injection of C14 amino acids into pupae. Further, in cell-free extracts, C14 tyrosine is converted at 0°C (60 minutes) to at least 25 labeled, low molecular weight compounds separable in the amino acid analyzer. Most of these substances, formed at 0°C, were found in the protein fraction after 15 minutes' incubation of the mixture at 30°C. One of the labeled compounds yielded, on hydrolysis, histidine and several mols of an amino acid with a spectrum in UV different from that of tyrosine but containing all of the label. It should be noted that none of the known amino acids becomes labeled in the system and that no melanin is produced. Results do not indicate protein synthesis as such but participation of peptides and protein in chitin synthesis is suspect. A detailed report for publication is in preparation.

10. ENZYME AND TOTAL PROTEIN CHANGES IN DROSOPHILA DURING DEVELOPMENT

Investigator: H. K. Mitchell
Support: National Science Foundation
National Institutes of Health, U.S. Public Health Service

As a basis for further studies on the metabolism of amino acids and peptides in cell-free extracts of Drosophila, enzyme activity (alkaline phosphatase) and enzyme and total protein stability in such extracts were studied in preparations from about 60 stages of the life cycle of the organism. Phosphatase activity (initial per mg of tissue) changes over about a tenfold range with low values in embryonic and pupal stages. During larval stages maximum activity was observed just prior to the two molts and pupation, with minimal activity at the two molts and a precipitous drop at pupation. Incubation of cell-free extracts at 30°C resulted in a loss in phosphatase and total protein in preparations from larvae or adults but, in general, in an increase in both during embryonic and pupal stages. A uniquely large increase in phosphatase activity just before hatching was observed, indicating a special mechanism of activation or synthesis during this period. The results reveal a variety of problems in the biochemistry of development and show clearly highly active but conserving metabolism in embryos and pupae where there is no exchange of materials with the environment. A manuscript covering details is in preparation.
11. PURIFICATION OF AN ALKALINE PHOSPHATASE

Investigator: Arthur D. Riggs  
Support: National Science Foundation  
General Funds

In connection with studies of protein synthesis in the pupae of the fruit fly Drosophila melanogaster it was necessary to isolate a specific protein in pure form. For this reason a procedure for the partial purification of the alkaline phosphatase of the fly's pupae has been worked out. Because of the small amount of protein remaining in the most pure fractions, accurate values for the protein content of these fractions have yet to be obtained. However, it can be estimated that about a thousand-fold purification has been obtained.

It has also been shown that there are at least two and probably more distinctly different alkaline phosphatases present in the pupae and a third present in the larvae that disappears during the pupal stage.

12. PEPTIDYL ACYLATING COMPOUNDS IN DROSOPHILA PUPAE

Investigator: Thomas A. Cole  
Support: Nutrition Foundation  
General Funds

The pupal stage of the fruit fly Drosophila melanogaster is a closed system in which the larval tissue is transformed into adult tissue in five days. Some studies on young pupae have been carried out; most of the work was done on low speed supernatants of cell-free homogenates. The supernatants can carry out chemical transformations which are sensitive to hydroxylamine, which traps high energy bonds. The resulting hydroxamic acids are formed faster at 25°C than at 0°C. Their formation is also enhanced by the addition of tyrosine.

Studies with the enzyme alkaline phosphatase show that hydroxylamine decreases the initial rates of the enzyme activity in timed aliquots from an incubation mixture of low speed supernatant. The hydroxamic acids formed with hydroxylamine in the Drosophila material show a strong coincidence with the ninhydrin-positive material on an Amberlite-120 column. Over 20 hydroxamic acids have been isolated by means of a 150 cm Dowex-50 column. The two compounds in highest concentration appear to be peptides for they give five or more ninhydrin-positive components after mineral acid hydrolysis. Both hydroxylamine and tyrosine affect the metabolism of ninhydrin-positive compounds as determined with an amino acid analyzer. The tentative interpretation of these data is that peptides are active in protein biosynthesis in Drosophila pupae. This does not appear to be the case in other systems that have been studied.

13. AVENACIN, AN ANTIMICROBIAL SUBSTANCE FROM OATS

Investigator: H. J. Burkhardt  
Support: National Institutes of Health, U.S. Public Health Service

The sapogenin constituent of avenacin (Biology 1961, p. 32) as obtained after acid hydrolysis of pure avenacin was shown to be a mixture of at least 12 chemically similar components, all arising from interactions of functional groups of the sapogenin in the presence of hydrochloric acid. A method has been developed to separate all 12 different sapogenins; 3 were obtained in a pure crystalline form and 1 was found to have about 30 to 32 carbon atoms and 6 functional groups, 5 hydroxyl and 1 keto group. The sapogenin therefore is most likely to be a pentacyclic triterpene.
Avenacinase, an enzyme known to separate the sugars from the avenacin molecule, has been isolated but was found to split off only one or two of the three monosaccharides.

Work is presently under way to obtain large amounts of the avenacin hydrolytic product for its characterization.

14. STRUCTURAL STUDIES ON NEUROSPORA TYROSINASE

Investigators: Marguerite Fling, Harlow H. Daron, N. H. Horowitz
Support: National Science Foundation

Wild-type strains of Neurospora crassa produce a series of allelic tyrosinases which differ in their thermostability and/or electrophoretic mobility. Isolation of two of these tyrosinases (types S and L) and a study of their physical properties was reported last year. The enzymes contain approximately 0.2% of copper, and this is in the cuprous state, as shown by electron spin resonance, spectrophotometric, and chemical analyses. Studies of the amino acid composition, tryptic fingerprints, and end groups of the enzymes are being carried out in order to learn how they differ structurally. Thus far, no significant differences have been found in their amino acid composition or in their tryptic fingerprints. Analysis of chymotryptic digests and of N-terminal and C-terminal amino acid residues are in progress.

Publications:


15. TYROSINASE SYNTHESIS IN VIVO

Investigators: Helen Macleod, Marguerite Fling, N. H. Horowitz
Support: National Science Foundation

Neurospora synthesizes essentially no tyrosinase while it is growing on minimal medium. If the mycelium is transferred to phosphate buffer at pH 6, however, an extensive synthesis of the enzyme occurs. After 48 hours in the buffer, tyrosinase accounts for as much as 5% of the extractable proteins of the mycelium. This system is a convenient one for investigating the conditions necessary for release of the tyrosinase-synthesizing mechanism. It has been found that the synthesis requires oxygen and is inhibited by divalent cations at 0.01 M, glucose, and actinomycin. It is not affected by chloramphenicol or puromycin. The pH optimum of the system is around pH 6. At pH 8.2, no tyrosinase synthesis occurs, nor is the synthesis restored if the mycelium is transferred back to a pH 6 buffer. This effect has been found to be due to the extraction from the mycelium, at pH 8 and higher, of substances essential for the synthesis. If the extract is adjusted to pH 6 and added back to the extracted mycelium, the synthesis is restored. Partial restoration of the synthesis occurs if a mixture of amino acids plus copper is added to the extracted mycelium, but this mixture does not account completely for the activity in the extracts. Fractionation of extracts has shown that the missing factor is an inorganic substance, and this has been identified as calcium. The function of calcium in the system is being investigated.
16. METABOLIC ROLE OF THE D-AMINO ACID OXIDASE

Investigators: Helen Macleod, N. H. Horowitz
Support: National Science Foundation

A study of the metabolic function of the D-amino acid oxidase has been completed. Five mutants of Neurospora deficient in the enzyme were used. With one exception, the mutants grow normally on minimal medium and are sexually fertile. The exceptional mutant is unable to utilize sulfate for growth; this effect appears to be due to the coincidental mutation of another gene closely linked to the gene governing D-amino acid oxidase synthesis. It has been found that the D-amino acid oxidase is essential for the inversion of exogenous D-amino acids in Neurospora, that it protects the organism against the toxic effects of D-aromatic amino acids, and that it attacks exogenous D-methionine at a rate sufficient to provide the sulfur, but not the nitrogen, needed for growth. It is concluded that the enzyme performs no essential function under laboratory conditions, although it appears to be useful to Neurospora in nature. A total of 13 D-amino acid oxidase deficient mutants of independent origin were obtained in this investigation. All of them appear to be alleles or closely linked in Linkage Group IV.

Publication:


17. THE EFFECT OF BASE ANALOGS ANDNUCLEIC ACID ON THE INDUCTION OF TYROSINASE IN NEUROSPORA

Investigator: C. Ronald Scott
Support: The National Foundation

Neurospora crassa may be induced to produce large amounts of the enzyme tyrosinase by transferring the organism after two days of growth to phosphate buffer (0.02 M at pH 6) and then extracting the enzyme two days later. When base analogs such as 6-mercaptopurine, 8-azaguanine, 2,6-diaminopurine or 5-fluorouracil are added to the inducing phosphate buffer there is no effect upon the production of tyrosinase. If, however, the base analogs are added during the growth period then there is growth inhibition which varies directly with the concentration of the base analog. The tyrosinase produced by the induction of these growth-inhibited cells is not obviously altered according to thermal stability curves and paper electrophoresis.

If the nucleic acid extracted from Neurospora or B. subtilis is added to growing cultures of Neurospora, tyrosinase production is inhibited following induction by phosphate buffer. The individual nucleosides, peptides and non-specific proteins do not appear to have a similar inhibitory effect, and the action of RNase or DNase on the nucleic acids does not completely abolish the inhibitory effect of the nucleic acids on tyrosinase production. This effect is being studied further.
18. THERMAL INACTIVATION OF ØX174 DEOXYRIBONUCLEIC ACID

Investigator: Walter C. Fiers
Support: Rockefeller Foundation
National Institutes of Health, U.S. Public Health Service

The thermal inactivation of ØX174 DNA was studied in order to obtain more information on the chemical structure.

The decrease in biological activity upon heating follows first order reaction kinetics, and is independent of the viral DNA concentration. At 80°C and an ionic strength of 0.03 the rate constants at pH 7.5, 8.1 and 9.0 are respectively $1.2 \times 10^{-3}$ sec$^{-1}$, $4.6 \times 10^{-4}$ sec$^{-1}$ and $8.2 \times 10^{-5}$ sec$^{-1}$. The increasing stability with increase in pH suggests that the lethal hit may be identified with the loss of a purine base. This conclusion is supported by the reasonable agreement between the observed inactivation rate at 80°C and pH 7.5 ($1.2 \times 10^{-3}$ sec$^{-1}$), and the rate calculated on the basis of the reported values for depurination under comparable conditions ($6.9 \times 10^{-4}$ sec$^{-1}$). At pH 9.0 the activation energy amounts to 40 kcal mole$^{-1}$.

Chain breakage, as assayed by the release of 3'OH end groups, becomes important only at temperatures higher than necessary for biological inactivation. This is consistent with a mechanism involving opening of depurinated sites through β-elimination.

Publication:

19. A RING STRUCTURE FOR øX174 DEOXYRIBONUCLEIC ACID

Investigator: Walter C. Fiers
Support: Rockefeller Foundation
National Institutes of Health, U.S. Public Health Service

Neither a 3'OH nor a 5'OH end group can be demonstrated in øX DNA by enzymological methods. Evidence was obtained suggesting that the unavailability is not due to blocking by hydrogen bonding or by a monoesterified phosphate group.

When centrifuged under conditions which cause stretching of the DNA molecules two homogeneous centrifugal components can be observed. $S_1$ amounts to 50 to 70% of the total, while $S_2$, which moves at a 10% slower rate, accounts for 20 to 30% of the viral DNA. This pattern is not modified by centrifugation in a medium in which conventional base pairing is excluded. The linear proportionality between biological activity and $S_1$ content in samples which have undergone different degrees of inactivation, suggests that the biologically active component is physico-chemically identical with the $S_1$ fraction. A single chain scission, either by pancreatic deoxyribonuclease or by thermal energy, converts $S_1$ into $S_2$. A second hit, which occurs with an equal probability, results in random fragmentation. These findings can best be interpreted in terms of a covalently linked cyclic structure for $S_1$, which when opened gives rise to $S_2$, a straight chain of unchanged molecular weight.

Degradation of the enzymatically formed $S_2$ component with Escherichia coli phosphodiesterase reveals, however, that the viral DNA chain contains one discontinuity, the nature of which is at present unknown (e.g. an odd base, an odd linkage, or a non-nucleotidic residue).

Publication:

20. PYRIMIDINE OLIGONUCLEOTIDES OF THE DNA OF THE BACTERIOPHAGE øX174

Investigator: John B. Hall
Support: National Institutes of Health, U.S. Public Health Service

Work has continued on the isolation and characterization of the pyrimidine oligonucleotides of the bacteriophage øX174. It is expected that the determination of the composition and sequence of nucleotides in these pyrimidine tracts will yield information about genetic coding in this organism.

The DNA of the virus was labeled to a high specific activity with $^{14}$C uracil. It was then degraded with formic acid and diphenylamine, and the resulting pyrimidine tracts were separated into fractions of uniform chain length by chromatography on a column of DEAE-sephadex. Although the number of possible isomers of the longest chain lengths is very large, the number of such isomers that is actually found is relatively small, and it is hoped that many of them will be readily resolvable into their component molecular species. From the distribution of radioactive material in the various fractions, it has been tentatively concluded that the DNA of øX174 contains one oligonucleotide 11 residues in length and two that are 10 residues in length. The latter two have been separated by paper chromatography and their composition is currently being determined.
Methods have been developed for the further fractionation of oligonucleotides of a given size into subfractions, each consisting of tracts containing the same number of cytidylic acid residues. This system appears to be effective for oligonucleotides as large as the heptanucleotide, and can apparently also be used, as mentioned previously for the decanucleotide, to separate two oligonucleotides of larger size, that differ in their cytidylic acid content by more than one residue.

21. STUDIES ON THE INTERACTION OF THE DNA OF BACTERIOPHAGE ØX174 WITH PROTOPLASTS OF ESCHERICHIA COLI K12

Investigator: George D. Guthrie
Support: Arthur McCallum Fund
National Institutes of Health, U.S. Public Health Service

Since the demonstration of the infectious nature of ØX174 DNA, the protoplast assay system has been characterized to determine optimum conditions for assay of infectious ØX DNA molecules and other infectious subviral particles of ØX174. The effect on infection levels of various suboptimal conditions often encountered during different assays has also been investigated. As a result of these experiments the efficiency of infection of the protoplasts has been increased approximately 10 times under optimum conditions. The fractional inhibition due to certain suboptimal conditions has been determined.

Subsequently the initial kinetics of interaction of ØX DNA molecules and protoplasts were investigated. The data resulting from studies of the influence of DNA and protoplast concentrations and the effects of temperature on infection have suggested, approximately, the following mathematical relationship for describing the level of infection after a 20 minute "infection period":

\[
[\text{infected protoplasts}] = Ke^{-A/T}
\]

where K includes the concentration of DNA molecules and protoplasts and T is the Kelvin temperature. Other parameters which may be included in K have not been investigated nor is the physical significance of A known. Further investigations leading to an understanding of the parameters involved in the constant A may offer direct evidence concerning the mechanism of uptake of DNA by protoplasts. Investigations are now in progress to determine the concentration limits of DNA molecules and protoplasts over which this mathematical relationship holds.

Reference:


22. STUDIES OF THE REPLICATIVE FORM OF THE DNA FROM ØX174

Investigator: Alice J. Burton
Support: National Institutes of Health, U.S. Public Health Service

During the replication of ØX174, the DNA of the bacteriophage passes through a stage in which its physical properties are different from those of the single-stranded DNA of the mature phage. The isolation and purification of sufficient quantities of this replicative form (RF) to allow the study of its chemistry has been undertaken. Growth conditions of bacterial cultures to be infected with ØX174 and methods of preparation of the DNA following infection and harvest have been studied to determine optimum conditions for the preparation of RF. In the best preparations,
yields of 25 to 40 units of biological activity per infective center are obtained. In terms of DNA content, such yields are relatively low, hampering the accumulation of significant amounts of RF and leading to preparations containing large quantities of host (E. coli) DNA relative to the amount of RF obtained.

The procedure which has been developed to purify the DNA preparations combines high speed centrifugation, isopropanol fractionation, and precipitation with cetyltrimethylammonium bromide. Two preparations of RF are obtained: one largely free of RNA and other impurities, but in which the ratio of host DNA to RF is of the order of 1,000; the other containing large amounts of RNA but in which the ratio of host DNA to RF has been reduced to about 20. RNA, single-stranded DNA and other impurities can be removed from DNA preparations by column chromatography, on kieselguhr coated with methylated bovine serum albumen. Cesium chloride gradient centrifugation and RNase digestion are also helpful. However, host DNA and RF have not yet been separated. Since the second preparation of RF contains much less host DNA relative to RF, it is being used to try other techniques—for example, other types of gradient centrifugation and counter-current distribution—to effect the separation of the two types of DNA.

In determining the biological activity of these preparations, the protoplast assay is used. Considerable inhibition of the assay by host DNA has been observed; for accurate assays, the solutions must be diluted until they contain less than 0.1 µg of E. coli DNA/ml. Criteria which distinguish RF from single-stranded DNA include column chromatography and the relative resistance of RF, when compared with single-stranded DNA, to inactivation by ultraviolet irradiation or formaldehyde. At the same time, no differences between the two RF preparations have been detected using these methods, though the possibility that some difference does exist will be investigated more fully as more material becomes available.

23. STUDIES ON THE MULTIPLICATION OF THE REPLICATIVE FORM OF BACTERIOPHAGE øX174

Investigator: Alan B. Stone
Support: National Institutes of Health, U.S. Public Health Service

At the present time there are two major theories concerning the structural complexity of DNA and of the conserved subunits involved in its replication. Watson and Crick have postulated that the parental DNA molecule consists of two antiparallel polynucleotide strands coiled helically around a common axis and joined together by hydrogen bonding between complementary bases, and that the subunits involved in its replication are the single strands. On the other hand, Cavalieri and Rosenberg have reported experiments which suggest that in E. coli the parental DNA molecule may be comprised of a number of Watson-Crick duplexes (possibly two) and that the conserved subunits may be these hydrogen-bonded duplexes themselves rather than single polynucleotide strands.

The present work is an attempt to determine by which of these alternative mechanisms the replicative form (RF) of phage øX174 (Sinsheimer, et al.) multiplies. This virus injects a single strand of DNA into its host bacterium; the RF is thought to consist of this parental strand, or copies of it, combined with further DNA. By studying the fate of an RF molecule in which the øX174 strand is density-labeled and the remaining component radioactively labeled, it ought to be possible to elucidate its mode of replication in vivo. The replication mechanism of RF is not necessarily the same as that involved in the multiplication of DNA in other organisms. Nevertheless it will be of interest if it can be demonstrated that at least in the case under study DNA replicates through the formation of a single strand on a single-stranded template, and that the duplex so formed is subsequently dissociated so as to allow the template to function again.
It is hoped to obtain clearer results by infecting cells in which the synthesis of bacterial DNA is inhibited, and initial experiments have been directed towards finding such an inhibited system in which RF is formed and will multiply. So far it has been found that E. coli infected with ØX synthesizes both DNA and RNA at rates similar to those of the uninfected cell. When starved bacteria are resuspended in nutrient medium and infected, 20 to 30% of the host DNA is degraded to acid-soluble fragments during the first 10 minutes of the latent period. The possibility is now being investigated that bacteria grown in D2O medium may not resume DNA synthesis for some minutes after having been chilled to 2°C and resuspended in H2O medium.

References:


24. STUDIES ON THE DEOXYRIBONUCLEIC ACID FROM THE BACTERIOPHAGE ØX174

Investigator: Thomas W. Thompson
Support: National Institutes of Health, U.S. Public Health Service

On infection of E. coli with bacteriophage ØX174, the single-stranded DNA of the bacteriophage is converted into another form known as the "replicative form" (RF). This new DNA, which is infective to bacterial protoplasts, has properties similar to those of a double-stranded DNA. The exact function of the RF DNA is not known, and a study of its chemical and physical properties is not possible until a method is obtained for the separation of RF DNA from the host DNA. The large excess of host DNA adds to the difficulty of the problem.

Present work is concerned with the incorporation of 5-bromouracil into the DNA of a thymine-requiring strain of E. coli. It is known that DNA containing 5-bromouracil instead of thymine denatures at a lower alkaline pH than normal DNA. Thus if cells with 5-bromouracil in the DNA are transferred to a medium containing thymine and infected with ØX174, normal RF DNA should be produced. After alkaline denaturation of the host DNA the RF could be obtained free of host DNA by separation on a column such as kieselguhr coated with methylated bovine serum albumin.

25. FURTHER STUDIES ON THE INTRACELLULAR DEVELOPMENT OF ØX174

Investigator: David T. Denhardt
Support: National Science Foundation

The technique of synchronization of ØX development in E. coli C by starvation, described in Biology 1961, has been further investigated. The effects of different growth media, different starvation buffers, and different temperatures have all been studied and optimal conditions defined. Once the ØX DNA enters the cell it readily forms a replicating factor (RF); in fact, it has so far not been possible to unambiguously demonstrate the single strand of ØX DNA inside the cell after infection. About 10% of the cells starved for one hour and then infected with ØX can form RF after 15 minutes in starvation buffer. Results of Luria-Latarjet experiments indicate
that formation of the first RF particle occurs 1 to 3 minutes after initiation of development. These studies have also shown that E. coli DNA inhibits the infection of protoplasts by RF when the coli DNA is present with an optical density of about 0.01/ml, and the RF is present at a concentration of about 10^7 particles/ml. The efficiency of infection of RF appears to be comparable to, or somewhat greater than, that of single-stranded DNA in the protoplast system.

With the synchronized system it has been possible to show that the appearance of the first mature ØX particle in the bacterial cell is exponential with time, while maturation is linear. An average of one mature ØX particle per cell appears 12 to 13 minutes after development begins, and further particles appear at a rate of two ØX particles per minute at 37°C. Growth was initiated in the starved and infected cells by the addition of an equal volume of prewarmed tryptone broth.

26. EFFECTS OF ULTRAVIOLET LIGHT ON ØX174

Investigator: Michael J. Yarus  
Support: Woodrow Wilson Foundation, Arthur McCallum Fund, National Science Foundation

A study of the effects of ultraviolet light on ØX174 bacteriophage and its associated infective nucleic acids has been undertaken. Action spectra of the free virus, extracted single-stranded nucleic acid, and a distinct intracellular form of ØX174 DNA (RF), have been obtained, using the protoplast assay for infective nucleic acid. There are a number of differences among these spectra, the most striking of which is that RF is ten times as resistant to UV as the single-stranded DNA. These differences allow characterization of a preparation of infective nucleic acid as to its relative composition in single strand and replicative form. A projected Luria-Latarjet type experiment, in which infected complexes are irradiated, may allow characterization of the replicative process in the same way.

Other questions which are susceptible to attack in this way are also being pursued. As an example, preliminary experiments indicate that much more damage must be done to the virus to prevent formation of RF than to prevent plaque forming.

27. GENETICS OF PHAGE ØX174

Investigator: Clyde A. Hutchison  
Support: National Institutes of Health, U.S. Public Health Service

A system of temperature sensitive (ts) mutants of ØX has been isolated for use in studying the physiological genetics of this virus. A purified ØX stock was nitrous acid treated and the survivors plated at 25°C on E. coli C. Plaques formed by single survivors were picked and replated at 25°C and 37°C. About 1% of the survivors were ts mutants which would plate at the low but not at the high temperature when the stock was inactivated to a survival of 10^-3 by HNO_2. Wild-type ØX grows normally at both temperatures. Other mutants have been isolated which form plaques at 25°C and 37°C but not at 42°C. In all, some 25 ts mutants have been isolated. These vary in degree of sensitivity, but it is possible to prepare stocks of a number of the mutants in which the relative titer at the high compared to the low temperature is less than 10^-6.

Thermal instability of the mutant particle is not responsible for the ts phenotype in the few mutants studied to date. Furthermore, the block in phage production at the high temperature seems generally to occur after injection since cells infected
at the low temperature will not produce virus if quickly transferred to the high temperature.

In crosses between two ts mutants it is possible to select the wild-type recombinant by plating at the high temperature. Typical recombination frequencies in crosses with an input of approximately 10 of each parental type are approximately 10\(^{-4}\). This is several orders of magnitude above the frequency of wild-type revertants observed in selfings. Crosses between ts and h mutations have also been made. Mapping of the mutants is in progress.

The number of cistrons in which the mutants lie is unknown. Complementation is not revealed in mixed spot tests with two ts mutants by clearing of the spot, but no conclusions may be drawn until more sensitive tests, such as burst size measurements on cells mixedly infected at the high temperature, have been made.

Mutants can be screened for production of coat protein at the high temperature by a serum blocking technique (see Stanley G. Krane, Biology 1962). Preliminary results with one mutant indicate that protein is synthesized.

28. THE PHYSICAL PROPERTIES OF THE PROTEIN SUBUNIT OF PHAGE \(\Phi X174\)

Investigator: Edward A. Carusi
Support: National Institutes of Health, U.S. Public Health Service

The protein moiety of bacteriophage \(\Phi X174\) can be dissociated into subunits of uniform molecular size by treatment with sodium dodecyl sulfate at elevated temperature and pH. Subsequent separation of protein from phage DNA may be effected by ammonium sulfate fractionation. About 60% of the protein of the starting material is recovered by this procedure.

The physical state of the subunit is markedly dependent upon solvent conditions. On removal of detergent or lowering of pH, the subunits aggregate. These aggregates may be dispersed by addition of detergent in alkaline (pH 10.5) solution.

Physical measurements of \(\Phi X\) protein were therefore conducted by the method of equilibrium centrifugation in three component systems consisting of protein, buffer, and detergent. In these systems the protein and detergent exist as complexes, the composition of which varies with the concentration of added detergent. The parameters observed are thus those of the complex. Accordingly, the composition of the protein-detergent complexes was determined by equilibrium dialysis and used to obtain corrected molecular weights for the \(\Phi X\) protein alone.

The lowest molecular weight obtained by physical methods for the protein subunit is 25,000. The molecular weight computed from the amino acid composition is in good agreement with this value.

A full report of this work has been submitted for publication in the Journal of Molecular Biology.

29. PHAGE PROTEIN SYNTHESIS IN \(\Phi X174\) INFECTED ESCHERICHIA COLI

Investigator: Stanley G. Krane
Support: National Institutes of Health, U.S. Public Health Service

The purpose of this study is to measure the kinetics of phage coat protein synthesis in E. coli C infected with the phage \(\Phi X174\).
The method of assay is to follow the development of serum-blocking power (SBP) during the course of intracellular phage growth (DeMars, 1955). This consists of lysing the cells, by treatment with lysozyme and versene, at various times after infection and absorbing an antiserum, prepared against whole ØX, with these lysates for 16 hours at 48°C. The residual inactivating titer against ØX, is then determined by adding a host range mutant of ØX ("tester phage"), and measuring its surviving fraction after an additional 2 hours at 48°C. A standard curve is constructed by absorbing antiserum with known concentrations of a purified wild-type ØX stock and measuring survival of "testers." That the assay is valid is demonstrated by the fact that dilution curves of lysates of infected cells are essentially identical to the standard curve with purified virus.

Artificial lysates from uninfected cells produce no SBP. Lysates from infected cells produce detectable amounts of SBP as early as 5 minutes after infection. The rate of SBP production between the 5th and 30th minute after infection is 36 phage equivalents per minute. The time at which the SBP growth curve levels off has not yet been accurately determined. Although the SBP and intracellular phage growth curves are roughly the same shape, the ratio of SBP phage equivalents to intracellular phage, at any given time, is greater than unity. At 5 minutes (when intracellular phage is still in eclipse) this ratio is 100; it decreases during the interval from 5 to 15 minutes after infection and then remains constant at a value of 20 from 15 to 30 minutes. These results are for a multiplicity of infection of 0.1.

Reference:

30. RESEARCH UPON BACTERIOPHAGES ØX174 AND MS2

Investigators: Robert L. Sinsheimer, Mary C. Nagler, Shirley N. Guthrie, Shizuko Yagi, Marianne Lawrence
Support: National Institutes of Health, U.S. Public Health Service

Studies of the physiology of the ØX temperature mutant (which forms plaques at 30°C but not at 37°C) indicate that at 37°C there is no mature phage synthesis and no replication of viral DNA. Adsorption is normal and the block appears to be on the "penetration" process. Even at 30°C, penetration is slow and very variable so that attempts to initiate infection at 30°C and study the effect of transfer to 37°C at various times after infection have not been successful.

Attempts to identify a free single strand ØX DNA stage early in infection, by infection of a thymine-requiring bacterium in the absence of thymine, or by infecting D₂O-grown cells transferred to H₂O-medium plus chloramphenicol, have not been successful. When free DNA is found it has the properties associated with the replicative form (RF). These results suggest that formation of RF may accompany release of the viral nucleic acid from the protein shell.

Preliminary studies have been initiated to look for an RNA-dependent RNA polymerase in MS2-infected cells.

Publications:

31. RESEARCH ON BACTERIOPHAGE MS2

Investigators: James E. Davis, James H. Strauss, Jr., Robert L. Sinsheimer
Support: National Science Foundation
N. Public Health Service

Bacteriophage MS2 is an RNA-containing phage similar in many respects to the phage f2 described by Zinder and Loeb. It was isolated by Dr. Alvin Clark at Berkeley by virtue of its unusual property of attacking certain F+ strains of E. coli but not the corresponding F- strains.

MS2 lyses the F+ strain of E. coli 3000 with a minimum latent period of about 25 minutes, and yields a burst size of 10 to 20 thousand. Lysates of extremely high titer, often 10^13 or better, may be grown routinely. The amount of viral RNA produced in such a burst is comparable to the total amount of RNA synthesized by an uninfected cell in the same length of time.

Electron micrographs of negatively stained purified virus taken by Dr. A. J. Hodge at Caltech indicate a polyhedral structure of mean diameter of just over 200 Å with some evidence of internal structure.

Radiotracer experiments with P32 labeled phage are in progress to determine the fate of the infecting particle inside the host cell.

References:

32. PURIFICATION OF BACTERIOPHAGE MS2 AND OF MS2 RNA

Investigator: James H. Strauss, Jr.
Support: National Institutes of Health, U.S. Public Health Service

Procedures have been developed for the preparation in purified form of large amounts of bacteriophage MS2 and of its RNA. Host bacteria are grown in large quantities and infected with the virus; the resulting lysate is precipitated with ammonium sulfate. This precipitate is spun out and extracted with a small volume of liquid containing equal parts of buffer and freon. Greater than 50% of the virus is recovered in the aqueous layer. Further purification and concentration of the viral suspension is obtained by equilibrium banding in a cesium chloride density gradient. The final virus suspension obtained is homogeneous as judged by its sedimentation behavior in the analytical ultracentrifuge with use of ultraviolet optics.

RNA is prepared from the purified MS2 by phenol extraction of the phage suspension, followed by ethanol precipitations. The RNA thus prepared is homogeneous, again as judged by its sedimentation behavior in the analytical ultracentrifuge with ultraviolet optics.

Studies are currently in progress on the physical-chemical properties of MS2 RNA. Preliminary results indicate that this RNA may possess a somewhat
unusual hydrogen bonded structure as compared with other ribonucleic acids. Upon heating, this material shows an increase in optical density in the ultraviolet only at unusually high temperatures.

33. AN INFECTIVE RNA FROM BACTERIOPHAGE MS2

Investigators: James E. Davis, Shizuko Yagi
Support: National Science Foundation
National Institutes of Health, U.S. Public Health Service

Phenol treatment of either MS2 lysates or of purified virus leads to a sub­

viral particle which is infective to protoplasts of E. coli. The infectivity is completely removed by treatment with minute amounts of pancreatic RNase (10^{-2} y/ml). The infective particle can be banded in a CsSO_{4} density gradient at a density of 1.6, which is known to be the appropriate density for RNA in solutions of this salt.

Under conditions similar to those used for the infection of protoplasts with DNA from bacteriophage φX174, MS2 RNA appears to be several orders of magnitude less infective than corresponding amounts of φX174 DNA. Efforts are now being directed toward optimizing the conditions for infection with MS2 RNA.

References:

34. LOW SPEED ANALYTICAL EQUILIBRIUM CENTRIFUGATION

Investigator: Edward A. Carusi
Support: National Institutes of Health, U.S. Public Health Service

Experiments have been undertaken which attempt to extend the useful range of equilibrium centrifugation beyond its existing practical limit of molecular size. Technical problems currently limit the method to molecules having weights of about 10^{5} or less. In principle it should be possible to extend this limit by an order of magnitude and thus include larger molecules of biological interest, e.g. proteins, nucleic acids, and small viruses.

The technical problems encountered are those concerned with stable operation of the analytical ultracentrifuge at low speeds, i.e. less than 1,000 rpm. At these speeds rotor precession becomes quite serious and fluctuations in the angular velocity of the rotor by as much as 10% about a desired mean are encountered.

We have found it possible to stabilize the rotor against precession at speeds as low as 500 rpm by immersing the lower end of the unmodified analytical rotor, equipped with a standard thermistor element, in a damping fluid. For this purpose silicone fluids having viscosities of 1,000 to 5,000 cstk. have proved most useful. It is necessary however to adjust the viscosity of the fluid for a given range of speed to avoid turbulence in the fluid. It was also necessary to redesign the dimensions of the cup containing the silicone fluid; probably because the smaller cup provided by the manufacturer is not necessarily placed concentrically with the rotor shaft.

With these modifications, preliminary experiments have been conducted with a virus sample (MS2). The results were encouraging and further experiments are in progress.
35. BASE COMPOSITION OF AMINO ACID CODING TRIPLETS FROM RNA VIRUSES

Investigators: John A. Petruska, Alan J. Hodge
Support: National Institutes of Health, U.S. Public Health Service

The coding unit in RNA that determines an amino acid residue in proteins is believed to be a triplet of the nucleotides uracil (U), cytosine (C), adenine (A) and guanine (G). Provided these four nucleotides are nearly randomly distributed, one can calculate the probability of occurrence of each possible triplet from the overall composition of the RNA. If nucleotide A makes up a fraction \( f_A \) of the total, the probability of finding the triplet ABC is simply \( f_A f_B f_C \). In the absence of any marked selection, then, one might expect to find in the proteins encoded by the RNA that the amino acid with a coding triplet of composition ABC will make up a fraction \( k f_A f_B f_C \) of the total amino acid residues, where \( k \) is some proportionality constant.

This argument has been applied to six different RNA viruses whose compositions are known, assuming that the part of the viral RNA that codes for the coat protein has a composition like that of the whole. By comparing the variation of the amino acid contents with the triplet probabilities calculated from the RNA composition, it was determined (by least squares) which triplet compositions gave a "good fit" for each amino acid; the chosen criterion for a "good fit" being that the average deviation from direct proportionality be less than 20%. For each of over half the twenty amino acids one or more "good-fitting" triplet compositions were found. In most of these cases one was like that found by the recent studies in the laboratories of Nirenberg and Ochoa on the cell-free synthesis of polypeptides stimulated by artificial RNA's; namely, UUU for phenylalanine, UUG for valine, UUC for leucine and serine, UCG for alanine, UCA for threonine, UAG and UAA for aspartic acid and asparagine.

36. POSSIBLE NATURE OF THE AMINO ACID CODE

Investigator: John A. Petruska
Support: National Institutes of Health, U.S. Public Health Service

A considerable amount of information has been accumulated over the past year, as a result of intensive work in a number of laboratories, regarding the composition of the amino acid coding units in RNA and their interrelationships. An attempt is being made to deduce from these data the structural framework of the code. Within the limitations of the data, the code appears to have many of the properties of a doublet code. However, there being only 16 unique doublets possible with the 4 different nucleotides present in RNA (U = uracil, C = cytosine, G = guanine, A = adenine) and at least 20 amino acids to be accounted for (with some almost certainly having more than 1 code word), a simple doublet code does not appear likely. More likely a triplet code is involved, as has been believed for many years.

It is interesting to note that a triplet code can be constructed, consistent with the present data, which is not far removed from a doublet code but which affords the opportunity of using all 64 possible triplet code words. One need only assume that in the triplet 1 of the 3 positions is not as highly specified as the other 2. For example, while a code word for a certain amino acid must begin with UG it may end with either of the two pyrimidines (U, C) or either of the two purines (A, G), i.e. in the third position the two pyrimidines may be essentially equivalent and the two purines may be essentially equivalent. Thus the code word UGU would be equivalent to UGC and UGA to UGG. In the following tabulation it is shown how the triplets may be assigned to the various amino acids on the basis of such a code. Heading the rows and columns are the nucleotides in the two highly specified positions in the triplet, arbitrarily assumed to be the first two positions. When the third position is occupied by a pyrimidine (U or C), the amino acid encoded is the upper of the pair entered; when by
leucine

a purine (A or G), the lower. Thus in the U row and C column the serine means that UCU and UCC stand for leucine, UCA and UCG for serine.

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37. THE FOLDING OF POLYPEPTIDE CHAINS IN GLOBULAR PROTEINS

Investigators: John A. Petruska, Alan J. Hodge, Richard Burger
Support: National Institutes of Health, U.S. Public Health Service

The polypeptide chain in globular proteins may be pictured crudely as a fairly tightly-wound helix (with only a few residues per turn) bent in a number of places to fit compactly into a roughly spherical volume. The fundamental helical pattern between the bends, where it has been identified, appears to be that of the right-handed α-helix, with 3.6 residues per turn. From 30 to 80% of the total residues may have this pattern, depending on the protein.

Except in the case of a few proteins, such as myoglobin and hemoglobin, whose structures have been unraveled by X-ray crystallography, it is not known which regions have the intact α-helices and which the bends. Knowing the amino acid sequence, however, one can get some idea from theoretical considerations. It is reasonable to suppose that from the N-terminal to the C-terminal end of the chain each quartet of amino acid residues will have the oxygens of its peptide amides bonded to the hydrogens of the peptide amides of the succeeding quartet, in the α-helical manner, unless the latter contains residues whose side chains interfere with this orderly hydrogen bonding. Proline is definitely such a residue. Its side chain being fused into a ring with its peptide amide, it lacks the hydrogen needed to propagate this hydrogen bonding and sterically prevents two of its neighbors from locking into the helix as well. Hence a break in this α-helix in the vicinity of a proline is virtually certain. Residues with hydrogen-bonding side chains, such as serine, threonine, glutamine and asparagin, when present in clusters, are also expected to interrupt the α-helix. The side chains of these residues can hydrogen-bond to nearby peptide amides and prevent them from going into the helix. On the basis of these considerations and others, plausible models have been derived for a number of proteins of known sequence, e.g. ribonuclease, TMV protein and cytochrome C.

In order to test the validity of these considerations and resultant models, certain experiments are being attempted. By using specific enzymes, attempts are being made to clip out those regions that are predicted to contain α-helix and to measure their helicity by optical rotation analysis. Preliminary experiments on ribonuclease, which is expected to have two 15 to 20 residue α-helical regions, using the enzyme trypsin, have as yet not given conclusive results. However, further studies continue with techniques, such as use of carbon disulphide to shield lysine residues from trypsin attack and thereby obtaining longer peptides.
38. **ULTRACENTRIFUGAL STUDY OF THE FORMATION OF COLLAGEN FIBRILS**

Investigator: John H. Fessler  
Support: National Institutes of Health, U.S. Public Health Service

Submicroscopic particles which are themselves aggregates of numerous macromolecules are still difficult to study by existing physico-chemical techniques. The initial success of studying collagen fibrils in ultracentrifugal density gradients was reported in Biology 1961 and prompted thorough exploration of the system. The biological problem is how collagen fibers are formed by aggregation of the macromolecular unit, called tropocollagen. The physico-chemical problem is the relationship of these 3,000 Å long tropocollagen rods to solvent and to each other, particularly as simply a change of temperature of some 10 to 20°C can reversibly precipitate fibrils out of solution. The present ultracentrifugal analysis has shown that when the tropocollagen molecules change from solution to fibril there is a definite increase of buoyant density which is independent of temperature and of the proportion of material which has changed. Thus solution and fibrils behave as separate phases. This poses the problem as to how large an aggregate of macromolecules has to be for it to behave as a separate phase. Investigation of the earliest detectable stages of fiber formation showed aggregates in the range of fifty macromolecules which have a density closely similar to that of the fibrils which are subsequently formed. These small aggregates are thus at the threshold of behaving as a separate phase and probably act as nuclei about which fiber formation occurs. Investigation of the reverse process, fiber dissolution on cooling, showed that the density of the system reverts to that of tropocollagen molecules in solution before dispersion proper of the aggregate occurs. Thus buoyant density is a measure of the state of the component macromolecules and the shifts of density must indicate changing, differential interaction of macromolecules and solvent. This has been shown to occur in D₂O as well as in H₂O and using various alkali halides to produce a density gradient. The results are consistent with a loss of "bound water" from the tropocollagen molecule when it changes from solution to fiber. The experiments show that salts are also bound to collagen, and while a change of bound salt could also cause a density shift, the results do not indicate this to be the most important factor.

39. **STUDIES ON SOLUBLE COLLAGEN**

Investigators: John H. Fessler, Nancy A. Martin  
Support: National Institutes of Health, U.S. Public Health Service

Although mature collagen fibers are insoluble tensile units, they are synthesized from macromolecules in solution. The question is: how is the insoluble condition achieved? From the results of the previous report it can be seen that dissolution of collagen fibers would occur in any skin, whose temperature is sometimes well below body core temperature, unless free interaction of extracellular fluid and the constituents of collagen fibers is hindered in some way. Metabolically older collagen is known to be less soluble.

Guinea pig skin was extracted at 4°C with phosphate buffer pH 7.3, physiological ionic strength, and the dissolved collagen was purified. From this were separated a fraction A and a fraction C. Both fractions formed typical collagen fibers on warming their solutions to 37°C, but only the fibers of fraction A dissolved readily again on cooling. A previous pulse labeling experiment, in vivo, had indicated that these fractions have the same metabolic age. Full amino acid analysis showed identical composition of the two fractions. Thus no evidence could be found that the change from the soluble condition towards the insoluble state is accompanied by the removal of some protective group. This suggests that the change in solubility must be due to some change in internal structure of the tropocollagen molecule.
Tropocollagen molecules are believed to consist each of three peptide strands, which on denaturation to gelatin are separated to various degrees. The resulting gelatin can be resolved into α, β, and γ fractions in the ultracentrifuge, and these are believed to contain, respectively, one, two and three peptide chains per molecule. It was found that gelatin prepared from collagen whose fibers readily re-dissolved on cooling consisted primarily of α fraction, while less soluble collagen also contained an appreciable amount of β fraction. Thus, decreasing solubility of tropocollagen seems to be associated with increasing internal linkage of peptide strands.

Separation of the three gelatin fractions by coerecvation with detergent has been studied with Drs. D. S. Jackson and J. Scott.

40. ELECTRON MICROSCOPE STUDIES OF TROPOCOLLAGEN STRUCTURES

Investigators: Alan J. Hodge, John A. Petruska
Support: National Institutes of Health, U.S. Public Health Service

The three-stranded, rod-like tropocollagen (TC) macromolecule can form a variety of crystalline aggregates under different conditions. In native-type fibrils adjacent macromolecules are displaced relative to one another by multiples of a fixed distance X, approximately one quarter the macromolecular length. This "quarter stagger" leads to a repeating band pattern with a period $X = 640 \text{ to } 680 \text{ Å}$ depending on the degree of hydration. In other aggregates, produced by adding foreign agents such as adenosine triphosphate, this displacement is entirely eliminated. The macromolecules pack together side by side in perfect register; the result being single segments of length equal to the macromolecular length (SLS) or fibrous structures with a repeat period of approximately this length (FSLS).

The SLS segments are of particular interest because they afford an opportunity to study a number of characteristics of the individual TC macromolecules. By virtue of the perfect, in-register packing, each staining locus on the macromolecule can be seen as a sharp line in the electron micrograph. The positions of non-polar, acidic and basic sites can thus be determined with considerable accuracy. Also, by using the negative contrast technique, i.e. by partially submerging the segments in an electron-opaque substance such as neutralized phosphotungstic acid, one can bring the ends of the macromolecule into relief, thereby establishing the actual length of the macromolecule and learning something about the nature of its ends.

By negative contrast it has been established that the macromolecular length is $4.40 \pm 0.02$ times $X$, the repeat period in the native-type fibrils. This means that in joining end to end to form the fibrils the individual TC macromolecules overlap by about 9% of their length. Studies are now under way to determine the character of this overlap region. In negative contrast the SLS segments show one end to be sharper than the other. This suggests that at one end the macromolecule may be single-stranded, at the other end double-stranded, as would be the result if all three strands were of length $4X$ with one strand being displaced relative to the other two by $0.4X$. Such an arrangement, which is presently being investigated, would give rise to a strong, three-stranded splice in the end overlap region in the native-type fibrils. The type of arrangement, which is also consistent with the effects of proteolytic enzymes on the polymerization behavior of TC, had already been postulated on other grounds (see reference below).

References:

Hodge, A. J. and F. O. Schmitt. Chapter in "Macromolecular Complexes."

41. REARRANGEMENT OF MACROMOLECULES IN PLASTIDS

Investigators: Albert Kahn, Alan J. Hodge
Support: National Science Foundation
National Institutes of Health, U.S. Public Health Service

The development of macromolecular structures in plastids of dark-grown bean plants during etiolation and subsequent illumination has been described by Kahn and collaborators. Only potassium permanganate fixation was used extensively in this electron microscopic investigation of lipo-protein structures. Transformation of vesicles to tubes ordered in a three-dimensional array is the most striking feature of dark development. When etiolated bean leaves are lighted, these tubes, which have a diameter of about 200 Å, are rapidly transformed into irregular vesicles in close correlation with the conversion of protochlorophyll to chlorophyll. Upon further illumination the vesicles become arranged in concentric layers, flatten, and pair to form grana.

The present study, utilizing a variety of fixation and staining techniques, allows the comparison of images of the described lipo-protein structures gained by diverse methods. Plants are grown and treated under the controlled conditions of the Earhart Laboratory. The intention is to assess the contributions of pretreatment, fixation, and staining to electron microscopic images of initially identical structures. In this way it is hoped to begin the characterization of the macromolecules under examination and the forces which determine their patterns of ordering.

Reference:

42. STUDIES ON THE STRUCTURE OF BACTERIOPHAGES

Investigator: Alan J. Hodge
Support: National Institutes of Health, U.S. Public Health Service

Preliminary electron microscopic evidence using the negative contrast technique suggests that the RNA of MS2 bacteriophage does not entirely fill the core volume enclosed by the protein shell (i.e. virion). When the virus is treated with formaldehyde prior to mixing with sodium phosphotungstate (PTA), electron micrographs show little if any penetration of the internal core volume by the relatively large PTA ions. On the other hand, uranyl ion can penetrate into the interior of the virus particles and is apparently bound by a peripheral zone immediately under the external protein shell under conditions in which the external environment is depleted of uranyl ion. The results suggest that the RNA content of the bacteriophage particles may be localized in a peripheral zone of the core, a situation unlike that so far found for other viruses. Confirmation must await a correlation between the calculated core volume and the molecular weight of MS2 RNA. Parallel studies on the DNA phage ΦX174 indicate that little if any of the internal volume is unoccupied, or that the protein shell does not permit penetration of relatively large ions.
43. PHYSICO-CHEMICAL PROPERTIES OF SEA URCHIN FERTILIZIN

Investigators: Albert Tyler, Edward A. Carusi
Support: National Institutes of Health, U.S. Public Health Service

Chemical properties of the fertilizins (egg-receptor substance for sperm) of sea urchin eggs have been discussed in previous reports. In several species of sea urchins the fertilizins are characterized as sulphated glycoproteins having approximately equal amounts of amino acids (of some 14 kinds) and sugars (usually 2 kinds). Previous studies by sedimentation velocity and diffusion methods with the fertilizin of Arbacia punctulata, have given a molecular weight of about 300,000 and an axial ratio of 20:1. The sedimentation coefficient for another echinoid Echinarachnius parma was in the same range.

Currently sedimentation velocity studies have been made with purified preparations of the fertilizin of the sea urchin Lytechinus pictus, which has been employed largely in the immunization of rabbits to produce antisera that block development of the fertilized egg, as noted below. The preparations show a single, hypersharp, sedimentation boundary. The sedimentation coefficients obtained at various dilutions extrapolate to an infinite dilution value of $S_{20} = 20$. This indicates a much higher molecular weight for this material than for Arbacia and is consistent with other properties of the gelatinous coat (composed primarily of fertilizin) of the egg in this species, such as its slower dissolution after the eggs have been shed into sea water. Studies on the products of degradation obtained by physical and enzymatic means are in progress, with reference also to the conversion of the molecule into a non-agglutinating (univalent) form.

Reference:


44. INHIBITION OF CLEAVAGE AND DEVELOPMENT BY ANTISERA PREPARED AGAINST FERTILIZIN AND OTHER MATERIALS OF SEA URCHIN EGGS

Investigators: Albert Tyler, Richard L. Clark
Support: National Institutes of Health, U.S. Public Health Service

As noted in previous reports (J. Reprod. and Fertil. 2:473-506, 1961) antisera prepared in rabbits against the fertilizin of sea urchin eggs can block development of the fertilized egg. During early cleavage the effect is manifest by an
inhibition of cell division. In strong antisera the inhibition is accomplished in a period of time that corresponds to about one-fourth of the division cycle. There is no evidence for particularly sensitive stages, and there is no other markedly visible deleterious effect on the egg until cytolysis sets in several hours later.

Antisera against sperm and against other tissue of adult sea urchins do not have this effect. Antisera against extracts of eggs that have been deprived of the gelatinous coat can block cleavage but generally such antisera are not as effective as the antisera prepared against fertilizin. The gelatinous coat of the egg is primarily composed of fertilizin but the latter is according to present evidence also a component of the plasma membrane (not the vitelline membrane) of the egg and is not removed by the mild procedures used for dissolving the gelatinous coat. The cleavage-blocking action of extracts of eggs that have been freed of their gelatinous coat might, then, be attributable to the presence of fertilizin (from the plasma membrane) in such extracts. Experiments in which the anti-developmental action of such antisera is removed or diminished by absorption with fertilizin indicates that the cytotoxic action is exerted primarily by the antifertilizin antibodies. The absorbed sera still possess high titers of antibodies against many other constituents of the egg, as shown on Ouchterlony plates. It would appear, then, that the antibodies against these other constituents do not penetrate the living egg or, if they do enter, they exert no cytotoxic action. Experiments with fluorescent-labeled antibodies support the former proposition. It appears then, that to be effective cytotoxic agents for developing sea urchin eggs the antibodies must be directed against a surface constituent of the cell. Also, it appears that antigenic structures of fertilizin which is on the plasma membrane of the unfertilized egg persist on the surface of the cells of the developing egg.

Publication:

45. RETRACTION OF MICROVILLI UPON FERTILIZATION IN EGGS OF URECHIS CAupo

Investigators: Albert Tyler, Kerry Donovan, Carol Dobrikin
Support: National Institutes of Health, U.S. Public Health Service

As part of a general theory of fertilization it has been proposed that initial specific adherence between egg and sperm occurs between receptors (fertilizin and antifertilizin respectively) on their plasma membranes, that the plasma membrane of the egg protrudes in the form of microvilli through pores in an overlying vitelline membrane and that all microvilli are retracted shortly after attachment of the fertilizing sperm as the vitelline membrane elevates to become the fertilization membrane and as cortical vesicles release their contents into the perivitelline space.

Electron microscopic observations by several investigators have shown the presence of microvillous extensions of the plasma membrane of eggs of many species of animals. These are figured as extending into the vitelline membrane but, generally, they have not been reported as penetrating the vitelline membrane completely; nor has their appearance at various times after fertilization been closely followed.

Investigations of the microvilli have been initiated with eggs of the gephyrean worm Urechis caupo. The preliminary results show that microvilli do penetrate completely through the vitelline membrane of the unfertilized egg. The frequency with which "protruding microvilli" are found varies with different preparations. It seems likely that in all cases there is some contraction of the surface, the extent of which varies with different methods of fixation, and that possibly all or most of the
microvilli protrude. This is to be explored further by use of low temperature and other methods of fixation. The electron micrographs also show the retraction of microvilli upon fertilization. The time course of the events is being studied in relation to their role in the block to polyspermy as proposed in the above-mentioned hypothesis.

Publications:


46. INITIAL EXPLORATION OF THE POSSIBILITY THAT PARENTAL STRAIN SPLEEN CELLS MAY DEVELOP AS TUMORS IN F₁ HYBRID HOSTS

Investigators: Albert Tyler, Delores R. Williams
Support: National Institutes of Health, U.S. Public Health Service

In the publications listed below a theory of cancer has been developed that assumes the initiating event to be the loss or inactivation of a gene or genes at one or more of the histocompatibility (H) loci of the animal. The affected cell and its descendants would thus come to lack an H-antigen, or antigens, possessed by other cells of the body. If immunologically competent it would react to subsequent exposure to this antigen, which other cells are probably releasing into the surroundings continuously at an irregular rate. This response would be proliferation and production of antibodies (circulating, or cell-bound, or both). One of the expectations of this hypothesis is that, under appropriate conditions, tumors should develop when tissues containing immunologically competent cells are transplanted from a parental pure-line animal to an F₁ hybrid host. As an appropriate condition for this effect it was supposed that it might be necessary to provide space in the host tissues where the transplanted cells could settle down. For this purpose, in the initial experiments, the hosts were treated with cortisone since its effectiveness in promoting metastases implied that it might act, like X-rays, in providing "cell-vacancies" in the host tissues.

In these experiments dissociated spleen cells of 216 mice of strain A/J, strain C57BL and their F₁ hybrid were injected subcutaneously into an equal number of young adult mice of these same strains, isologously and homologously. Half of the hosts received cortisone (5 mg hydrocortisone hemisuccinate, subcutaneously) on the previous day. Of the 48 F₁ hybrid mice that received parental spleen cells, tumors have arisen in five (three A/J into F₁ and two C57 into F₁) at 9 to 12 weeks after the injections. Only one of these was in the cortisonized group. After an initial period of rapid growth for about a month three have regressed and two (A/J cells into F₁) have persisted with little change for 3 months. It is planned to test the transplantability of the persistent tumors and to explore this problem further with other methods.
Publications:


47. A HOMOGRAFT REACTION AGAINST F1 HYBRID CELLS THAT AFFECTS HOST TISSUE

Investigators: Albert Tyler, Delores R. Williams
Support: National Institutes of Health, U.S. Public Health Service

Among the various controls of the experiments described above a striking reaction was observed in the situation where the parental strain animals received (subcutaneously) F1 hybrid dissociated spleen cells. At about 6 to 9 weeks after the injection the skin at the injection site broke down and sloughed off completely so that the underlying muscle layer was exposed. The area involved varied considerably in different animals from a barely visible spot to a diameter of some 2 centimeters. The reaction occurred abruptly without any marked preliminary sign other than a moderate swelling in the area several days in advance. The animals with the most severe reactions (10/24) failed to heal and died after periods ranging from 1 to 4 months. In the others healing was apparently complete. None of the other controls showed this effect, except that the homologous combinations (A/J cells into C57BL and C57BL cells into A/J) showed slight to moderate swelling in the injection area, at 3 to 5 weeks, which soon regressed.

As a tentative explanation it is proposed that the "isohomologous" spleen cells, being not quite as foreign to the host as the homologous cells are able to persist longer in the host and settle down interspersed among the host's skin cells. Then, when they are later destroyed in the homograft reaction the associated skin cells of the host are sloughed off along with them. The host's skin cells may also be affected by secondary products of the homograft reaction.

Gene-dose effects have been noted in a number of situations in tissue-transplantation (cf. Kallman, 1960; Billingham and Silvers, 1961). In the present instance one interesting feature is the superficial resemblance of this effect to the separation of the placenta in parturition. Also, it may be noted that the fetus is genetically "isohomologous" with respect to the mother.

This finding may, then, offer some support to theories of parturition that have been proposed on the basis that separation of the placenta resembles a homograft reaction. It could account also for the long delay and abrupt occurrence of parturition. However, the fact that inbred animals can have female young would still be enigmatic on this basis. Possibly differences in the time of development of various histocompatibility antigens in different tissues, and/or in the development of the immune system of the fetus (cf. Dancis, et al., 1962, for evidence that the fetal placenta is immunologically competent) may provide a basis for further exploration of such hypotheses.
References:


48. IMMUNOLOGIC ASPECTS OF HUMAN INFERTILITY

Investigators: Nicholas Nakabayashi, Albert Tyler
Support: National Institutes of Health, U.S. Public Health Service

Infertility in the human may be due to a number of causes, some of which, such as anatomical abnormalities, hormonal imbalance, and certain other physiological disturbances, can be identified. However, in most cases the exact cause of the infertility is not readily determined. Recently, there has been some evidence to indicate that an auto-sensitization to sperm may occur in infertile males, and sperm agglutinating antibodies may appear in the blood serum and seminal secretions.

In extensive study conducted by Drs. Rümke and Hellinga in Amsterdam, sperm agglutinins were detected in the blood sera of 4% of sterile men. They also noted that obstruction of the seminal tubules with normal formation of sperm occurred in approximately one third of the positive cases. It was the opinion of these investigators that the absorption of the spermatozoa from the tubules brought about the formation of the sperm agglutinins.

In a collaborative study with Drs. Rümke in Holland, Rao in India, Segal in New York, and E. T. Tyler in Los Angeles, similar results were obtained with the blood sera collected from sterile individuals in Los Angeles. In the analyses, it was also noted that the sperm agglutinin titers of the sera varied with test cells from different donors. The basis of this variability is being explored and methods that may provide a more uniform system are being studied.

In order to explore further the antigenic structure of spermatozoa, rabbits have been immunized against human sperm, seminal fluid, and blood serum. Antisera against all of these antigens agglutinated spermatozoa, those against blood serum being of relatively low titer. Physical and chemical extraction methods are being employed to obtain and characterize the antigenic components of sperm responsible for the formation of the agglutinating antibodies. For further determination of the location and specificity of the antigenic substances in the spermatozoa, globulin fractions of the antisera have been labeled with fluorescein isothiocyanate and these are being studied by fluorescence-microscopy.

A general review of work on "Immuno-Reproduction" has recently been published.

Publications:


Reference:


49. REPEATED FERTILIZATION OF FERTILIZED RAT EGGS AFTER TREATMENT WITH VERSENE (EDTA)

Investigator: Lajos Piko
Support: Lalor Foundation

Tyler and Schultz (1932) were the first to show in Urechis caupo that repeated fertilization was possible. More recently, demembranated sea urchin eggs could be refertilized even at the two-cell stage particularly if exposed to calcium- and magnesium-free media.

In mammals, there is no good evidence of repeated fertilization in spite of the natural occurrence, in several species, of "supplementary spermatozoa" in the perivitelline space. It is found, however, that versene treatment can provoke a later penetration into the vitellus of these spermatozoa.

Sherman rats were mated towards the end of estrus. The right tube was exteriorized under Nembutal narcosis 2.5 to 3 hours after mating and 1 μl of 1:500 M versene was injected into the ampulla; the left side served as control. The females were sacrificed 20 to 50 hours later and the eggs examined by phase microscopy before and after acetocarmine staining.

Of 399 eggs from 38 females, most were fertilized, and developed normally on both sides. The incidence of "typical" dispermy (simultaneous penetration of two sperm into the cytoplasm) did not differ significantly (2.8% experimental side; 4% control side). But the number of eggs with supplementary spermatozoa increased twofold on the treated side (46.2% vs. 22.6%; P<0.001) and one fifth of such eggs contained in their cytoplasm spermatozoa of a secondary penetration. The refertilizing sperm head either developed into an extra nucleus or, more often, degenerated and showed up as a darkly staining mass up to the time of second cleavage. Judging from the appearance of the sperm, refertilization occurred at various times up to the first cleavage.

These observations suggest that the so-called "vitelline block to polyspermy" (prevention of the penetration of more than one sperm into the egg cytoplasm) in mammals has basically an underlying mechanism similar to that described in the sea urchin and some other lower animals.

Publications:


FERTILIZATION AND ASSOCIATED PHENOMENA IN THE CHINESE HAMSTER

Investigator: Lajos Piko
Support: Lalor Foundation

The Chinese hamster Cricetulus griseus has several unique features as a laboratory animal (cf. Yerganian, 1958). Its low chromosome number \(2n = 22\) and well defined karyotype make it particularly suitable for cytogenetical research. Data on its reproductive physiology, however, are lacking.

Stock originally obtained from G. Yerganian, has been maintained on an artificial day-night schedule with 14 hours' light and 10 hours' darkness. Estrous females were selected by vaginal inspection and added to the males according to one of the following schedules: 1) One hour after the beginning of the dark period when mating usually took place in 1 to 2 hours ("normal matings"); 2) at the end of the dark period ("delayed matings"). The females were then sacrificed at different intervals for the study of sperm transport, ovulation, sperm penetration, pronuclear development and cleavage (by phase microscopy).

Ovulation is spontaneous and it occurs, in the above conditions, during the last 2 hours of darkness. The eggs are at metaphase II when ovulated. In normally mated females, the ampullae are usually void of sperm at the time of ovulation; at the time sperm numbers are also low in the middle third of the tube but there are large numbers (from several hundred to about two thousand) in the lower third.

After normal matings, 610 eggs were obtained from 89 females, ranging from pre-fertilization stages to the 64-cell blastocyst (6th day after fertilization). Sperm penetration takes place 2 to 4 hours after ovulation in the lower part of the ampulla. The cleavage rate, about 24 hours per stage during the first 6 days of development, is among the lowest in laboratory rodents.

Some peculiar features of fertilization are the behavior of the fertilizing sperm tail (it remains in the perivitelline space in most eggs but may occasionally penetrate into the cytoplasm and also may be entirely missing from the egg) and the high incidence of female subnuclei at the pronuclear stage (about 13%). Dispermy occurred in at least 1.2% of fertilized eggs; such eggs seemed to undergo a normal early cleavage. The frequency of so-called "giant" eggs (double the normal size) was a relatively high 0.7%. Direct evidence was obtained that "giant" eggs contain twice the normal complement of chromosomes and may develop, after fertilization by one spermatozoon, into a triploid embryo. (One unfertilized egg was found that had a diploid number of dyads; and one 8-cell stage was found that showed triploid metaphase.)

After delayed matings, 241 eggs were obtained from 36 females. The only major effect appeared to be a considerable decrease in the fertilization rate (to about 50% as compared to about 90% after normal matings). Those eggs which were fertilized, however, appeared normal. There was no evidence of second polar body suppression and the frequency of dispermy remained unchanged.

Publications:


51. STUDY OF THE ANTIGENIC STRUCTURE OF CHINESE HAMSTER SPERM

Investigator: Lajos Piko
Support: Lalor Foundation

As an initial step toward an immunological analysis of fertilization, antisera were produced against sperm and certain sperm constituents and other materials of the Chinese hamster. To this end, adult female rabbits were injected with the following antigenic materials combined with the complete Freund adjuvant:

1. Whole washed epididymal sperm.
2. First supernatant of epididymal washings ("sperm plasma"); dialyzed and concentrated by pervaporation (through the dialyzation tube).
3. Alkaline extract (by N/100 NaOH) of washed epididymal sperm ("acrosome extract"); dialyzed and concentrated by pervaporation.
4. Sperm residue of alkaline extraction.
5. Spleen homogenate.

Antigen-antibody reactions were studied by the agar gel diffusion method, using fresh antigens prepared with additional care to keep cross-contamination of fractions as low as possible.

Among the sperm materials examined, whole washed epididymal sperm appears to have the most complex antigenic structure. Extracts of this material prepared by freeze-thawing produced at least 10 precipitation lines with the corresponding antiserum in repeated tests. Tested with the same antiserum, the "acrosome extract" produced 6 lines while the "sperm plasma" gave 5 lines. However, "sperm plasma" antigen produces with anti-sperm-plasma antiserum a heavy precipitation line due to a slowly diffusible antigen which may well be characteristic for this material. Antisera against whole sperm and "sperm plasma" each gave 2 weak, apparently identical, lines with spleen antigen.

Preliminary tests showed that the antiserum against epididymal sperm of the Chinese hamster cross-reacted strongly with the frozen-thawed extract of epididymal sperm of the golden hamster and, to a lesser degree, with that of the mouse and the rat.

Further investigations are planned as to the identification and localization of sperm antigens and their possible role in fertilization.

General Reference:


52. SEA URCHIN EMBRYONIC ALKALINE PHOSPHATASE

Investigator: Paul C. Denny
Support: National Institutes of Health, U.S. Public Health Service

Alkaline phosphatase in the developing sea urchin Lytechinus pictus has been investigated with respect to activity at various stages, ionic requirements and intracellular localization. The activity per embryo remains the same in the
unfertilized egg, fertilized egg and cleavage stages. At a time just prior to gastrulation (about 10 hours after fertilization) the activity begins to rise and increases 300 times during the next 60 hours over the activity in the cleavage stages. Embryos that are fed show a greater and more prolonged increase in activity than the forementioned non-fed embryos.

The optimal ionic strength for enzymatic activity shows a wide peak at 0.6 to 1.0. The divalent ions, calcium and magnesium, show an additional optimum at 0.02 to 0.07 M. Versene at concentrations of 0.0001 M and higher shows definite inhibition of activity.

Intracellular localization of alkaline phosphatase in the 72 hour embryo has been studied employing the differential centrifugation method. In washed preparations the major portion of the total activity of the initial homogenate was found in both mitochondrial and microsomal fractions with less than 5% in the nuclear and cell debris fraction and less than 2% in the final supernatant. The activity could be released from these fractions by treatment with 1% sodium desoxycholate.

53. MECHANISMS OF FLAGELLAR MOVEMENT

Investigator: Charles J. Brokaw
Support: Max C. Fleischmann Foundation of Nevada
National Institutes of Health, U.S. Public Health Service

Cilia and flagella use energy provided by adenosine triphosphate (ATP) to execute bending movements, and they therefore share with muscle the ability to transform chemical energy into mechanical energy. The bending movements are also periodic and are coordinated along the length of the flagellum, so that smooth bending waves pass along the flagellum, resulting in a propulsive thrust. All these features can be studied with flagella isolated from the orange alga Polytoma uvella by stirring in 50% glycerol at low temperatures. In addition, the preparations of isolated flagella are pure enough for biochemical study.

Analysis of the movement of the isolated flagella, by means of photographic recording, is continuing. Methods for extracting and purifying the protein possessing the ATP-splitting activity of the flagella have been developed, so that detailed characterization and localization of the macromolecular components of flagella can now be attempted.

Reference:


54. NEGATIVE STAINING OF POLYTOMA FLAGELLA

Investigator: Vincent C. Hascall
Support: National Institutes of Health, U.S. Public Health Service

Although electron microscopy of whole flagella has been less fashionable since the advent of thin-sectioning techniques, the simplicity of this type of preparation permits the trial of a large variety of treatments which may informatively alter the appearance of flagella structures. Negative staining by phosphotungstic acid clearly shows the two subfibers in each of the nine peripheral fibers of flagella of whole cell suspensions of Polytoma uvella. One of the two subfibers is usually less dense, and more frequently partially disintegrated, than the other; this difference is consistent along the length of the flagellum. Faint bridges between the peripheral fibers were seen, with a regular longitudinal spacing of about 800 Å.
Flagella isolated and stored for several days in 50% glycerol at -20°C had lost the bridges, and frequently the central fiber pair and the weaker subfiber of the outer fibers had also disintegrated.

The fibers of flagella treated with 0.1% lead nitrate appeared to be composed of a number (10 to 20?) of parallel longitudinal filaments.
The way in which the development and differentiation of an organism is controlled by the genes is a central problem in biology. We are studying it with Drosophila by making use of a series of mutations which cause the fly to be transformed in various degrees from a two-winged, six-legged organism towards a four-winged, eight-legged one. Thus far, we have found that a total of five different genes are directly involved in the control of these body segment transformations.

A variety of methods of analysis are employed. In the first place by making crosses between the various mutant genes and studying the progeny it has been possible to show that the five genes are clustered together within a very short segment of the third chromosome. From such studies it is possible to infer that each gene in this so-called "bithorax" cluster controls a specific step in a series of five reactions. Moreover, the order in which at least 3 of the 5 genes control the reactions is the same as the order in the chromosome. This can be represented as follows: C → D → E → F, where c, d and e signify three of the genes and D, E, and F the respective hypothetical products of those genes.

Not only is it possible to study interactions between the five different genes by deriving different combinations but it is also possible in some cases to study a variety of different kinds of mutations or "alleles" of the same genes. Until recently the analysis has been handicapped because only a single and very extreme mutant form of the "c" gene was known. A new mutation discovered by Dr. Hans Gloor, who kindly has placed it at our disposal, has now been tested for its position in the series and has been found to be a probable new allele of the "c" gene and one that is much less extreme than the original one. Tests show that the new mutant allele is also less extreme in its inactivation of the normal "d" and "e" genes than is the original "c" mutant and this is in accord with the postulated sequence of gene-controlled reactions.

A second method of analysis is to construct mosaic individuals in which part of the body contains a set of normal genes and the remaining part contains the mutant genes. Somatic elimination of special ring chromosomes is the mechanism used to produce these mosaics in the present case. In this way several three-winged flies have been produced as well as numerous flies with much smaller areas of mutant tissue. Careful study of these specimens indicates that the mutant and normal tissues develop independently or "autonomously." This is taken to mean that the chemical substances involved in effecting the body segment transformations do not diffuse appreciably from cell to cell.
In another approach, X-rays are being used to produce additional mutant alleles belonging to the bithorax cluster. Most of such changes prove to be also accompanied by violent rearrangements of the chromosomes and are of limited usefulness compared to the available spontaneous mutants. However, the X-ray studies have produced a new set of mutations which act as specific suppressors and enhancers of the bithorax mutants belonging to the bithorax cluster. These may be said to constitute a separate class of genes which act in an indirect and unknown way on the primary reactions controlled by the bithorax cluster. Thus far only the localization of these enhancers and suppressors has been accomplished. None is physically very close to the bithorax cluster. Indeed three are located in other chromosomes, two in the X or first chromosome; one in the second and three in the third. It is hoped that such modifying genes can eventually be used to give clues as to the biochemical nature of the reactions controlled by the bithorax cluster.

56. ANOMALOUS CHROMOSOME BEHAVIOR IN AN INBRED STRAIN OF DROSOPHILA

Investigator: A. H. Sturtevant
Support: General Funds

A strain of Drosophila melanogaster that has been closely inbred for many generations has been found to give anomalous results on outcrossing. Females from the inbred strain regularly give some sons that receive their X from their fathers—i.e., these females produce offspring that get no maternal sex-chromosome. The frequency with which these exceptional males occur is variable, ranging from 1 to 3% of the male offspring up to 20 to 30%. The condition has been transferred to other strains, but it has not proved possible to fix either the high or the low frequency of exceptions by inbreeding and selection. There is evidence that the peculiar behavior is dependent on two recessive genes—one in X and one in the second chromosome—that must both be present and homozygous. However, there are still complications that are not understood, since experiments designed to locate these genes usually give a few cultures that do not agree with the main body of data.
GENETICS

PART B. -- IMMUNOGENETICS

STAFF:
Proфессor:
Research Fellows: Ray D. Owen, David Dresser, Raymond Gilden, Mary C. Henry, Max Houtchens, John B. Loefer, Margaret MacGillivray, Grace L. Rosenquist, Seikichi Tokuda
Graduate Students: Alan P. Roberts, Donald C. Shreffler
Student Assistants: Nancy Couse, Randall Griepp, Charles F. Leonard, Roy J. Riblet
Research Assistants: Flora M. Alvin, Gilly Campbell

57. IMMUNOGENETIC BASES OF TISSUE TRANSPLANT TOLERANCE AND REJECTION

Investigator: Ray D. Owen
Support: U.S. Atomic Energy Commission
Max C. Fleischmann Foundation Fund

Studies of the population genetics of tissue graft compatibility, described in Biology 1961, were reported at a conference in Prague, Czechoslovakia, and will appear in the volume resulting from that conference. A detailed survey of methodology in mammalian immunogenetics is also in press.

Publications:


58. MAMMALIAN BLOOD GROUPS AND THEIR USE AS MARKERS FOR ERYTHROCYTE CHIMERISM

Investigators: Ray D. Owen, Grace L. Rosenquist, Gilly Campbell
Support: U.S. Atomic Energy Commission
Max C. Fleischmann Foundation Fund

Studies of radiation-induced, bone-marrow injected rhesus monkeys, reported in Biology 1961, have continued in collaboration with the School of Aviation Medicine of the United States Air Force. The most interesting development this year has been the recognition of a transient chimerism in monkeys given homologous bone marrow after being injected with heavily radioactive material in a vehicle that appears to affect lymphatic tissues, and therefore immune responses, preferentially.

Two papers, on blood group studies in rhesus monkeys and in rats respectively, appeared during the year.

Publications:


59. **PARABIOTIC INTOXICATION IN MICE**

**Investigator:** Seikichi Tokuda  
**Support:** National Institutes of Health, U.S. Public Health Service  
U.S. Atomic Energy Commission

Studies on parent to F1 parabiotic intoxication in A/J mice parabiosed with BAF1(C57Bl/6J x A/J) mice and in AKR/J mice parabiosed with AKD2F1(AKR/J x DBA/2J) have been continued (Biology 1961). In the current studies it was established that immunologically competent A/J cells colonize the intoxicated BAF1 spleen during parabiosis. This was shown by the ability of "intoxicated" BAF1 spleen cells to induce splenic enlargement in 4 to 10-day-old BAF1 mice. However, this type of reactivity was not seen when "intoxicated" AKD2F1 spleen cells were introduced into 4 to 10-day-old AKD2F1 mice. In another series of experiments, AKR/J mice were irradiated prior to parabiosis with the F1 hybrid mice. In six such combinations, these mice had a mean survival time of 126 days with a range of 57 to 206 days. One pair is still alive after 11.5 months in parabiosis. (The mean survival time of AKD2F1 mice parabiosed with AKR/J mice is 14 days.) Thus, if one considers (a) the genetics of parabiotic intoxication, (b) the induction of splenomegaly in 4 to 10-day-old BAF1 mice with "intoxicated" BAF1 spleen cells, and (c) the abrogation of parabiotic intoxication by irradiating the parent strain mouse prior to parabiosis with a hybrid mouse, then one can conclude that parent to F1 parabiotic intoxication in mice is the result of a parent versus F1 immunological reaction and that this disease is another manifestation of "homologous" or "runt" disease.

A working model to study the cause of the anemia and polycythemia during parabiotic intoxication has been devised and is under investigation.

**Publications:**


60. **SURVIVAL OF Cr51-LABELED RED BLOOD CELLS IN IMMUNIZED AKR/J MICE**

**Investigator:** Seikichi Tokuda  
**Support:** National Institutes of Health, U.S. Public Health Service  
U.S. Atomic Energy Commission

In collaboration with Dr. M. H. MacGillivray, it was noted that AKR/J mice surgically separated after one week in parabiotic union with F1 hybrid mice did not show immune clearance of isotopically labeled F1 hybrid red blood cells. It was also noted that during parabiotic intoxication Cr51-labeled F1 hybrid red cells do not undergo immune clearance (Biology 1961). The present report concerns studies on the survival of Cr51-labeled red blood cells in immunized AKR/J mice. AKR/J mice were immunized with skin grafts and with whole blood from F1 hybrid (AKR/J x DBA/2J) donors. The presence of specific anti-F1 antibodies were determined by the human serum-dextran technique of Gorer and Mikulska. The results of these studies
indicates that the AKR mice are capable of demonstrating the accelerated clearance of F1 red blood cells at a time when these mice have detectable anti-F1 red cell agglutinins. This investigation is being continued with CBA/J donor red blood cells in specifically immunized AKR/J recipients.

Although the significance of this finding is not readily understood, it must be stated that three other groups of investigators have recently noted the same phenomenon in different strain combinations (J. W. Goodman, Oak Ridge, L. Herzenberg, Stanford University, and A. Mitchison, Mill Hill, London, personal communications).

61. STUDIES ON IMMUNOLOGICAL MECHANISMS

Investigator: David Dresser
Support: National Institutes of Health, U.S. Public Health Service
U.S. Atomic Energy Commission

I. The role of adjuvants

It was previously shown that an intravenous injection of bovine gamma globulin into CBA mice would not immunize these mice if the solution was centrifuged free of all particulate matter. However, it was also shown that an intraperitoneal injection of this protein would induce the production of antibody if a subcutaneous injection of adjuvant was made at the same time. This system was used to test several substances for adjuvanticity (adjuvant activity). Freund's adjuvant (made up without antigen) mycobacterial suspension, oleic acid and some non-ionic detergents were all found to have adjuvanticity (Dresser, 1961). This system has been studied in greater detail. For instance, an investigation has been made on the adjuvanticity of substances which stimulate the phagocytosis by the reticuloendothelial system of colloidal material injected into the circulation; the results seem to indicate that there is no relationship between adjuvanticity and stimulation of the RES. Recently it has been shown that adjuvanticity controls which of two mutually exclusive states an immunologically competent cell can attain after contact with antigen; namely the ability to produce antibody or a specific state of immunological paralysis.

Reference:


II. Immunological paralysis

It has already been mentioned that adjuvanticity controls whether or not a state of immunological paralysis can be induced. A centrifuged solution of bovine gamma globulin has been shown to lack adjuvanticity and previous experiments have shown that this antigen will induce paralysis in adult mice even in very small quantities (Dresser, 1962). The induction of paralysis in neonatal animals (tolerance) has been studied and compared with overloading paralysis of adults. It seems likely that overloading paralysis (Medawar, 1961) is the only way to inhibit antibody production when smaller doses of the same antigen induce antibody formation. However, bovine gamma globulin (BGG) never induces antibody formation unless an adjuvant is injected at the same time as the antigen; it is clear that in the absence of adjuvanticity BGG induces paralysis, even in an amount as small as 50 micrograms, or about $10^{-4}$ to $10^{-5}$ of the dose required to prevent antibody synthesis in a mouse injected with adjuvant at the same time as the antigen. It has been possible to measure the induction time of the paralysis response. The significance of these results, in relationship to all immunological unresponsiveness phenomena and to a tentative working hypothesis, is at present being investigated in considerable detail.
References:


III. The secondary response

A previous attempt has been made to study the cellular inheritance of the induced state of cellular differentiation associated with a secondary immune response (Dresser, 1961). In adoptive immunization experiments cells from immunized mice were injected into lethally irradiated isogenic mice and there allowed to divide in the absence of antigen. The ability to give a secondary response decreased rather than increased, indicating one of two major possibilities: the cell capable of giving a secondary response does not divide in the absence of antigen [recent evidence of Dutton and Pearce (1962), shows they synthesize DNA in the presence of antigen] or that they do divide and that the mechanism responsible for antibody synthesis does not replicate. In subsequent experiments these results have been confirmed and it has also been shown that in the presence of antigen the ability to give a secondary response increases. Further experiments are being carried out to determine if the cells responsible for the secondary response can only divide when stimulated by antigen.

References:


62. ANTIBODY RESPONSES AFTER SUCCESSIVE INJECTIONS OF RELATED PROTEIN ANTIGENS

Investigators: Raymond Gilden, Seikichi Tokuda
Support: National Institutes of Health, U.S. Public Health Service

The striking difference in the "quality" of the antibodies formed after one and two injections of the same antigen provides a useful means of evaluating antibody responses after various experimental treatments. Although the biological basis of this difference is unknown, kinetic data obtained by Talmage (1960) show that antibody formed late in immunization dissociates from combination with antigen at a much slower rate than early formed antibody. This difference is being used to study antibody responses after successive injections of related antigens. The data obtained should aid in interpretation of recent results based on the quantity of antibody formed in this situation.

References:


63. ANTIBODY ACTIVITY OF THE MICROSOME FRACTION FROM THE SPLEEN OF IMMUNIZED CHICKS

Investigator: Grace L. Rosenquist
Support: U.S. Atomic Energy Commission

Antibody produced in the cytoplasm of the cell would be expected to be associated with the microsomal fraction before its release into the circulation. The main problem is to develop an assay sensitive enough to pick up the very small amount of antibody associated with this fraction. The assay of antibody at this level is especially complicated by an exchange of antibody between the microsomes and the serum, as well as by the minute amounts of antibody bound to this fraction of the cell.

Spleens from chickens immunized with bovine serum albumin (BSA) were removed at various times after injection of antigen and studied for antibody activity. The microsome fractions were prepared in either 0.001 M Tris buffer (pH 8.0) or 0.88 M sucrose with 0.001 M MgCl$_2$. No antibody could be detected associated with the resuspended microsomes either by precipitation with BSA or by a modified Boyden hemagglutination technique. The use of binding of $^{131}$I labeled BSA to the microsome fraction shows some promise as a sensitive assay method for checking antibody production at the subcellular level.

Reference:

64. THE IMMUNE RESPONSES OF THE FOWL TO BOVINE SERUM ALBUMIN

Investigators: Grace L. Rosenquist, Gilly Campbell, David Dresser
Support: U.S. Atomic Energy Commission
National Institutes of Health, U.S. Public Health Service

Antibody production to bovine serum albumin (BSA) in the fowl starts 5 days after the first injection of antigen, reaches a peak 8 days later and slowly declines until 20 to 25 days later when precipitating antibody (precipitin) can no longer be detected. After a second injection, the response is quicker, greater and lasts somewhat longer.

Because the lower quantitative limit of the precipitin assay is about 6 micrograms of antibody protein per ml of serum, the possibility of using the more sensitive antigen binding capacity method (Farr, 1958) was explored. This technique has already been used by other workers as a quantitative and qualitative test with rabbit and mouse sera. The method relies on the insolubility of serum globulins in half-saturated ammonium sulphate at which salt concentration serum albumins are completely soluble. Antibody to a serum albumin can be measured by labeling the antigen with a radioactive isotope and then determining the amount of antigen which is bound to globulin when the latter is precipitated in half-saturated ammonium sulphate. Furthermore, decreasing the concentration of antigen in the test system results in a decrease in the ability of any one molecule of antibody to bind a molecule of antigen. The ratio of the dilution of antigen used to the decrease in the binding obtained is called the avidity of the antibody. Avidity, which is a measure of the strength of the "bond" between antigen and antibody, changes progressively during the course of the immune response in the sera of those mammals that have so far been investigated.
In our experiments the antigen binding capacity (ABC or Farr) method has been extended to the fowl. Preliminary results show that there is a very close positive correlation between relatively high precipitin titers and the results of the ABC tests carried out with an arbitrarily chosen concentration of antigen. However, it has been shown that antibody persists in measurable amounts for very much longer than had previously been thought (the lower limit of an accurate quantitative estimate with the ABC test in the system was 0.02 micrograms of antibody protein per ml of serum). It has also been shown that the avidity of the serum progressively increases after a first injection of antigen and remains high after a second injection.

Reference:


65. CELLULAR CAUSES OF THE GRAFT-VERSUS-HOST REACTION IN INFANT MICE

Investigator: Ann M. Dresser
Support: Max C. Fleischmann Foundation Fund

It has been found in chickens, rats and mice that there is a relationship between the number of lymphoid cells in an inoculum and the appearance of the various symptoms of the graft-versus-host reaction (GVHR) in young animals. There is some conflict in the literature as to whether the large or the small lymphocyte is the cell which actually mediates the reaction. The results of previous preliminary experiments had suggested that the large lymphocyte might be the cell involved. It was found, on culturing spleen cells in a variety of tissue culture media, that only when large lymphocytes survived the period of culture could the harvested cells induce splenomegaly (one of the symptoms of the GVHR) in infant mice. These experiments have been repeated here with the same result. Other experiments are also in progress in which the proportion of different lymphocyte types in the tissues of donor animals has been altered by the treatment of donor animals, or their cells, with biologically or pharmaceutically active substances (thymus extract, phytohemagglutinin, cortisone, colchicine and antigenic materials).

66. THE SERIAL TRANSFER OF MOUSE BONE MARROW IN IRRADIATED RECIPIENTS

Investigators: Ann M. Dresser, David Dresser
Support: Max C. Fleischmann Foundation Fund
National Institutes of Health, U.S. Public Health Service

The role of cell division and environmental conditions in the expression of a genetically controlled character of a cell is of considerable interest both developmentally and genetically. One system in which such features can be studied is found in the transplantation of bone marrow to irradiated recipient mice. The hemoglobin of different strains of mice have been shown to move either as diffuse or single bands during electrophoresis, and the expression of this character has been shown to be under genetic control. Furthermore bone marrow cells are known to divide during the recolonization of irradiated recipient animals. By transplanting bone marrow to mice of different strains the milieu of the marrow cells can be varied, while serial transfer of bone marrow from one radiation chimera to another irradiated recipient will induce extensive cell division.
In the experiments which have been carried out so far $6 \times 10^6$ or $6 \times 10^6$ bone marrow and spleen cells from BALB/c H-2d mice have been injected into C57Bl/Ks H-2d mice which have received a dose of 700r. The animals were bled at intervals throughout the course of the experiment and the amount of donor type hemoglobin was determined by using the technique of Popp and Cosgrove (1959). Four weeks after this first marrow transfer, $6 \times 10^6$ marrow and spleen cells from the first group of mice or $6 \times 10^6$ cells from the second group of mice were transferred to further irradiated recipient C57Bl/Ks mice. The same procedure was repeated after another four weeks, using the secondary hosts as donors for the tertiary recipients.

Preliminary results suggest that there is a difference in behavior of the transplanted marrow in the two series of recipients. This could be a reflection of the degree of recolonization of the primary and secondary hosts at the time at which they are used as donors for the secondary and tertiary recipients. There is some indication however that other factors may be active. Further experiments using this system are in progress.

Reference:


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### 67. STUDIES ON INSULIN TOLERANCE IN KL MICE

**Investigator:** Mary C. Henry  
**Support:** National Institutes of Health, U.S. Public Health Service

The genetic background of an animal is important in determining its insulin sensitivity. Adult mice of the C57Bl and BUB strains tolerate 3 to 8 units and 30 to 50 units respectively, whereas the KL strains (KLBUB and KLC57Bl) will tolerate insulin doses above 200 units. The decreased insulin sensitivity of KL mice may be due to an exaggerated insulin-binding system in the liver or vascular system. This possibility was investigated by studies on the rate of insulin-131 removal from the circulation. The results indicated no correlation between high insulin tolerance and insulin retention in the vascular system, although there are different rates of insulin elimination among the strains. By electrophoresis on starch gel, the distribution of insulin-131 among the serum fractions was seen to be similar for the four strains, indicating that the KL mice have no unusual insulin-binding factor in their sera. Further work includes studying the insulin-binding capacities of the liver and the insulin sensitivity of peripheral tissues.

### 68. STUDIES OF PROTEINS IN NORMAL MOUSE URINE

**Investigator:** Alan P. Roberts  
**Support:** National Institutes of Health, U.S. Public Health Service  
U.S. Atomic Energy Commission

It has been known for some time that the urine of normal mice contains a relatively high concentration of low molecular weight protein. In studying further characteristics of this material it has been shown to be electrophoretically separable into at least six bands on starch gel. The slowest moving component exhibits genetic variation, its position being altered in animals of the C57Bl inbred lines as compared to animals of all other strains tested.

Tests of F2 and backcross progeny from the cross C57Bl/6 × CBA for the urinary protein type as well as for transferrin, a $\beta$-globulin variant of the serum
proteins, have shown linkage between the two markers. A further test-cross series has demonstrated linkage between the urinary protein variant and the dilution locus. Thus the urinary protein locus is placed in linkage group II of the mouse.

Immunodiffusion and immuno-electrophoresis studies using rabbit antisera prepared against whole mouse urine following dialysis have indicated cross-reactions of the urinary proteins with the serum α₂-macroglobulins and γ-globulins. However there is clearly no identity between the proteins of the serum and of the urine, the electrophoretic mobilities of all the urinary components being much greater than any of the serum proteins. It is therefore suggested that the urinary proteins may be small fractions of larger serum protein molecules which still contain cross-reactive combining groups. Antibody combining sites, however, appear to be absent; urinary proteins from hyperimmunized mice show no antibody activity as determined by inhibition tests.

69. BACTERIAL CLEARANCE IN MOUSE RADIATION CHIMERAS

Investigators: Charles F. Leonard, Alan P. Roberts
Support: U.S. Atomic Energy Commission
Max C. Fleischmann Foundation Fund

A test in which P³₂-labeled E. coli was used was designed to determine the bacterial clearing activity of both fixed and circulating phagocytes of mice as a function of X-radiation and tissue chimerism. In the strain combination used, BAF¹ with BALB/c, only mild homologous disease was noted. All irradiated animals displayed much more rapid and thorough clearance of bacteria from the blood than did unirradiated controls. Detectable, though small, differences appeared between homologous- and isologous-marrow injected irradiated hosts; any transplant of erythropoietic tissue, however, hastened bacterial clearance compared with the already rapid process in irradiated but not marrow-injected mice. Irradiation resulted in a decrease in clearance activity per unit weight of spleen; in spite of spleen enlargement after irradiation, the net effect of irradiation was a less efficient spleen with regard to bacterial phagocytosis. There were no conclusive overall differences in spleen effects between homologous- and isologous-marrow-injected irradiated hosts, but those homologous animals with greatest increase of spleen weight relative to body weight also exhibited the greatest degree of weight loss, bacterial clearance speed-up, and other secondary disease symptoms.

70. TISSUE ANTIGENS AND THE B LOCUS IN CHICKENS

Investigator: Randall Griepp
Support: U.S. Atomic Energy Commission

Recent work by Gilmour and by Schierman and Nordskog suggests that a relationship between hemagglutinating and transplantation antigens, similar to that in the H-2 system in mice, may exist in chickens. The erythrocyte antigens involved are those controlled by the B locus. Experiments undertaken to test this hypothesis have yielded the following results:

Hemagglutinins against B locus antigens obtained by erythrocyte isoimmunization are not specifically absorbed by liver cell membrane extracts prepared by the method of Herzenberg. Lyophilized crude liver extracts do exhibit specific absorption.
The injection of erythrocyte-free liver homogenate induces the formation of hemagglutinins against the erythrocytes of the liver donor.

Hemagglutinins against the skin donor appear in the serum of a skin graft recipient at about the time of sloughing of the graft.

Experiments are in progress to answer the following questions:

Are hemagglutinins resulting from immunization via liver homogenate injection and via skin grafting directed against erythrocyte antigens of the B blood group locus?

Are hemagglutinins resulting from immunization via skin grafting specifically absorbed by liver extracts?

References:


71. THE STUDY OF SELF INCOMPATIBILITY IN PETUNIA

Investigator: Nancy Couse
Support: National Science Foundation
Arthur McCallum Fund
U.S. Atomic Energy Commission

In Petunia inflata, a reaction between the diploid stylar tissue and the haploid pollen tube results in the failure of otherwise fully functional gametes from the same flower to effect fertilization. The specificities concerned are under the control of a multiple allelic series, designated as S. Clones of plants of the following genotypes: S2S5, S1S2, S5S5, and S1S1 were kindly provided by Dr. James Brewbaker, of Brookhaven National Laboratory. Extracts of styles were prepared and injected into rabbits to obtain antisera for the study. Pollen extracts, prepared from pollen germinated for 24 hours, were subjected to starch gel electrophoresis. No bands were observed with an esterase stain, but an amido black stain showed five distinct bands for each of two pollen genotypes observed. These bands appeared to be exactly the same in the two types of pollen. Stylar extracts did not display bands when an esterase stain was applied, but amido black stain revealed several bands. An extra band, which was absent in all the other types, was obtained with virgin S1S2 styles. No "interaction protein" as reported by Linskens (Z. Bot. 48:126-135, 1960), was observed in crossed styles, distinguishing such styles from either self or virgin styles. Useful antiserum was obtained for virgin S2S5 stylar extracts. Gel diffusion tests revealed several bands, differing only in quantity among tests from different stylar extracts. Diffusion plates, but no diffusion slides, revealed at least one component common to pollen and stylar extracts. Preliminary immunoelectrophoretic studies suggested that two bands were characteristic of S1S1 and not found in S5S5 stylar extracts.
Serological studies on the 78 strains of Tetrahymena pyriformis, variety 1, as noted in Biology 1961, have continued particularly on the serotypes expressed at 15°C and 35°C in comparison to those exhibited at 25°C.

Establishment of several dozen of these strains in axenic culture and their propagation at temperatures of 15, 25 and 35°C have enabled extension of studies initiated several years ago on variety 1 serotypes expressed in axenic cultures (cf. reference below). In general, results show that most but not all strains exhibit the same or a similar serotype at 35°C as at 25°C, and that most of them at 15°C have immobilizing antigens different from those expressed at 25°C. In a number of cases the 25°C serotype is also found in the same strain cultured at 15°C. These studies also show that there is a greater correlation existing between the serotypes expressed in axenic and bacterized cultures than was apparent from initial studies in reports in 1959.

Publication:


Reference:

GENETICS

PART C.--NEUROSPORA

STAFF:

Professor: Sterling Emerson
Visiting Professor: D. G. Catcheside
Research Fellows: P. C. Huang, Mary B. Mitchell
Student Assistant: Daniel Rifkin
Research Assistant: Mary R. Emerson
Research Aide: Françoise Cousin

73. FURTHER GENETIC STUDIES OF SLIME

Investigators: Sterling Emerson, Françoise Cousin
Support: National Institutes of Health, U.S. Public Health Service

As reported earlier (Biology 1960, 1961) three independent genes, os, fz, and sp, are necessary for the production of the slime phenotype in isolates obtained from outcroses of the original slime variant (1207-1A)UV#1, and the original slime still carries A, arg-1, cr, aur, and os on the mating type chromosome, all genes present in Stanford strain 1207-1A from which the original slime was derived. The cross fz os X (1207-1A)UV#1 yielded about equal proportions of fz os and of cr phenotypes among the usual 18% of spores germinating. Outcrosses of each of the isolates with the cr phenotype segregated for fz, showed that this gene is present in the original slime variant and, further, that cr is epistatic to fz.

In these outcrossoes cr, aur, and fz segregated in the expected proportions, but segregation for os varied in different crosses from 15% to 80% (germination 80% or higher in these crosses). Furthermore, some segregants did not give clear tests for the os character, not being strongly inhibited on sorbose medium, as is wild-type, nor on 5% KCl medium, as is os. Some of the isolates giving an indeterminate os reaction were found to be heterocaryons as judged by distinct phenotypes which could be separated vegetatively. The heterocaryotic condition has, in at least one instance, been transferred to some descendants through a second sexual cycle. Aberrant tetrad ratios appear to be unusually frequent in some of these crosses. The cytology of ascus development in slime crosses is now being undertaken in the hope of finding a basis for the unusual segregations observed.

74. PHYSIOLOGICAL AND DEVELOPMENTAL GENETICS
OF NEUROSPORA

Investigator: P. C. Huang
Support: National Institutes of Health, U.S. Public Health Service

I. Recombination and complementation studies with albino mutants

By methods previously described (Biology 1961), the various albino mutants collected at Stanford University could be classified into two closely linked recombination groups. Such a grouping is consistent although incomplete when based on complementation tests for carotenoid pigment formation. The noncomplementarity between members of different groups may be genetic or due to an unbalanced nuclear ratio. The inference that the albino mutants of N. crassa are located at only one or the other of these two loci has been further sustained by genetic tests with 16 more albino mutants provided by the Oak Ridge National Laboratory.
II. Characteristics and properties of slime, a plasmodioid mutant

a. Morphogenesis and ultrastructure. With phase microscopy (Emerson, Biology 1961), it has been shown that the marginal cells of a slime colony, when grown on agar, are apparently devoid of cell walls. Old cells or liquid-cultured cells may or may not contain the typical wild-type cell wall. Slime is deficient in phospholipids (Nyc, unpublished) and in an unknown substance from the acid hydrolysates of cell wall residue (Olson, Huang and Elliott, Biology 1962). Both compounds are present in the wild-type and may be related to the formation of cell membrane and cell wall. Present studies with electron microscopy on cultures with the slime growth habit indicate that while there exist double layered membranes and hexagonal ergosterol crystals, as in the wild-types, there is no evidence of ordinary cell wall structure. In slime, there is an excessive number of irregular to spherical-shaped electron dense bodies with sizes ranging from 0.1 to 1.2 µ diameter. An irregularity in the morphology of mitochondria of slime is suggested, but not established. Further observations are being made with slime cultures developed under various growth conditions and of different ages. These will be compared with wild-types and heterocaryons involving slime. (S. Emerson also participated in this part of the studies.)

b. Heterocaryosis involving slime. A heterocaryon between slime, which requires arginine for growth, and a non-slime mutant requiring lysine and nicotinic acid was made originally by Mary Emerson. This and subsequent isolations of the nuclear components provide supportive evidence for the conclusion that slime is a genuine Neurospora mutant and that the slime character is determined solely by the nucleus and not cytoplasmically. Conidia from this heterocaryon, when plated on variously controlled media, give rise to correspondingly different proportions of slimy versus mycelial colonies. Some colonies from heterocaryotic conidia begin development with a typical slime morphology and later produce a parenchymatous mass of cells from which hyphae develop. This change in morphology is thought to be due to a change in nuclear ratio ranging from an excess to a deficiency of slime nuclei. Hyphal heterocaryons have so far had a preponderance of non-slime nuclei. A study on heterocaryosis may thus lead to a better understanding of a comparable phenomenon of dominance-recessiveness occurring in diploid organisms. (Francoise Cousin also participated in a part of the work.)

Publication:


c. Growth. Slime is found to be sensitive to cold (-12°C, 48 hours), chloroform (2%), vibration (186-4" strokes/min., 120 minutes), extreme osmotic concentrations, and rapid freeze-thawing (three times or more). All these and similar treatments lead to a drastic reduction in viability, probably due to rupturing of the cells. The slime colonies, when developed on agar, cease to enlarge after a while; hence linear measurements are not feasible. Also each colony may originate from one or more nuclei, making colonial counts inaccurate. Ordinary procedures for DNA and RNA determinations are inaccurate because of interference by the culture medium. Slime cannot be washed without considerable loss of material. Turbidity measurements are also not reliable due to the heterogeneity of the material. It is thus difficult to accurately measure growth. On the basis of preliminary determinations of changes in protein contents, it can be shown that the growth of slime in liquid is not logarithmic as in many unicellular microbes, nor a function of cubes or square roots as in wild-type Neurospora. Radioactive tracers are used in these studies.
d. Possible association of viruses with slime. Several viruses studied in the Division of Biology, such as bacteriophages MS2 (assays by James Davis) and \( \phi X174 \) (assays by Mike Yarus and repeated by the author), animal viruses Sinbis and Vaccinia (assays by Robert Ting), and plant virus TMV (assays made in collaboration with Philip Filner) have been tested. Under conditions that are optimal for assay of the respective viruses in the absence of slime, instantaneous partial inactivation is observed upon addition of slime to either plant or animal viruses, while no change in bacteriophage infectivity occurs even after 12 hours of incubation with slime. However, neither whole bacteriophage nor its infectious nucleic acid (MS2 RNA; \( \phi X174 \) DNA) produced detectable amounts of new infectious material when incubated in this intact slime system. Among the viruses inactivated by incubation, the animal viruses Sinbis and Vaccinia showed no recovery of infectivity, while TMV infectivity decreased during the first 8 hours to less than 1/100 of the zero time level, followed by a period of infectivity increase, but with less than a 10-fold gain over the 8-hour minimum. The recovery phenomenon is not observed under conditions which are optimal for slime growth. It is not clear whether the recovery of TMV infectivity is due to production of new infectious material or to reactivation of previously inhibited virus. The inactivation, however, seems to be a purely physical phenomenon, because zero time virus inactivation can be correlated with total amount of protein in the slime, whether actively growing or freshly lysed. All the viruses tested have no apparent effect on the morphology of the slime.

75. REPLICATE SEGREGATION PATTERNS AMONG RELATED ASCI OF NEUROSPORA

Investigator: Mary B. Mitchell
Support: National Institutes of Health, U.S. Public Health Service

In pursuing an investigation of the possibility that the regular formation of pairs of morphologically similar or identical asci, developing concurrently and in close association, means that each ascus has an identical twin, a more complex cross has been utilized in order to observe coincidences of identical segregation patterns among a larger number of infrequent classes. Clusters of asci were classified with respect to segregation patterns of 3 markers, 2 of them linked. For a given marker there are 6 regular patterns, hence, for 3 markers, 216, of which 141 occurred at least once among 1010 asci. Although improbable duplicates from the same cluster were found, their frequency is judged to be too low to permit the conclusion that each pattern occurs in duplicate. The results are thought to suggest, rather, that the reason for these and for the marked structural similarity of twins may lie in relationships among larger groups of asci. Coincidences of several replicates or partial replicates of infrequent patterns indicate a tendency towards repetition of patterns, possibly among pairs of asci formed consecutively from the same initial point in the ascus cluster. This would not seem to bear directly on the question of why asci are produced in pairs but rather to constitute a further complication in connection with evaluation of the genetic significance of segregation patterns as well as with the overall mechanics of ascus formation.

76. H-TYPE PERITHECIA IN NEUROSPORA

Investigator: Mary B. Mitchell
Support: National Institutes of Health, U.S. Public Health Service

In heterothallic species (those regularly showing mating type reaction) of the red bread mold Neurospora, two modes of fruiting body initiation have been recognized. Parent A may produce incipient fruiting bodies which are then fertilized by parent a, or the reverse (Dodge, Mycologia 27:418, 1935; Mitchell and Mitchell, P.N.A.S. 38:442, 1952). Initiation of fruiting bodies, or perithecia, by a
heterocaryotic mycelium (one containing genetic components of both parents), formed by prior interaction of the two parents, is a third possibility and recent observations indicate that this also occurs. Relative frequencies of the three types of perithecia appear to vary widely, depending upon the strains crossed and, to some extent, on culture conditions. The h-type (arising from heterocaryons) is thought to be, more usually, rare, but is of special interest because of observations suggesting that it allows greater opportunities for recombination. Further study of it holds promise, therefore, of contributing to a better understanding of the recombinational capacities of mutant strains.

77. STUDIES ON INTERALLELIC COMPLEMENTATION

Investigator: D. G. Catcheside
Support: General Funds

The collection of 389 tryptophan and 238 histidine mutants, newly made in the Division of Biology last year, was transferred to the University of Birmingham, where further analysis has been continued. Several related objectives have been in mind in the course of this study, the immediate purpose of which has been to find suitable material for the study of various aspects of the relation between complementation and enzyme structure and function. One of these is to find cases in which absence of complementation is highly probable and in which the function controlled by the gene is accessible to study. This will help to decide whether or not such genes specify some function other than the structure of enzymes. The numbers of tryptophan-2 and of tryptophan-4 mutants, respectively 53 and 28, with no pair showing interallelic complementation, are now large enough to be fairly sure that no complementation would be found in an even larger sample. The function controlled by tryptophan-4 is known approximately and is enzymic.

The complementation maps of tryptophan-1 (120 mutants) and tryptophan-3 (233 mutants) have been further studied, especially with a view to correlation of the maps with residual enzymic functions possessed by the mutants. For tryptophan-1 the map divides neatly into two parts, one region being concerned with the formation of anthranilic acid, while the other is concerned with the utilization of anthranilic acid. For tryptophan-3 the map is more complicated, being divisible into three regions A, B and C in that order. Regions A and C together are concerned with the conversion of indole glycerol phosphate to indole, while regions B and C together are concerned with the conversion of indole to tryptophan. Further advance along these lines promises to show how structure is integrated to determine function.

Arising from the work done with the new histidine mutants there are two points especially worthy of note. One is the discovery of a new histidine-4 mutant, which is less leaky than the previously known one (C141). This gene controls the specific histidinol dephosphorylase and efforts are now being made, by a special method, to obtain more mutants of it. The other new point is that, amongst the 51 histidine-3 mutants analyzed, there are two new groups which may throw further light on the functions controlled by the histidine-3 gene. This gene appears to control two functions, one early in biosynthesis of histidine and the other the terminal step, namely the dehydrogenation of histidinol to form histidine. Previously, the evidence had suggested that these functions were organized separately, but one of the new kinds of mutants appears, subject to confirmation, to indicate a more integrated organization.
GENETICS

PART D.--OTHER

STAFF:
Research Fellows: Oliver E. Nelson, Jr., Yair Parag
Laboratory Assistants: R. W. Atwood, Evelyn J. Robbins

78. INVESTIGATIONS ON THE WAXY MUTANT IN MAIZE

Investigator: Oliver E. Nelson, Jr.
Support: National Science Foundation

The waxy locus in maize is the only known system in a higher organism where it is feasible to investigate genetic fine structure. As such, it constitutes an invaluable link between the genetics of higher and lower organisms.

Although the net effect of the waxy mutant (amylopectin as the only carbohydrate storage product in the endosperm and pollen grain as opposed to both amylopectin and amylase in normal maize) has been known for many years, the specific block in amylose synthesis has never been elucidated. Investigations to date make it unlikely that a transglycosidase such as amylomaltase or the D-enzyme is responsible for the difference. Phosphorylated compounds are apparently implicated since extracts of normal endosperms show a small but consistently higher level of phosphorus present as inorganic phosphate. Total phosphorus levels are apparently the same in extracts of both types. In addition, grinding starch granules from developing seeds liberates approximately twice as much as an adenine-containing nucleotide from normal preparations as from waxy preparations.

In the past several months, twenty Wx alleles arising by recombination between the alleles wxCoe and wx90 have been tested for their ability to support amylase synthesis. Only recombinants carrying one or both of the recessive outside markers from the wxCoe parent were used in order to be certain that no Wx contaminants were tested. The results show no indications that any of the recombinants can be considered significantly less effective than the controls in supporting amylase synthesis.

79. GENETIC STUDIES OF THE SELF INCOMPATIBILITY IN SCHIZOPHYLLUM: MUTATIONS IN THE B FACTOR

Investigator: Yair Parag
Support: National Institutes of Health, U.S. Public Health Service

In a previous report (Biology 1961, No. 110), studies on a mutation in the B incompatibility factor of the tetrapolar mushroom Schizophyllum commune were reported. These studies have now been extended.

Preliminary results reported earlier indicated that the spontaneous mutation in study occurred in the β subunit of the B factor. Segregants from additional crosses gave more support to this postulation. Among a total of 780 segregants, 4 recombinants were found; all were those expected if the mutation occurred at the β subunit, e.g. B43l × B3m → B3c, and no B43. An effort was made to induce more mutations with mutagens: base analogs, nitrous acid, nitrogen mustard, proflavin and UV irradiation. So far, two more mutations were obtained by nitrogen mustard: both were found to be also located at the β subunit. All the three mutations lead to the loss of the function of the B factor, i.e. they lead to the loss of the control of self incompatibility by the B factor. This fact is very relevant to the understanding of the
mode of action of the incompatibility factors. Two genetic models for the mode of action were suggested (Raper & Parag and Parag & Raper, in preparation). A differentiation between these two models may be made possible by the obtainable types of mutations: mutations resulting in the loss of factor-function would be expected by either model, whereas subunit specificities, hence factor specificities, would be unique to one of the models. The three mutations, therefore, did not give the answer, and the differentiation can be achieved only after new active alleles are obtained via mutations or after enough information is accumulated to suggest that new active alleles cannot be obtained. A large scale mutant hunting is therefore continued, with an emphasis on the use of base analogs and nitrous acid.

Any one of the strains carrying a B-mutation has the properties of a common-A heterokaryon, among them being unilateral (i.e. when mated with another mycelium it donates only nuclei but does not accept nuclei from the other mycelium). Matings $B^m \times B^m$ were, therefore, not feasible. It was now found, however, that if the matings are made among young mycelia, 24 hours after germination of the basidiospores, they are successful, and the material is ready for the use of the B mutations as selective markers for detecting intragenic recombination in the $\beta$ subunits.

Publication:

80. INTEGRATED SIGNALS FROM THE OPTIC GANGLIA OF THE HAWAIIAN CRAB

Investigators: C. A. G. Wiersma, B. M. H. Bush
Support: National Science Foundation

In previous preliminary investigations in Bermuda, it had been observed that from the "optic nerve" running from the eyes of different Crustacea to the brain, impulses could be obtained in single units which showed a number of remarkable integrative properties.

The Hawaiian crab Podophthalmus was used as the experimental animal. Its long, completely external, proximal segments of the eye stalk enable use of the experimental techniques by which single unit responses are obtained in the optic bundle, a method extensively used in studies on the central nervous system of the crayfish. The arterial blood supply to the eye was maintained, the venous supply being first replaced with a perfusion fluid. Under these conditions the light reactions could be maintained for several hours and a sample of reacting units made in a number of preparations. In this series of experiments, the nerve bundle, which is about one inch in total length, was centrally ligated, and only units coming from the eye registered.

The eye was exposed to light flashes of different durations (both polarized and natural), to illumination with small light spots, to moving targets manipulated by hand, to moving shadows and to small moving lights. A considerable number of differently reacting units were revealed in this way. A full account of all observations will not be given here, in part because the analysis of the data is not complete. Some classes of fibers will be shortly described. Among the large interneurons, a good many react especially to moving objects in the visual field, but these fall into quite distinct classes, of which the main ones are: a) large fibers with very extensive visual fields, reacting with the highest number of impulses on quick moving objects, b) medium sized fibers with visual fields of a few decades of degrees, responding mostly to speeds of movement which elicit optomotor reflexes, and c) large fibers with fairly large visual fields, responding best to very slow movements just visible to the human eye. Whereas fibers in the b class show but little adaptation on repeating the stimulus, both a and c class fibers adapt immediately for a considerable
time in any part of their fields exposed, but not in others. They have therefore the quality of "novelty" fibers. All these fibers respond to switching lights on and off. Also present are fibers which continue firing during light exposure, and fibers activated by darkness, as well as small field fibers with special properties. These experiments, which must be considered as still of a preliminary nature, demonstrate the types of data analysis occurring in the optic ganglia and the way the messages are coded towards other parts of the central nervous system.

This work was done in collaboration with T. H. Waterman of Yale University.

81. "CROSS-TALK" BETWEEN THE OPTIC GANGLIA OF THE HAWAIIAN CRAB, PODOPHTHALMUS

Investigators: C. A. G. Wiersma, B. H. M. Bush
Support: National Science Foundation

It has already been noted in experiments conducted in Bermuda that impulses in the optic nerve not only travel from the eye to the brain but also in the opposite direction. In the present series of experiments this traffic was studied by removing the eye of the optic nerve from which the lead was taken, and exposing the other eye to light stimuli. The preparations lasted even longer than those described in the preceding report, because it was possible to ligate the artery and "plug-up" the exchange of the venous blood with the perfusion fluid, thus greatly diminishing mixing. The lead-off nerve was exposed in a small, separate chamber with the exposed eye fixed in position. In this way it was possible to more accurately measure the visual fields of different fibers by surrounding the eye with a ping-pong ball and projecting a light spot onto it. Reacting units can be divided into two main classes, those that react to visual stimuli only, and those that react, in addition, to tactile or proprioceptive stimulation. The latter will be described in the following report also. It may be noted here that among the present fibers are also included those which in addition respond to visual stimuli of the removed eye. For instance, there is good reason to believe that fast movement fibers (see preceding report under a) can respond to stimulation of both eyes as well as to touch anywhere on the body. It can be stated with fair certainty that all fibers reacting to more than one modality have large visual fields, but which do not necessarily cover the whole eye. Thus, one fiber was shown to react to the heterolateral body and to the whole lateral, but not to the medial, visual field. Among fibers reacting only to visual stimulation were found those reacting to medium and slow movements (see preceding report under b and c). The sampling was not extensive enough to be certain which types of fibers found in the report above do not have branches to the other eye, but the present impression is that small field fibers may fail to do so.

This work was done in collaboration with T. H. Waterman of Yale University.

82. CENTRIFUGAL IMPULSES IN INTERNEURONS OF THE OPTIC NERVE OF THE HAWAIIAN CRAB

Support: National Science Foundation

With the same preparation as mentioned in the preceding report a survey was made of interneurons reacting to tactile and proprioceptive stimulation of different parts of the head and body. Because the preparation had to be immobilized to a great extent, which involved binding the legs, it was not a favorable one for a more precise study of such aspects as determining which leg joints, and what type of movement of them, caused discharges in the specific interneurons.
The following observations may be extracted from the data. 1) Representation of small fields is more abundant for segments closer to the eyes than for more distant ones. Thus antennules and antenna are separately represented, but single legs are not. 2) Representation of purely homolateral, purely heterolateral, asymmetric and bilateral fields all were found, as they are in other parts of the crustacean central nervous system. 3) Interneurons responding exclusively to a single modality are in the majority, but it appears that the number responding readily to several modalities is larger here than at other levels. A considerable number appear to respond readily to both light and other modalities. 4) Individual interneurons can show very distinct firing patterns, typical for themselves, and differing from the patterns of others. All these findings are in good accord with previous observations in various crustaceans at other levels and tend to show that these types of interneurons, besides carrying each a modified aspect of the sensory input along parallel lines, also make parallel connections in a number of ganglia. The latter point is illustrated by such observations as that a crab with both eyestalks cut, still reacts to other than light stimuli in an apparently normal manner though, of course, all integration in the optic ganglia is lost. These experiments emphasize the facts that the optic ganglia are part of the brain, from which they embryonically arise, and that even between high order integrating centers, communication purely by nerve impulse traffic, without the opportunity for a significant role of intracellular or extracellular electric fields, is realized.

This work was done in collaboration with T. H. Waterman of Yale University.

83. MOVEMENTS OF EYESTALKS IN CALAPPA

Investigators: C. A. G. Wiersma, Alison A. Bush
Support: National Science Foundation

Crustaceans with movable eyestalks have almost invariably the added problem that in the withdrawn position of the eye the orientation in space is completely different from that in the extended position. Hence a great change in orientation of the visual field goes together with the protection offered by the withdrawal. It was noted that, in Calappa hepatica the outer segment of the eye, which bears the ommatidia, is elevated and withdrawn like a periscope (with at most a compulsory rotation of only a few degrees) through a kind of hole, formed at three sides by the carapace and at the fourth by a part of the antenna. It was observed, by operations, that this possibility is realized by the presence of a large cavity, surrounded by the carapace, in which the proximal segment of the eyestalk moves forward for extension and backward for retraction. The distal segment is very flexibly attached to the proximal one and takes up the slack resulting from the difference in position. By careful opening of this chamber from the back, leaving the "funnel" standing, normal movements could be observed in chronic preparations for a period of several weeks.

84. "DESCENDING" INTERNEURONS IN THE CIRCUMESOPHAGEAL COMMISSURES OF THE CRAYFISH

Investigators: C. A. G. Wiersma, Peter J. Mill
Support: National Science Foundation

Impulse traffic in interneurons in the commissures of the crayfish has been rather intensively investigated in older work (Wiersma, Ripley and Christensen, 1952; Wiersma, 1958). However, it was realized that in comparison with the "ascending" interneurons, the "descending" ones have been less intensively studied. The present investigation was undertaken primarily to obtain more information about interneurons activated by visual stimuli. In this respect the results have remained, so far, rather meager; the reason being, that notwithstanding a number of precautions, light reactivity disappears rather soon after preparation. In addition there is a
further difficulty (caused by changing thresholds) in determining, between preparations, whether or not the same unit reacts. It is certain that two fiber types are present, one reacting to light only, the other to light stimuli in combination with mechanoreceptive receptors. The total number of such fibers does not appear to be very different from the number of interneurons which react, e.g. to antennal stimulation. In some cases fibers reacting specifically to movement of objects in the visual field have been noted, but very few "light" units have so far become definitely established; that is to say, show consistent enough reactions in a number of preparations to allow confidence in their reaction to definite inputs.

A fair number of other interneurons from the head, from combinations of head and body and from the body alone, all giving descending impulses, have become established. Further evidence for most of the established fibers from the first series has been obtained. In addition, a considerable number of bilaterally reacting fibers have been found, most of which are "activity" fibers. Indications for some additional primary sensory fibers of specific regions, e.g. the antennal flagellum and anterior carapace, have also been obtained.

85. THE STRUCTURE OF THE EYES OF CRUSTACEA

Investigator: Peter J. Mill
Support: National Science Foundation

The structure of the crustacean eye received a certain amount of attention at the beginning of the century (e.g. Parker, 1891; Bernhards, 1916) but so little information has been obtained since then that it was thought necessary to investigate the eye structure of, specifically, Procambarus clarkii and Podophthalmus vigil, as a basis for the physiological work being carried out on the visual systems of these two species.

The eyes of both these species are composed of numerous ommatidia, which have certain features in common, e.g. the same number of retinula cells. The principal differences concern the shape of the facets of the ommatidia and the structure of the zone between the basement membrane and the lamina ganglionaris (first optic ganglion). The facets are square-shaped in Procambarus and hexagonal in Podophthalmus, but a square grid system will fit both. The cornea covering the eye is composed of at least two layers, secreted by the underlying corneal cells. Beneath the cornea each ommatidium consists of four crystal bodies, equal in size in Procambarus, but two small and two large in Podophthalmus. The crystal cells taper proximally to form the crystal stalk and, in Procambarus, become vacuolated in this region. Proximal to the crystal stalk lies the rhabdome, surrounded by the seven retinula cells. It has a laminated structure, this being especially noticeable in Podophthalmus, and appears to end on the basement membrane, the proximal processes of the retinula cells passing through the membrane and then running to the lamina ganglionaris. In Podophthalmus these processes run together in bundles to the lamina ganglionaris through a highly vascularized region. In Procambarus this region is much less vascularized.

86. CRAYFISH MULTIPLE INPUT INTERNEURON MODEL

Investigator: C. A. G. Wiersma
Support: National Science Foundation
National Institutes of Health, U.S. Public Health Service

A computer analysis program of crayfish interneurons with multiple inputs has been written for the IBM 7090. The program includes a quite realistic assumption about the effect of antidromic impulses upon the synaptic region. With this
assumption it has been demonstrated on the computer and analytically that for inputs all firing near a single unvarying frequency, an extremely strongly patterned output occurs. Furthermore, the number of impulses lost by collision is extremely small for frequencies even quite close to the maximum possible frequency. The program also computes the autocorrelation of the output to measure the regularity and type of patterning which occurs in the output.

87. SOME PRELIMINARY OBSERVATIONS ON THE EFFECT OF ASYMMETRIC VISUAL INPUT ON TURNING PREFERENCE IN THE CRAYFISH

Investigator: Edward Eisenstein
Support: National Science Foundation

Invertebrate behavior provides ample evidence of stereotypy. The marked uniformity seen in complex instinctive patterns leaves no doubt that the coupling of sensory input with motor output in these behaviors is, to a great extent, invariant.

In order to examine in greater detail the relationship existing between sensory input and motor output in the crayfish, a relatively simple unlearned behavior, viz. direction of turning at the choice point in a T-maze, was selected for study.

Following an initial training procedure in which the turning preference for each animal was determined, the animals were divided into four groups so that the number of right and left turns was as nearly equal as possible for all groups. Small pieces of transparent tygon tubing were then placed over the eyestalks of all crayfish. Three experimental groups were distinguished by whether the tubing covering the right eye, the left eye, or both eyes, was coated black, while the tubing over the eyestalks of the control group remained transparent.

Following the application of the tubing, the animals were again tested for side preference in the T-maze. The preliminary results indicate a marked tendency for the animals with one eye darkened to turn in the direction of the darkened eye at the choice point. Although the tubing remained on the eyestalks for the remainder of the experiment, no evidence of circling behavior was noted in these animals either in the home quarters or in the alleys of the maze. It was also noted that animals with both eyes darkened appeared to delay longer in leaving the start box, and took longer than the control animals to traverse the maze.

These results, although preliminary, suggest the importance of visual input in regulating the behavior of the crayfish.

88. CONDUCTIVITY CHANGES IN SOME ORGANS AFTER CIRCULATORY ARREST

Investigators: A. van Harreveld, Michael Biber

The relationship between extracellular electrolytes and tissue impedance has become a subject of interest since the latter provides a convenient method for the study of electrolyte shifts which occur under various conditions. In the past the impedance of central nervous tissue during spreading depression and after asphyxiation, and of the submaxillary gland during secretion have been studied. This examination has been extended to a number of other organs in which conductivity changes were measured after circulatory arrest. The liver showed a drop in conductivity of 85 to 90% during the first 30 minutes after circulatory arrest. In the kidney the loss was somewhat
less severe. Salivary glands lost 70 to 80% of their conductivity in 1.5 hours. In skeletal muscle the conductivity loss was small and developed late. The losses in conductivity were correlated with shifts of the extracellular ion, chloride, which can be studied in preparations treated with a histochemical method for this ion. In this context the electrolytes in the plasma of the vessels have to be included in the extracellular compartment. Extracellular electrolytes are lost either because they leave the tissue with blood which flows out of the organ after circulatory arrest, or because they are transported from the extracellular into the intracellular space.

Publication:


89. ASPHYXIAL IMPEDANCE CHANGES IN THE SPINAL CORD

Investigators: A. van Harreveld, Pieter Biersteker
Support: U.S. Navy, Office of Naval Research

Asphyxiation of the cerebral cortex produces after a latency of 2 to 3 minutes a sudden drop in electrical conductivity which may be as large as 30 to 40% of the pre-asphyxial value. This drop is accompanied by the development of a surface negativity of the cortex. The gray matter of the spinal cord also develops an asphyxial potential which, depending on the method used to produce O2 lack, has the much shorter latency of 8 to 20 seconds. Impedance measurements of the spinal gray matter have demonstrated an asphyxial conductivity drop of 10 to 15%. The latency of this change corresponded with that of the asphyxial cord potential, a fact supporting the postulate that the asphyxial changes in cortex and cord are essentially similar, notwithstanding the difference in latency. In the cortex the impedance rise is accompanied by a histochemically demonstrable transport of chloride into apical dendrites and a swelling of these structures. A search for comparable changes in the spinal cord has been made. Although the anatomical conditions for the application of the histochemical method for chloride are less favorable in the cord it appears that dendritic changes similar to those found in the cortex do occur during asphyxiation.

90. CORD POTENTIALS PRODUCED BY ACUTE OXYGEN DEPRIVATION

Investigators: A. van Harreveld, Pieter Biersteker
Support: U.S. Navy, Office of Naval Research

Arrest of oxygen supply to the spinal cord is followed after a short latency (8 to 10 seconds) by development of a large negativity of the gray matter. The potential field generated in the cord has been investigated. It can be described best by a system of two dipoles in the gray matter, one in the dorsal, the other in the ventral gray. The negative ends of the dipoles face each other in the center of the cord. Potentials recorded from the outside of the spinal cord support this two-dipole concept. The maximum negativity with respect to an outside electrode is measured in the center of the gray matter and is very considerable (of the order of 25 mV). Changes in pH of the cord and in concentration of other ions have been excluded as causes of this potential. It seems likely that the asphyxial potential is a function of neuronal elements since it cannot be demonstrated in spinal cords in which the nerve cells have been destroyed by a preceding asphyxiation. The best explanation is perhaps that it is due to a speedy depolarization of dendrites. There is evidence that the somas of nerve cells depolarize quite slowly (see below). Such a differential depolarization
could account for considerable potentials in the cord if it is assumed that the resulting microscopic dipoles of individual cells act to produce the two main dipoles found.

91. EFFECT OF OXYGEN LACK ON SOMA POTENTIALS

Investigators: Pieter Biersteker, A. van Harreveld
Support: U.S. Navy, Office of Naval Research

Microelectrode technique has been used to measure soma membrane potentials in the cerebral cortex and in the spinal cord. Although it has been possible to maintain a micropipette in a soma for the first minutes of oxygen deprivation, such a procedure is inadequate for an investigation of the slow depolarization of the somas. A statistical method has therefore been worked out in which a microprobe is slowly pushed through the entire spinal cord or cortex of rats and all sudden potential jumps indicating penetration of cell membranes are noted. In this way the membrane potentials of both nerve and glia cells are recorded. By repetitively exploring the cord or cortex in this way it is possible to determine the rate of depolarization of the cell membranes. It has been found that the spinal cord cells, after an initial speedy depolarization of the order of 10 mV, depolarize very slowly. It takes 60 to 80 minutes to reach a low value. The initial fast depolarization may be caused by the dendritic depolarization postulated above, conducted electrotonically to the soma. Even after an hour, reoxygenation of the cord restores the membrane potentials in 15 to 20 minutes. In the cortex depolarization occurs faster, low values being reached after 15 minutes. Also in the cortex reoxygenation causes a speedy recovery of membrane potentials, even after oxygen lack for 45 minutes.

92. CHANGES IN THE ELECTRICAL CONDUCTIVITY OF CEREBRAL CORTEX DURING SEIZURE ACTIVITY

Investigator: A. van Harreveld
Support: National Institutes of Health, U.S. Public Health Service

The rabbit may respond to Metrazol-induced cortical convulsive activity with a long-lasting depression of the spontaneous electrocorticogram, a slowly developing and declining cortical negativity and a marked drop in the electrical conductivity of the cortex. This reaction strikingly resembles spreading depression. However, since it is elicited simultaneously over a considerable cortical area, it may lack one of the features of spreading depression, namely the slow propagation. The rabbit may also respond to convulsive activity with negativity of much smaller magnitude, which is suggestive of an "abortive" spreading depression. Such a convulsion is followed by a short depression which may be ended by a new seizure when sufficient Metrazol has been injected.

The response to Metrazol convulsions of the cat resembled the second reaction forms observed in the rabbit. A moderate drop in cortical conductivity was recorded during the seizure, but no evidence for typical or even abortive spreading depression was found in this species. The drop in conductivity during typical and abortive spreading depression seen in the rabbit, as well as the drop during convulsive activity observed in the cat, may be indicative of a transport of electrolytes from an extracellular compartment into neuronal elements. The resulting change in the ionic composition of the cells and fibers thus affected could account for a decrease in cortical excitability which arrests convulsive activity.

This work was done in association with J. P. Schade of the Netherland Central Institute for Brain Research.
93. REFLEX NATURE OF ASPHYXIAL RIGIDITY

Investigators: A. van Harreveld, Pieter Biersteker
Support: U.S. Navy, Office of Naval Research

Reflex activity has been studied in spinal cords modified by asphyxial death of a considerable part of the interneurons and a smaller percentage of the motoneurons. The destruction of interneurons tends to enhance the excitability of monosynaptic reflexes, resulting in hypertone of certain muscle groups. It has been claimed recently that the rigidity is no reflex activity but is due to a spontaneous discharge of hyperexcitable motoneurons. This possibility has been investigated by examining the effect of stretch and relaxation of a muscle on its action potentials. Since the afferent impulses for the postulated monosynaptic reflex arise in stretch receptors in the muscle, manipulation of the muscle should affect its action potentials. If the rigidity is based on spontaneous discharge then no such effect could be expected. The experiments showed in all preparations exhibiting the rigidity a major increase of muscle action potentials by stretch of the muscle, indicating that monosynaptic reflex activity is the principal mechanism of the hypertone. As an alternate way of demonstrating the reflex nature of the rigidity dorsal spinal roots were transected, which interrupts the reflex arc. The rigidity can indeed be prevented by completely deafferentiating the relevant section of the spinal cord. Even a small rootlet left intact, however, is able to carry a sufficient sensory influx to produce rigidity.

94. SUBSTITUTION FIXATION OF NERVOUS TISSUE

Investigator: A. van Harreveld
Support: National Science Foundation

There is evidence suggesting that the distribution of water and electrolytes in the living central nervous system is different from that shown in electron microscope pictures of this tissue fixed by conventional methods. An attempt is being made to adapt substitution fixation of frozen tissues in alcoholic silver nitrate solutions to electron microscopy. Such a procedure allows determination of the location of chloride precipitated as its silver salt in the normal tissue. The technical difficulties are considerable since after vitreous freezing all procedures have to be carried out at low temperatures to prevent ice formation.

Publications:


95. AUDIO-MOTOR INTEGRATION IN SPLIT-BRAIN CATS

Investigator: Douglas B. Webster
Support: National Institutes of Health, U.S. Public Health Service

The present experiment was designed to explore the pathways and mechanisms utilized in the integration of sensory with motor areas of the cerebral cortex. Cats were trained to use the right paw (controlled by left motor cortex) to push either of a pair of pedals. Soft touch on either pedal activated a speaker to emit a 2,000 cps tone. Further depression of the pedal then resulted in food reward if the tone was on the animal's left, but only a bright light and withdrawal of the pedals if the tone came from the animal's right. Electrical connections of right and left pedals to the left (rewarded) speaker were randomly interchanged as were also speakers and sound intensity.

Midline sectioning of the forebrain commissures and thalamus following learning caused, at most, transient loss in performance. Additional removal of left auditory cortex caused complete loss of the discrimination but relearning proceeded as fast as original learning. Subsequent ablation of right sensorimotor cortex had no effect on the performance. Finally the right auditory cortex was removed, resulting in permanent loss of the directional discrimination. These data strongly suggest that integration of sensory and motor cortical areas can occur through subcortical (probably midbrain) neural pathways.

96. BEHAVIORAL CHANGES FOLLOWING HIPPOCAMPAL REMOVALS

Investigators: Douglas B. Webster, Theodore J. Voneida
Support: National Institutes of Health, U.S. Public Health Service

The split-brain preparation is utilized in the present investigation to study the role of the hippocampus, which continues to remain something of a functional enigma. Cats with unilateral hippocampal removals and midline sections of the corpus callosum and anterior commissure were trained to perform discrete tactile discriminations. This learning, involving the correct selection of one of two pedals by touch only, has previously been shown in split-brain cats to be learned only in the hemisphere contralateral to the forepaw used in pedal pushing. Split-brain cats with one hippocampus removed showed normal learning curves with either paw, but in reversal learning (positive pedal becomes negative and vice versa) the contralateral paw affected by the hippocampal removal took four to six times longer to reverse than the paw controlled from the intact hemisphere. Extinction of the response (neither pedal rewarded) took place readily with either paw and also transferred from one side of the brain to the other. Following extinction each paw was again trained to a high level of performance and a different type of extinction procedure was begun, in which the previously negative pedal, as well as the positive, was now rewarded. When subjects used the paw governed by the intact hemisphere they rapidly reached a chance level of performance but with the other paw they continued indefinitely to push the previously
positive pedal. Cats with bilateral hippocampal removals show difficulty in reversing the habit with either paw, although they too can learn a new problem as readily as normal animals. Extinction testing of the bilateral cases is in process.

These results support the suggestion that the hippocampus may play a major role in maintaining a type of functional plasticity, specifically the ability to make adaptive changes in an already stabilized habit.

97. INTERACTION BETWEEN CORTICAL AND SUBCORTICAL STRUCTURES IN THE PERFORMANCE OF A TACTILE DISCRIMINATION

Investigators: Theodore J. Voneida, Douglas B. Webster
Support: National Institutes of Health, U.S. Public Health Service

Cats with midline sections of all forebrain commissures, massa intermedia, and commissures of the superior and inferior colliculi, in addition to a massive unilateral cortical removal which spares only the frontal cortical region, can learn and retain discrete tactile discriminations with the paw contralateral to the cortical removal. A number of subcortical structures on the side ipsilateral to the cortical lesion have also been removed in these animals in order to ascertain what cortical-subcortical relationships are critical in the performance of a problem of this type. To date the learning has survived the combined removal of hippocampus, frontal half of the thalamus, head of the caudate nucleus, claustrum, and superior colliculus in addition to the cortical lesion. The caudate nucleus and anterior thalamus are the only structures the ablation of which resulted in an appreciable impairment in performance, and this was transitory. All surgery has been confirmed histologically. It would appear, therefore, that none of the above structures plays a critical role in the performance of a discrimination of this type.

On the other hand, complete loss of the habit was observed whenever any substantial damage was imposed on those structures involved in the classical sensorimotor pathways for tactile sensation, such as the posterior thalamus, internal capsule, or sensorimotor cortex.

98. CENTRAL MECHANISMS IN CONDITIONING

Investigator: Theodore J. Voneida
Support: National Institutes of Health, U.S. Public Health Service

An attempt is being made to analyze the means by which cats perform a conditioned withdrawal of one forelimb in response to a flashing light signal.

It has been demonstrated in an earlier phase of this problem that animals can be trained to perform at high levels of accuracy when the sensory input is restricted to one hemisphere and the motor output is restricted to the other, even though the crossed optic fibers, the forebrain and dorsal midbrain commissures, and the thalamus have been separated by midline section. Continued investigation has shown that there is only a slight, transient loss in performance levels when normal, fully trained animals are subjected to the above surgery. Furthermore, midline sections have now been extended caudally through the tegmentum and into upper pontine levels. All surgery has been confirmed histologically.

Tests of the untrained eye for transfer of the habit have proven completely negative in the above animals, suggesting that the conditioned signal remains on the side of the input. Removal of frontal cortex from the hemisphere opposite to the side of input completely abolishes the conditioned response, while other motor abilities are only slightly affected and show rapid recovery.
These data suggest that the conditional response may be organized in the contralateral frontal cortex on the basis of the unconditioned stimulus and general sensory information pertaining to the conditioning situation. It is further postulated, on the basis of the above results, that the information necessary for a motor response is constantly passing by way of efferent cortical pathways to opposite subcortical regions caudal to the midbrain where the final connection between conditioned stimulus and conditioned response is made.

99. CENTRAL CROSS-INTEGRATION OF VISUAL INPUTS PRESENTED SIMULTANEOUSLY TO THE SEPARATE EYES

Investigators: John S. Robinson, Theodore J. Voneida
Support: National Institutes of Health, U.S. Public Health Service

Cats were trained under normal visual conditions to select the brighter of two lighted windows. An attempt was then made to determine the capacity of various commissures to mediate perceptual integration of brightness inputs to the separate eyes. Initially, the inputs to the two eyes were separated by using red and blue filters over the light sources and in goggles over the subjects' eyes. Both inputs could still enter each side of the brain, however, since some of the optic fibers from each eye cross to the opposite side via the optic chiasm. Inputs to each hemisphere were then separated by sectioning the optic chiasm. Later, brain bisection was carried out in two stages, the first involving the corpus callosum and the habenular and hippocampal commissures; and the second the thalamus, anterior and posterior commissures, and the commissures of the superior and inferior colliculi. Tests of the animal's ability to integrate the brightnesses were made after each operation (following a 7-12 day period for convalescence). The results for the 4 animals which received the complete train-operate-test sequence, and for which histology has been completed are as follows:

1. With all central commissures intact, but with cross-integration via crossed optic fibers precluded by chiasm section, efficient integration of the two brightnesses is still possible. One animal showed only a very transient loss following the operation.

2. The capacity for integration survives section of the chiasm and the first stage of brain-bisection described above. The results from one animal indicate that integration does not require the corpus callosum, hippocampal or habenular commissures.

3. The second bisection results in a severe and prolonged depression of the capacity for integration. One animal with all forebrain and tectal commissures sectioned (a remnant of the commissure of the inferior colliculus remained intact) dropped to a chance level following surgery. With extended training, however, criterial performance (90% correct on each of 3 successive days, 60 trials per day) was again attained.

4. The results reported under (3) are due to the specific effect of interrupting structures mediating integration between brain-halves. If the chiasm is left intact when all forebrain and tectal commissures are sectioned (two animals), performance remains at a high level.

Additional animals are being given the same train-operate-test sequences to verify these initial findings.
Publications:


100. VISUAL LOSS FOLLOWING MIDLINE TEGMENTAL SECTION IN THE CAT

Investigator: Theodore J. Voneida
Support: National Institutes of Health, U.S. Public Health Service

Midline section of the optic chiasm, anterior, posterior, hippocampal and habenular commissures, corpus callosum, massa intermedia and commissures of the superior and inferior colliculi, even when performed in a single operation, has surprisingly little effect on an animal's activity. Deficits related to this surgery require special testing methods for their demonstration. If, however, the bisection is extended through the midbrain tegmentum, there is a complete loss of vision which lasts for 40 to 60 days postoperatively. There is a gradual but incomplete visual recovery following this loss, so that within approximately 90 days following surgery the animals show visual placing responses and can walk through complex mazes with very little difficulty. The maze must be well lighted, however. The visual deficits remain pronounced in dim lighting.

It is difficult to explain these results on a neuroanatomical basis, since there are no descriptions of a critical visual area in the midbrain tegmentum. Possibly the visual loss is due to a complete lack of visual integration between the two brain halves rather than to the destruction of a specific visual center. A surgical approach is presently being developed in which only the tegmentum will be bisected as a test of this possibility.

101. AN ANATOMICAL STUDY OF THE CONNECTIONS OF THE POSTERIOR COMMISSURE IN THE CAT

Investigator: Theodore J. Voneida
Support: National Institutes of Health, U.S. Public Health Service

Connections of the posterior commissure are being traced microscopically by the Nauta-Gygax technique for degenerated axons. Two approaches have been used for sectioning this structure. In the first, a direct surgical exposure is made, and the tract is sectioned by aspiration of its fibers in the midline. The second approach involves exposing the commissure as above, but instead of cutting it directly, a fine wire is looped around it. This wire is brought out through an opening in the skull, and the skin is closed around it. Four to six weeks later the wire is pulled, thereby cutting the commissure. This waiting period allows for degeneration to occur in any other structures which may have been damaged during surgery. The animal is sacrificed fourteen days after section of the posterior commissure, and the brain is stained according to the above technique for degenerated axons. It is now possible to differentiate between the recently degenerated axons and those which began degenerating at the time of the original surgery.

Fibers have been traced laterally into the tegmentum and to several structures surrounding the central gray regions of the midbrain. Further operations are planned, in which small electrolytic lesions will be placed in this structure, in addition to the complete sections as described above.
102. INTEROCULAR TRANSFER OF ALTERED VISUO-MOTOR COORDINATIONS IN SPLIT-BRAIN MONKEYS

Investigators: Joseph Bossom, Charles R. Hamilton
Support: National Institutes of Health, U.S. Public Health Service

Experiments described in last year's report have shown that normal Rhesus monkeys readily adapt the aim of their reaching to a lateral displacement of the visual world produced by wearing wedge prisms. The degree of adaptation is measured by the amount of overcompensation (prism aftereffect) that follows the removal of the prism. If adaptation is induced monocularly by occluding one eye and placing the prism before the other, it is found upon testing the unexposed eye that full adaptation is still present. The present experiment is designed to study the effect of split-brain surgery on this transfer.

Three Rhesus monkeys have been studied, two with midline section of the optic chiasma and forebrain commissures and the third with additional section of the midbrain commissures, massa intermedia, and the cerebellum. Histological verification of the surgery has been obtained for one of the animals. Since other reports had indicated strong contralateral eye-hand preferences following split-brain surgery, the animals were allowed free choice of hand use during all the experiments.

Complete transfer of the prism aftereffect to the unexposed eye was found in all three animals. In addition there was no contralateral eye-hand preference. All the monkeys chose to respond with their right hands regardless of which eye was initially exposed, and continued to use that hand when the other eye was tested, even though the right eye-right hand pairing presumably required visual and motor areas which were surgically separated. The animals were perfectly capable of using their left hands and often did so in other situations.

103. INTERMANUAL TRANSFER OF PRISM-INDUCED AFTER-EFFECTS IN SPLIT-BRAIN MONKEYS

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

In earlier experiments on normal monkeys the presence of partial intermanual transfer of prism-induced aftereffects of the kind described above was observed. Human subjects have also been reported to transfer such adjustments intermanually as well as interocularly. Three split-brain monkeys were tested for transfer in a series of experiments involving forced eye-hand combinations. All four combinations of eye-hand pairs were adapted in different experiments, and all combinations tested for aftereffects in each experiment. Good transfer was obtained from one eye to the other but there was no significant carry-over from one hand to the other. Additional normal controls are now being run to insure that intermanual transfer is significant in the normals under identical testing conditions.

The lack of intermanual transfer precludes the hypothesis that prism adaptation results from an altered judgment of eye position since such information should be equally available to either hand. Furthermore, a brain stem motor center receiving a downward flow of visual information from each hemisphere seems less likely since commissure section should not affect this type of information transmission. It now appears more probable that cortical motor centers capable of communicating with each other through the commissures but also receiving information from the opposite visual cortex via brain stem paths are involved.
104. INTERMANUAL TRANSFER OF PRISM-INDUCED AFTERRIGHTS IN HUMAN SUBJECTS

Investigators: Charles R. Hamilton, Joseph Bossom
Support: National Institutes of Health, U.S. Public Health Service

Helmholtz, quoting experiments of Czermak, states that intermanual transfer of prism adaptation occurs in man, and cites this as evidence for an altered judgment of eye position being the mechanism for the adjustments. Recently evidence for a lack of complete intermanual transfer in humans has been observed in this laboratory. In view of the importance of this to monkey experiments and the lack of precise data on human subjects, a systematic investigation has been started. To date eight subjects have been tested three times each. They were adapted by viewing one hand, which they moved back and forth, through binocular wedge prisms for fifteen minutes. Following this exposure, the aftereffects of each hand were determined and compared.

The mean intermanual transfer of altered aim in reaching from the exposed to the unexposed hand is around 50%. However, adaptation of the left hand (unpreferred in all subjects) leads to larger aftereffects in the exposed hand and smaller aftereffects in the unexposed hand than does the reverse (right hand) exposure. Whether this phenomenon is due to a right-hand preference or to the use of only base-left prisms in an otherwise balanced experimental design is currently being studied.

105. DECAY OF PRISM-INDUCED AFTERRIGHTS

Investigators: Charles R. Hamilton, Joseph Bossom
Support: National Institutes of Health, U.S. Public Health Service

Recent investigations from other laboratories have shown that voluntary movement and the resulting visual feedback (termed reafferent stimulation) are necessary for adaptation to prisms. A scheme involving a correlation between the outgoing motor signals and incoming visual stimulation has been presented to explain these results. Further extension of this mechanism to explain the initial acquisition of visuo-motor coordinations and their disruption in sensory deprivation experiments has been suggested.

The importance of reafferent stimulation for the disappearance of prism after-effects was tested in the present experiment. Eight subjects were adapted to the prisms and their aftereffects measured. The changes in the aftereffects after fifteen minutes of normal reafferent stimulation were compared with those obtained after fifteen minutes of sitting in the dark. Both conditions were accompanied by significant shifts back toward the normal coordination, though the reafferent decay was somewhat greater than the decay in the dark.

The shifts back toward normal coordination both with and without reafferent stimulation indicate that reafferent exposure is not necessary for the disappearance of an aftereffect, but that it apparently accelerates the process. The results suggest that reafferent stimulation is not the sole mechanism involved in maintaining visuo-motor coordinations.

106. BINOCULAR DEPTH DETECTION IN MONKEYS

Investigator: Colwyn Trevarthen
Support: National Institutes of Health, U.S. Public Health Service

An important part of visual depth or contour detection depends upon small differences in the images of the two eyes. It is not known where the images are
compared in the brain, but impulses from the two eyes are known to converge in the cerebral cortex. The binocular visual field is sharply divided in the vertical mid-line, the left field projecting the right cerebral hemisphere and vice versa. In the visual cortex of each hemisphere the half fields of the two eyes are neatly mingled in register. When the optic chiasm is cut all nerve fibers from the left eye die in the right side of the brain, and vice versa, and so the double representation of each half field is destroyed.

Three normal monkeys have been trained to choose the one of two black dots which appeared closer than the other. The apparent difference in the positions of the dots was caused by projecting differently spaced pairs of dots to the two eyes with the aid of polarized stimuli and polarizing filters in front of the eyes. When all three had reached high proficiency, the crossed optic fibers of the chiasma were cut in two cases, while in the third the anterior commissure and the corpus callosum were cut in addition. In all cases the operations resulted in complete loss of the ability to choose, and the score remained low for more than 500 trials of training. All showed good postsurgical retention by both eyes of a pattern discrimination task trained before surgery.

The loss of function in these animals is consistent with the view that visual patterns are compared in the cortex of each hemisphere. Apparently other ways of interocular comparison, either via the corpus callosum or in the brain stem, are not sufficient to maintain binocular depth detection.

107. TRANSCALLOSAL TRANSFER OF VISUAL LEARNING IN THE MONKEY

Investigator: Colwyn Trevarthen
Support: National Institutes of Health, U. S. Public Health Service

Experiments with chiasma-sectioned cats have established that simple pattern discrimination skills transfer from one hemisphere to the other as long as the corpus callosum is intact. More complicated tasks transferred imperfectly. There is one report of a comparable study in monkeys which includes the somewhat surprising conclusion that pattern discrimination learning usually fails to transfer across the corpus callosum in chiasma-sectioned subjects.

An assortment of visual tasks is being trained monocularly to two chiasma-sectioned monkeys, with subsequent tests of retention by the other eye providing the measure of interhemispheric transfer. The chiasma operations were made by a new approach from above between the frontal lobes. Both subjects have shown immediate and complete transfer of a color discrimination task (violet spot versus green spot) and a simple pattern discrimination task (black ring versus black square). There was imperfect transfer of a task requiring discrimination of a difference in orientation (T versus inverted T).

A control subject with corpus callosum and anterior commissure sectioned in addition to the optic chiasma has failed to transfer learning of the ring versus square task. A savings in the learning by the second eye is attributed to familiarization with the training situation with sharing of non-visual information between the cerebral hemispheres. This subject has also failed to show transfer of a color discrimination task although similar color tasks have previously shown a transitory transfer in animals with the same extent of surgery but with a different testing procedure (see Biology 1960 and 1961).
Though split-brain monkeys seem to move about with normal coordination of body movements and without postural abnormalities, and though they can coordinate the movements of the two hands in delicate tasks under visual guidance, they are quite unlike normals when they are trained with visual cues presented separately to the two eyes. Experimentally naive and recently operated subjects soon develop a hand preference in performance of visual discriminations as normals do, but when they are presented with two sets of stimuli simultaneously to the two eyes they generally learn with only one eye. In this case the eye which shows evidence of learning is always the one opposite to the preferred hand, a coupling which is obviously related to the crossing of motor fibers from each cerebral hemisphere to the contralateral hand. The active visual and motor areas thus come to lie in one hemisphere. If the other eye is forced to be used alone, by covering the favored one, the previously favored hand becomes poorly coordinated and makes misdirected movements. In a short time the subject "discovers" the use of the other hand and, after about 50 trials of practice, awkward conflicting moves of the first hand disappear and the shift of hands is complete. Learning by the second eye accompanies the change of hands, or follows shortly on it.

Even after both visual systems have completed independent learning with their respective contralateral hands, the split-brain subject is at first unable to perform well when forced to direct the right hand with the right eye and vice versa. These ipsilateral combinations are consistently malcoordinated in a way which suggests that the intention to move is intact but that visual monitoring necessary for the shaping of consistently correct responses is absent or imperfect. When a responding subject hesitates and looks for visual guidance his subsequent hand movements are awkward and arbitrary and they often appear cramped. From the start, familiar objects, such as peanuts, can be quickly recognized and grasped with fairly accurate but impulsive movements aided by any accompanying nonvisual cues. It is probable that recognition of the previously learned visual cues occurs from the start of this testing, but that it is blocked from motor expression by way of the ipsilateral hand.

With continued training the ipsilateral combinations of eye and hand come to function better and this improvement is transferred to new tasks in the same apparatus. During learning of this new coordination the movements are characteristically uncertain with periods of excellent discrimination followed suddenly by periods of vague, random responses. There is a preponderance of intermediate levels of choice from time to time. The sudden shifts of level of performance and the plateaus of intermediate score may reflect competition between the functions of the two cerebral hemispheres. Experiments are under way to seek more direct evidence of this kind of competition.

The defects of eye-hand coordination in split-brain monkeys are not found in cases in which the chiasma alone is sectioned. In the latter both visual areas and motor areas of the two hemispheres have cross-communication through the corpus callosum. Observations will continue with subjects in which the posterior, visual connections of the corpus callosum are cut while the anterior parts, more directly concerned with motor functions, remain intact.
Two split-brain subjects, one with surgery extended to include division of the roof of the midbrain, have been trained with both eyes to choose the larger of two circles. They were then able to choose correctly when one circle was presented to each eye. One of the subjects has also been able to choose the more tilted of two simple figures presented in the same way as the circles, but this same monkey failed to relate the orientation of a black bar seen by one eye to a striped background seen by the other eye. Both eyes had been trained to high proficiency with the bars and backgrounds projected overlapping and both visible to each eye.

In a recent experiment a split-brain monkey had been trained to push one of two colored screens on the basis of a match to the color of a third screen. When able to do this task well using either eye alone, the subject also obtained significant scores with the visual information divided between the two eyes.

The binocular visuomotor coordination involving visual mechanisms of both hemispheres is less efficient in these animals than intrahemispheric coordination. Even after extended training the performance is erratic and the average level of proficiency is about midway between random and perfect performance. Hand movements in responding are hesitant as the decision is made, and sometimes they are distorted. It is possible that, once a scheme for attending to the two hemispheres has been learned, a large part of the difficulty of this task arises from the unequal connections between the two eyes and the hand which makes the responses.

Previous experiments have demonstrated concurrent double learning of conflicting visual discrimination tasks presented simultaneously to the separate eyes of split-brain monkeys. The following was aimed at the question of whether the separated hemispheres work simultaneously in parallel in the above situation or whether the double learning is achieved by rapid alternation of attention from one hemisphere to the other.

Two split-brain Rhesus monkeys (one forebrain, one fore and midbrain) were taught visual discrimination problems, first binocularly and then monocularly, until the problem could be performed at a high level when presented in random alternation to either eye. The same patterns were then presented tachistoscopically at brief exposure times, again on an alternating schedule that prevented anticipation of the eye to be used. Under these conditions the subjects were able to attain significant levels of performance for exposure times as short as 0.1 seconds. The results indicate that the subjects were capable of attending simultaneously to visual stimuli with both of the separated hemispheres.
111. SOME EFFECTS OF SECTIONING THE CEREBRAL COMMISSURES IN MAN

Investigator: Michael S. Gazzaniga
Support: National Institutes of Health, U.S. Public Health Service

It has been possible in studies of callosum-sectioned cats and monkeys in recent years to obtain consistent demonstration of a variety of interhemispheric integrational functions mediated by the corpus callosum. These animal findings have stood out in marked contrast to the apparent lack of corresponding functional deficits produced by similar surgery in human patients. The general picture of callosal functions based on the animal studies tends to be supported in current early testing of a 48-year-old male war veteran with recent complete section of the corpus callosum, anterior and hippocampal commissures.

Tests of tactual, visual and motor functions have been carried out both pre- and postoperatively. The results indicate that commissurotomy in the adult human gives rise to a genuine callosal syndrome including a left agnosia and anomia for both visual and tactual tasks. The subject locates accurately points of tactile stimulation on the fingers of either hand by touching with the thumb of the same hand, but is quite unable to cross-locate from either hand to the other. Responses to symbolic visual stimuli are appropriate only when the stimuli are projected to the hemisphere effecting the response. The subject also demonstrates a pronounced left apraxia to verbal commands. While individuated finger movements with good hand and arm coordination are exhibited in automatic-type movements, these same movements became impossible to carry out in response to a verbal request. More detailed analysis of the impairments is in progress.

Dr. J. E. Bogen of the White Memorial Hospital is collaborating in this study.

112. ATTENTION AND CEREBRAL DOMINANCE IN SPLIT-BRAIN MONKEYS

Investigator: Michael S. Gazzaniga
Support: National Institutes of Health, U.S. Public Health Service

I. Effects of commissurotomy on preoperative acquired visual discriminations

In the normal human it is apparent that the two cerebral hemispheres do not carry out identical tasks nor is their organization similar. The presence of handedness in the monkey has been reported, but there has been no systematic analysis of this in relation to the cerebral hemispheres, nor of any other asymmetrical cerebral characteristic. Indeed, very little is known concerning the physico-anatomical factors involved in the development and organization of lateral dominance.

In the present study two normal monkeys forced to use each hand an equal number of times during training, learned binocularly a visual discrimination problem. As soon as these animals eventually acquired the task, every eye-hand combination was able immediately to perform at criterion. After one animal had been overtrained and the other not, both underwent midline section of the optic chiasm and forebrain commissures. Thereafter these same animals were able to perform at criterion with only one of the four eye-hand combinations that formerly were proficient. The one that survived in both cases involved visual and motor centers within the same hemisphere.
The results suggest that the monkey tends to learn visual discriminations in one dominant hemisphere instead of bilaterally as indicated in earlier results with the cat.

II. Unequal facility for visual learning in each half brain

In a previous study it was indicated that a normal monkey may retain a visual discrimination with only one eye-hand combination subsequent to midline section of the optic chiasma and forebrain commissures. Postoperatively, these animals, when trained new discriminations binocularly using each hand equally, demonstrate that one dominant eye-hand combination acquires the task with greater facility and speed than the other three. Furthermore, when the deficient eye-hand combinations are force-trained, it is the opposite mirror-image intrahemispheric combination which learns next, followed later by the interhemispheric eye-hand pairs. It is also of interest to note that the mirror image eye-hand pairs do not learn at equal rates. This finding seems to be in marked contrast to the superimposable learning curves that have been found in the cat and in some of the tactual learning studies in split-brain monkeys.

The data suggest that split-brain monkeys during initial visual training seem to favor one hemisphere and that under forced training conditions the other hemisphere learns with much less facility than the hemisphere naturally preferred by the animal.

III. Capacity of split-brain monkeys to attend with non-dominant eye

The concepts of attention, eye dominance, and preparatory set are poorly understood in physiological terms. By using the split-brain preparation new questions may be asked that might possibly give some insight into the neural organization. Can split-brain animals voluntarily attend with one eye and can they voluntarily choose which of the separated hemispheres to use? If so, where in the brain is such a decision made and through what pathways is it effected? On the other hand, if such a volitional decision is not possible, why so?

As an approach to the above, a split-brain monkey was trained on a visual discrimination to criterion with every eye-hand combination. Subsequently, using the Polaroid technique of Trevarthen, these same visual stimuli were simultaneously presented to both eyes. However, the reward for the dominant eye was then randomized so that the problem was insolvable in this hemisphere while the test conditions for the non-dominant eye remained unchanged. The results suggest that it is extremely difficult for the split-brain monkey to voluntarily attend through the non-dominant eye. However, with continued training (4,000 trials) the animal eventually learned to do this. However, this ability to attend to and respond to stimuli entering the non-dominant hemisphere while simultaneously ignoring the same stimuli entering the dominate hemisphere did not generalize to discriminations other than the one so trained. Under the above testing conditions in which the split-brain case continued to work with apparent enthusiasm, a normal control has become totally frustrated and refuses to work.

113. OPERANT TECHNIQUES OF STUDYING INTERHEMISPHERIC TRANSFER OF VISUAL DISCRIMINATION LEARNING IN SPLIT-BRAIN MONKEY

Investigators: Herman Teitelbaum, Joseph Bossm
Support: National Institutes of Health, U.S. Public Health Service

In this experiment subjects are trained to perform a particular response in order to obtain a food reward from an automatic dispenser. When this behavior has been learned a high and steady rate of response is obtained by presenting the reward
irregularly according to a variable response interval schedule. Three distinctive visual stimuli are sequentially presented while the animal is "working" for food. Initially equal rates of response are emitted during the presentation of the various stimuli. By selective reward and punishment one can alter the rates at which responses are emitted during the presentation of the various stimuli. This change in frequency may then be taken as a measure of the efficacy with which the animal discriminates between the various visual forms.

By restricting visual input to one eye and responses to the hand opposite that eye it is possible to compare the interhemispheric transfer as well as rates of learning for two kinds of discrimination. In the present experiment discrimination based upon brief shock is compared to discrimination produced by not rewarding responses to a particular stimulus. The interhemispheric transfer of each of these discriminations is being studied in monkeys with midsagittal section of optic chiasma, corpus callosum, anterior, posterior hippocampal and habenular commissures. Previous reports have indicated that in cats with only the optic chiasma and corpus callosum sectioned, discriminations based upon selective food rewards do not transfer to the untrained hemisphere, whereas discriminations based upon shock do. With the preparation used here it will be possible to determine whether any of the additional structures here sectioned affect the interhemispheric transfer of discriminations based upon punishment.

114. EXPERIMENTAL ANALYSIS OF THE EFFECT OF UNILATERAL TEMPORAL LOBECTOMY IN SPLIT-BRAIN MONKEYS

Investigators: Joseph Bossom, Herman Teitelbaum
Support: National Institutes of Health, U.S. Public Health Service

Bilateral removal of the temporal lobes in monkey produces a dramatic and complex syndrome of behavioral changes including increased tameness, sexual activity and appetite in addition to certain deficits in gnostic functions. Aspects of this syndrome may also be observed in the split-brain monkey with unilateral temporal lobectomy when visual input is restricted to the operated hemisphere. In the experiments here described operant conditioning techniques for analyzing behavior are employed to examine some of these behavioral changes in further detail.

The tameness ascribed to this syndrome is in actuality a raised threshold for fearful responses such that the animal does not react to visual stimuli which frighten normal monkeys. The purpose of this experiment is to determine whether this increased "tameness" is a result of a motivational change of simply another manifestation of a gnostic defect such that the monkey no longer recognizes the significance of the stimuli.

The rate at which the monkeys learn to discriminate between two stimuli will be studied when the discrimination is based upon shock avoidance for incorrect responses and when the discrimination is based upon food reward for response to only one of the stimuli. The extent to which either of these forms of discrimination learning invokes emotion in the subjects will be measured by obtaining a continuous record of changes in electric conductivity of the skin (G.S.R.) during training. By comparing the rate at which these discriminations are learned in the operated and unoperated hemispheres, it will be possible to separate out gnostic from emotion deficits. In addition, the interhemispheric transfer to and from the operated hemisphere will be tested for both types of discrimination learning.
115. EFFECTS OF UNILATERAL HIPPOCAMPAL AND ORBITOFRONTAL LESIONS UPON PERSEVERATIVE BEHAVIOR IN THE CAT

Investigator: Herman Teitelbaum
Support: National Institutes of Health, U.S. Public Health Service

The most characteristic change in behavior after removal of the frontal poles in animals has been shown to be an exaggerated tendency to perseverate in learned behavior which in the past was rewarded, but which no longer yields any positive reinforcement.

In work performed at McGill University, this author has shown that bilateral hippocampal lesions in rats result in a similar deficit. Webster and Voneida have repeated and extended those findings with unilateral lesions in the split-brain cat. Experiments are under way in an effort to compare these two types of lesions, i.e. frontal and hippocampal, one in each hemisphere in split-brain cats. The severity and qualitative nature of the effects of the two lesions can thus be compared in the same animal, thereby removing any variation due to individual differences in ability and motivation. Thus far the animals are still involved in the series of pretraining surgical operations including sections of the corpus callosum and anterior commissure, and the unilateral hippocampal and orbital frontal removals.

116. OPTIC NERVE REGENERATION AFTER SURGICAL CROSS-UNION OF MEDIAL AND LATERAL OPTIC TRACTS

Investigators: Harbans L. Arora, R. W. Sperry
Support: National Institutes of Health, U.S. Public Health Service, Frank P. Hixon Fund

In fishes and amphibians excellent visual recovery with respect to color, directionality, and acuity has been observed to follow section and regeneration of the optic nerve. It has been shown that the regenerating fibers not only find their former terminal loci in the optic lobe, but that they reach these terminals by preferentially following their original central pathways. In the present experiment, the medial and lateral tracts of the optic nerve in the cichlid fish, Astronotus ocellatus, were teased free, sectioned separately, and cross-directed each toward the opposite central stump in order to determine whether the nerve bundles, if deliberately directed into the wrong channels, would grow into the foreign tracts and perhaps establish connections in foreign regions of the tectum. After visual recovery in 30 to 40 days, the fish were able to follow moving objects and to localize them correctly. Histological study of the crossed regenerated tracts indicates that each fiber bundle, instead of growing ahead into the foreign channel, crossed back toward its original pathway and entered its own sector of the tectum. The results indicate the strength of the tendency on the part of the regenerating optic fibers to avoid foreign channels and to follow their own central pathways. Additional studies are under way to further determine the degree of precision with which the growing axons may be directed to specific "loci" in the tectum.

117. ROLE OF COMMISSURAL FIBERS IN INTEROCULAR TRANSFER OF VISUAL DISCRIMINATIONS IN FISHES

Investigator: Harbans L. Arora
Support: National Institutes of Health, U.S. Public Health Service

Investigations on the localization of learning and memory in the brain of fish, reported in Biology 1961 have been extended to include the role of commissural fibers in interocular transfer. It was reported that extirpation of the optic lobe of the
brain connected to the trained eye had no effect on transfer of the previously trained visual discriminations. It was inferred that such transfer may be due to the simultaneous establishment of two memory trace systems, one in each optic lobe of the brain; or alternatively, to the drawing by the untrained side upon traces remaining in the sub-tectal centers on the trained side after removal of the trained tectum. The present experiment involves one series of fish in which the two principal commissures (tectal and posterior) connecting the midbrain were sectioned prior to the training of one eye and another series in which sectioning followed training. The fish are being tested for indications of interocular transfer of color and pattern discriminations.

Publications:


118. STACKING INTERACTION OF THE BASES IN NUCLEIC ACIDS

The physico-chemical principles concerned with the interaction, structure, and stability of nucleic acids are of importance to molecular biology. Attention in the past has been focused on the hydrogen-bonding scheme, the base pairing specificity (A to T and G to C) and the relative strengths of the base pairs (G-C, A-T). In recent years, the significance of the contribution of the hydrophobic and stacking interaction of the bases in the nucleic acids has been recognized. Early work in this laboratory on the behavior and denaturation of nucleic acids in organic solvents was related to this aspect of nucleic acid chemistry. More recently, more direct and quantitative data related to the stacking interaction of the bases in nucleic acids have been obtained. This new information is briefly summarized below.
I.  INTERACTION OF BASES AND NUCLEOSIDES IN HOMOGENEOUS SOLUTION

Investigators:  Paul O. P. Ts'o, Ingelore S. Melvin, Alfred C. Olson
Support:  National Institutes of Health, U.S. Public Health Service
          U.S. Department of Agriculture

Osmotic coefficients of purine, uridine and cytidine at 25°C in the concentration range of 0.1 to 1.0 molar have been determined by thermoelectric measurements of vapor pressure lowering of these homogeneous solutions. Through the help of the computer (IBM 7090), fourth degree polynomials were constructed by the method of least squares to correlate the molar concentrations and the osmotic coefficients. Direct integration of these polynomials in the Gibbs-Duhem equation yields the activity coefficients of purine, uridine and cytidine from their respective osmotic coefficients. Both the activity coefficient and the osmotic coefficient data indicated that these solutes have a strong tendency to associate in aqueous solution.

Analysis of the data indicates that the association process does not proceed simply to the dimer stage, but continues, and can be approximated by a situation in which the association processes have equal equilibrium constants for all successive steps in the formation of polymers. Equilibrium constants at 25°C and standard free energies for association of purine, uridine and cytidine were found to be 2.1, 0.6, 0.8 and -440, 290, 80 cal/mole respectively. The equilibrium constant and standard free energy for the association of urea in water are, respectively, 0.04 and 400 cal/mole.

The mechanism of the association of bases and nucleosides in solution is most probably a vertical stacking interaction and is not due to horizontal hydrogen-bonding interaction. This concept is supported by the fact that the equilibrium constants for the association of these ring compounds are much larger than that of urea, which is an excellent hydrogen-bonding agent. In summary, two conclusions can be drawn from the data.

(a) The vertical stacking interaction of base and nucleosides is substantial and is stronger than the hydrogen-bonding interaction of urea in water.

(b) Purine-purine interaction is stronger than pyrimidine-pyrimidine interaction.

II.  INTERACTION BETWEEN VARIOUS BASES AND NUCLEOSIDES IN HETEROGENEOUS SOLUTION

Investigators:  Paul O. P. Ts'o, Ingelore S. Melvin, Alfred C. Olson
Support:  National Institutes of Health, U.S. Public Health Service
          U.S. Department of Agriculture

In studying the interaction of one base with another, such as purine and pyrimidine, a different approach was adopted. This interaction was examined by measuring the increase of solubility of the sparingly soluble adenine-C^14 or thymine-C^14 in the presence of varying concentrations of a variety of interacting substances (I), such as purine, pyrimidine, cytosine, uridine, cyclohexanol, adonitol, urea, etc. at 25.5°C. Adenine and thymine were found to be much more soluble in solutions of purine, nucleosides, pyrimidine and phenol, while additions of cyclohexanol, adonitol and urea had negligible effect. The decrease of activity coefficient of adenine and thymine in such solutions of these substances ranges from 30 to 60% at 0.2 M and from 50 to 85% at 0.6 M. The data were analyzed on the assumption that the concentration of the total base-C^14 in solution consists of the contribution from the free base as governed by the solubility of the solid and the contribution from the water-soluble
base-I complex governed by the association process. Though definite equilibrium constants for these association processes cannot be assigned because of uncertainty in the stoichiometry, minimum equilibrium constants can be estimated. These minimum equilibrium constants clearly indicate the comparative strengths of the interactions between purine and pyrimidine in the following series:

Purine-purine (adenine-purine) > purine-pyrimidine (adenine-cytidine or adenine-uridine) > pyrimidine-pyrimidine (thymine-uridine or thymine-pyrimidine).

Experiments carried out at higher temperature (38 °C) indicate a lowering of the minimum equilibrium constants and thus a lowering of the minimum standard free energy change. A negative entropy contribution is therefore indicated in these association processes.

III. INTERACTION OF NUCLEOSIDES AND RELATED COMPOUNDS WITH NUCLEIC ACIDS AS INDICATED BY THE CHANGE OF HELIX-COIL TRANSITION TEMPERATURE

Investigators: Paul O. P. Ts'io, Christian Sander
Support: National Institutes of Health, U.S. Public Health Service

A series of compounds has been tested for effectiveness in lowering the melting temperature of helical poly A and of thymus DNA by measurements of optical rotation. The order of increasing activity was found to be: adonitol, methyl riboside (both negligible) < cyclohexanol < phenol, pyrimidine, uridine < cytidine, thymidine < purine, adenosine, inosine, deoxyguanosine < caffeine, coumarin, 2,6-dichloro-7-methylpurine. Urea was ineffective with poly A and only slightly effective with DNA. At a concentration of 0.3 M, purine lowered the melting temperature of DNA about 9 °C.

These results suggest that the mechanism of the interaction of these bases, nucleosides, and related compounds with the helical nucleic acid is not necessarily involved with hydrogen-bonding but rather with hydrophobic and stacking interactions. This interpretation now receives further support from the above experiments (reported in sections I and II) concerned with vapor pressure lowering and solubility measurements. The tendency of these compounds to associate in water correlates positively with the effectiveness of these compounds in reducing the stability of helical form of nucleic acids. For example, the preference of the following compounds to associate as indicated by osmotic coefficient and equilibrium constants measurements is: Caffeine > purine, inosine > uridine, cytidine. This same relationship also holds true for the effectiveness of these compounds in lowering the melting temperature of DNA and helical poly A as reported in the preceding paragraph.

This work was done in association with G. K. Helmkamp, University of California, Riverside.

IV. BINDING OF STEROIDS AND BASES ONTO NUCLEIC ACIDS

Investigators: Paul O. P. Ts'io, Ponzy Lu
Support: National Institutes of Health, U.S. Public Health Service

Results in the above sections suggest that those aromatic, ring compounds may have a tendency to bind with nucleic acids by means of stacking and hydrophobic interaction. This notion is now being pursued by studying the binding of tritium-labeled steroids to nucleic acids by the method of equilibrium dialysis at 5 °C. Estradiol-β-17 (female sex hormone) was chosen because of the aromaticity of the "A" ring which makes that portion of the molecule planar. Significant binding was
found between this steroid and the poly A coil and the denatured DNA. The binding constant of estradiol-$\beta$-17 to poly A is approximately 10-fold higher than that of thymine, again indicating the importance of hydrophobic interaction in comparison to the hydrogen-bonding interaction. The relationship of the configuration of the steroids and the conformation of the nucleic acids to this binding process is now under active investigation.

Publications:


119. STRUCTURE OF HELICAL POLYCYTIDYLIC ACID

Investigators: Ezekiel Akinrimisi, Christian Sander, Paul O. P. Ts'o
Support: National Institutes of Health, U.S. Public Health Service

In acidic aqueous solution, synthetic polycytidylic acid assumes a compact and helical conformation. Information with regard to the hydrogen-bonding scheme and structure of the helical poly C is of value and interest.

This matter has been studied by optical absorbancy, optical rotatory dispersion and viscosity at various temperatures, pH's and ionic strengths. The findings can be summarized as follows:

1. At a given ionic strength (0.1 M, sodium acetate), the melting temperature ($T_m$) of poly C increases with lowering of pH until the pH reaches 4.0. The $T_m$ then decreases as the pH decreases to lower values. Thus, the stability of poly C in response to proton concentration can be classified into two zones, i.e. proton stabilization zone (pH 5.5 and higher to pH 4.0) and the proton destabilization zone (from pH 4.0 to pH 3.5 and lower). The same value of $T_m$ is obtained from the O.D. versus temperature profile and from the specific rotation at 436 m$\mu$ versus temperature profile.

2. In the proton stabilization zone, increase in ionic strength of the solution lowers the $T_m$ and reduces the stability of the helix, as is the case with the acidic poly A helix. In the proton destabilization zone, increase in ionic strength raises the $T_m$ and enhances the stability of the helix, a response similar to that of DNA.
3. Optical rotatory dispersion studies indicate a major change in $\lambda_C$ and in $K_m$ (obtained in the one term Drude plot) of poly C as the material goes from the neutral or alkaline form to the acidic form. On the other hand, all constants of the one-term Drude plot of poly C are essentially identical in the proton stabilization zone and in the proton destabilization zone. The data fit well with one-term Drude equation.

4. Viscosity measurements indicate that the acidic form of poly C has a low intrinsic viscosity (0.25 dl/g) and that the reduced viscosity is essentially polymer concentration and ionic strength (0.05 to 0.5 M NaCl) independent. The alkaline form of poly C, on the other hand, has an intrinsic viscosity (at 0.05 to 0.57 NaCl of 0.77 to 0.69 dl/g). The reduced viscosity of alkaline poly C is markedly concentration and ionic strength dependent.

The data suggest that poly C in acidic solutions forms a compact and helical structure. The initial addition of protons to the ring nitrogen tend to stabilize the helix. After a certain number of protons have been added further additions to the helix tend to destabilize it without apparent or gross change in its structure. This information supports the idea that the hydrogen-bonding scheme of poly C helix is similar to that of cytosine-5-acetic acid in crystal form as determined in this institution by Marsh, Bierstedt and Eichhorn (Acta Cryst. 15, 310, 1962). This scheme consists of one pair of hydrogen bonds between two strains, A and B, i.e. the $-\text{NH}_2$ of cytosine$_A$ to $\text{CO}$ of cytosine$_B$ and the $\text{CO}$ of cytosine$_A$ to $-\text{NH}_2$ of cytosine$_B$. Furthermore, a proton is located between and shared by the N$_1$ atoms of two cytosines in the center of the helix. Thus, this helix is stabilized by both hydrogen bonding and the charge effect of the added proton.

120. PURIFICATION OF TYROSINE AND METHIONINE ACTIVATING ENZYMES

Investigators: Joan M. Wallace, Paul O. P. Ts'o
Support: National Institutes of Health, U.S. Public Health Service

Purified amino acid activating enzymes from baker's yeast are being prepared for use in studies of the nature and properties of amino acid binding by the enzymes in the presence of their specific soluble ribonucleic acids. Baker's yeast contains large amounts of the tyrosine and the methionine activating enzymes. These two enzymes have been purified more than 20-fold by ammonium sulfate fractionation. Further purification is in progress. The partially purified preparations when frozen retain their enzyme activities for at least three months.

Enzyme activity has been determined by colorimetric measure of the amino acyl hydroxamate formed from adenosine triphosphate and the specific amino acid in the presence of hydroxylamine. Other compounds which contain activated acyl groups react with hydroxylamine to form hydroxamates and therefore interfere. By the use of radioactively labeled amino acids in the same assay procedure, to yield labeled hydroxamates, the sensitivity of the method has been increased. The amino acids and their hydroxamates can be separated by selective binding on cation exchange resin. This principle is the basis for the assay procedure being developed, the preliminary results of which are promising. This more sensitive and specific assay will facilitate the fractionation and isolation of the activating enzymes.
121. STUDIES ON DENATURATION AND RENATURATION OF T7 DNA

Investigator: F. William Studier
Support: McCallum Fund
General Funds

Conditions for optimal renaturation at 25°C in a formamide-water-salt system have been determined. It has thus been possible to study the complete cycle of denaturation and renaturation under conditions which do not degrade DNA.

Contrary to expectations the kinetics of denaturation of the homogeneous T7 DNA (as followed by melting profile and cesium chloride density gradient banding of samples removed from the denaturing conditions) are not first order. Depending on conditions of formamide, salt, pH, and temperature the high initial rate of denaturation rapidly decreases, and in some cases the denaturation reaction appears to stop at a partially denatured condition. All attempts to demonstrate equilibrium between native and fully denatured DNA under these conditions have failed. The kinetics of denaturation in dilute aqueous salt solution at elevated temperatures are similar to those observed in the formamide system.

Studies on CsCl banding molecular weight of T7 DNA at low concentrations give a value of 16-19 x 10^6 for the anhydrous sodium molecular weight. The total DNA content of the T7 phage as determined by Davison is 19 x 10^6 grams per mole of phage. Thus the value of the molecular weight obtained by the CsCl density gradient banding method is very close to the best estimate of the true molecular weight for this DNA.

122. RESOLUTION OF CHROMATIN INTO ITS CONSTITUENT ENTITIES

Investigators: R. C. Huang, James Bonner, Hedi Imfeld
Support: National Institutes of Health, U.S. Public Health Service

It has earlier been reported that isolated chromosomes of pea embryos possess the ability to carry out DNA-dependent RNA synthesis. Such synthesis requires the simultaneous presence of the four riboside triphosphates and in the synthesis inorganic pyrophosphate is eliminated.

Chromatin, as isolated in this laboratory from pea embryos, consists of DNA, RNA (in part at least recently synthesized), histone and non-histone chromosomal protein. The enzyme, RNA polymerase, which catalyzes DNA-dependent RNA synthesis, appears to be bound as an integral part of the chromosomal structure. An elegant procedure has been found for the separation of nucleic acid from the protein of chromosomes; it consists in the dispersal of chromatin in 4 M cesium chloride (CsCl). At the ionic strength of 4 M cesium chloride (CsCl), protein and nucleic acid become dissociated from one another. At the density of 4 M CsCl, 1.4, nucleic acid sinks while protein floats. The suspension is therefore centrifuged and the nucleic acid collected as a pellet and the chromosomal protein as a skin which floats upon the surface of the solution.

It is of interest that dispersal in 4 M CsCl results in resolution of the chromosomal structure which originally consisted of large aggregates sedimentable at 4,000 x g. After the 4 M CsCl treatment, the DNA of the chromosomal structure is recovered as molecularly dispersed material of sedimentation constant approximately 18S. The bonds which hold the chromosomal structure together appear therefore to be ionic ones. Non-histone chromosomal protein is recovered from the skin protein, from which it may be extracted with low ionic strength neutral buffer. The chromosomal histones which are insoluble in this medium may be dissolved in either acid solution
(0.2 N HCl) or in solution of high ionic strength (1 to 2.5 M NaCl). These methods have been applied to the investigation of the functions of the several chromosomal entities.

123. PURIFICATION AND PROPERTIES OF CHROMOSOMAL RNA POLYMERASE

Investigators: R. C. Huang, James Bonner, Hedi Imfeld
Support: National Institutes of Health, U.S. Public Health Service

Non-histone protein constitutes 15 to 20% of the mass of chromatin as prepared from pea embryos. Such non-histone protein includes the enzyme RNA polymerase, which is responsible for the DNA-dependent synthesis of RNA.

Separation of the bulk of chromosomal protein from the bulk of the chromosomal nucleic acid by centrifugation in 4 M cesium chloride yields a nucleic acid pellet, which, although molecularly dispersed, still contains bound to it approximately 4% by weight of protein. This DNA-bound protein includes a portion of the chromosomal RNA polymerase, since the preparation is active in DNA-dependent RNA synthesis. By cesium chloride fractionation the specific RNA synthesizing activity of the nucleic acid bound RNA polymerase is increased approximately 1,000-fold above that of the initial crude chromatin.

Although a portion of the chromosomal RNA polymerase remains associated with DNA during high salt concentration-induced disaggregation of chromatin, a further portion of the enzyme is released and accompanies the histone. Non-histone protein may be extracted from the whole chromosomal protein with low ionic strength (0.05 M tris) buffer (pH 8.0), in which the chromosomal histone is insoluble. The thus extracted non-histone chromosomal protein contains RNA polymerase activity, and this has been purified by streptomycin treatment and ammonium sulfate fractionation. By these means the enzyme has been increased in specific activity approximately 135-fold above the level found in crude chromatin itself.

Purified soluble chromosomal RNA polymerase contains bound to it DNA, and the activity of the purified enzyme, although responsive to added exogenous DNA, is not wholly dependent upon such addition. All methods thus far examined for the separation of the DNA bound to chromosomal RNA polymerase have resulted either in no separation or in denaturation of the enzyme. Such methods include, for example, centrifugation of the purified enzyme in 4 M CsCl (which results in pelleting of the enzyme-bound DNA), treatment with DNase followed by exhaustive dialysis, and DEAE fractionation with sodium chloride gradient elution.

124. HISTONE A REPRESSOR OF CHROMOSOMAL RNA SYNTHESIS

Investigators: R. C. Huang, James Bonner, Hedi Imfeld
Support: National Institutes of Health, U.S. Public Health Service

Although chromatin prepared according to methods used in this laboratory possesses the ability to carry out DNA-dependent RNA synthesis, it is nonetheless relatively ineffective in this process. Thus DNA as it is present in chromatin is less effective in promoting RNA synthesis than is an equal amount of pure DNA in the presence of soluble RNA polymerase. It appears, therefore, that there is in the chromosome some factor which renders the chromosomal DNA not fully effective in RNA synthesis. That the factor inhibitory to RNA synthesis is the chromosomal histone is indicated by the fact that chromatin, from which the histone has been removed by centrifugation in 4 M cesium chloride, followed by recombination of the extracted non-histone protein with the nucleic acid, is approximately five-fold more active in RNA synthesis than the original chromatin itself.
To further elucidate the role of histone in DNA-dependent RNA synthesis, nucleohistone has been reconstituted from its purified components, DNA and pure chromosomal histone. For such reconstitution, DNA and histone are mixed in a mass ratio of approximately 1:1.3 in solution of high ionic strength, and slowly brought to low ionic strength by gradient dialysis over a period of several hours. That the histone actually becomes bound to DNA during this procedure is indicated in a separate report. The thus reconstituted nucleohistone is totally ineffective in the support of DNA-dependent RNA synthesis in the presence of purified chromosomal RNA polymerase. That the DNA is not irreversibly influenced by combination with histone is shown by again separating the reconstituted nucleohistone into histone and DNA by centrifugation in 4 M CsCl. The recovered DNA exhibits the melting profile of pure pea chromosomal DNA, and is fully effective in the support of DNA-dependent RNA synthesis.

These facts indicate that DNA fully complexed with histone is inactive in the support of DNA-dependent RNA synthesis. Since chromatin as obtained from the pea embryo possesses some ability to conduct DNA-dependent RNA synthesis, it appears probable that a portion of the DNA of pea chromatin is not fully occupied by histone, and that it is this fraction of the DNA which is active in RNA synthesis. In the sense that it suppresses the ability of a DNA molecule to participate in DNA-dependent RNA synthesis, histone is a suppressor of genetic activity.

Publications:


125. EVIDENCE THAT CHROMOSOMAL DNA IS IN PART COMPLEXED WITH HISTONE AND IN PART NOT SO COMPLEXED

Investigators: James Bonner, R. C. Huang
Support: National Institutes of Health, U.S. Public Health Service

Chromatin as obtained from pea embryos is composed of DNA and histone in approximately 1:1 ratio. The histone is intimately and ionically bound to the DNA of the chromatin. Thus it has been shown by electron microscopy (Philip Filner and Alex Lielausis) that the DNA molecules of native chromatin do not possess the 20 Å width characteristic of pure DNA, but rather are approximately 35 Å in width, a dimension characteristic also of nucleohistone made by suitable combination of pure DNA and purified chromosomal histones. That the histone of nucleohistone intimately and uniformly covers the surface of the DNA molecules of chromatin has been indicated earlier by Zubay and Doty for the case of thymus nucleohistone. That the histone is in all probability bound spirally around the DNA molecule, occupying one of the two grooves of the latter has been indicated also by Zubay, Wilkins and Blout.

Binding of histone to DNA stabilizes the structure of the latter as is evidenced by the fact that the melting temperature of chromosomal nucleohistone is higher than that of the corresponding pure DNA. Thus, in the case of pea chromatin, the temperature at which half melting (as followed by increase in optical density) has
occurred is 84°C for pea chromatin nucleohistone under conditions (0.016 M salt) for which the half melting temperature of deproteinized pea DNA is 70°C.

These facts have been used to establish a method for the estimation of the extent to which the DNA of chromatin is not complexed with histone. Melting profiles have been determined for highly dispersed, purified, native chromatin of pea embryos. Such melting profiles exhibit a sharp melting at the 70°C characteristic of pure (non-histone complexed) DNA, and a further less sharp melting with a melting temperature of 84°C characteristic of chromosomal nucleohistone. From the ratios of the hyperchromicities elicited at the two melting temperatures, it is estimated that approximately one-fifth of the DNA of pea embryo chromatin is present in non-histone complexed form.

126. STABILIZATION OF DNA STRUCTURE BY HISTONE AS A FUNCTION OF HISTONE COMPOSITION

Investigators: James Bonner, R. C. Huang
Support: National Institutes of Health, U.S. Public Health Service

The binding of histone to DNA stabilizes the structure of DNA as evidenced by the fact that melting of the nucleohistone complex occurs in general at a temperature higher than that of DNA itself. It is known, however, that chromosomal histone includes a wide variety of proteins which although they have in common a high (25 to 27%) content of the basic amino acids arginine and lysine, differ from one another in the ratio of lysine to arginine, in amino acid sequence, and in end groups. Exhaustive study and separation of the histone components of calf thymus have been reported, and such purified histone constituents of calf thymus are available. The purified individual thymus histones have now been used to make a series of artificially reconstituted nucleohistones. For such reconstitution the histone and DNA, in approximately 1:3:1 mass ratio, are dissolved in 2.5 M sodium chloride in which both are soluble, and the salt concentration gradually lowered by gradient dialysis to 0.016 M (pH 8.0). At high ionic strength, association of DNA and histone is minimal. As the salt concentration of the DNA-histone solution lowers during gradient dialysis the histone molecules bind ionically to the DNA. The gradient dialysis encourages binding to take place in such a way as to yield complexes of maximal stability. That this reconstituted nucleohistone actually contains bound histone is evidenced by two facts: 1) The sedimentation constant of the DNA is increased by the presence of histone, and 2) the melting profiles of the reconstituted nucleohistones are characteristically different from that of DNA itself.

The melting profiles of the artificially reconstituted nucleohistones have been followed by optical density measurements at increasing temperature. Under the circumstances, pea DNA as well as calf thymus DNA exhibits a sharp increase in optical density in the region 65 to 75°C with a half melting temperature of 70°C. The temperatures of half melting of the artificially reconstituted nucleohistones of our series vary from 71 to 84°C, each having its characteristic melting profile.

The most obvious correlation between nucleohistone melting temperature and chemical composition of the histone involved is with lysine and arginine content. Thus, nucleohistone reconstituted from DNA and from histone containing only lysine as the basic amino acid possesses in this series a melting temperature of 84°C. A nucleohistone analog made by reconstitution of DNA with clupein, a protamine from herring sperm, and containing only arginine as the basic amino acid, possesses a melting temperature of 70°C, identical with that of DNA itself. The other members of the reconstituted nucleohistone series possess intermediate melting temperatures. The relation between histone lysine content and melting temperature of the reconstituted nucleohistone made with that histone is an approximately linear one.
This work was done in collaboration with Kenneth Murray, Department of Chemistry, Stanford University.

127. BASE COMPOSITIONS OF PEA CHROMATIN RNA AND DNA

Investigators: Joon Hee Rho, R. C. Huang, James Bonner
Support: National Institutes of Health, U.S. Public Health Service

Chromatin as it is isolated from pea embryos contains both DNA and RNA. The endogenous RNA represents, in part at least, RNA recently synthesized from the four riboside triphosphates by the DNA-dependent RNA polymerase of chromatin. An investigation has therefore been made of the base ratios of chromosomal DNA and RNA to learn the relation, if any, between them.

The base composition of pea embryo DNA was determined by hydrolysis in a concentrated formic acid at 175°C and subsequent separation of the bases by paper chromatography. Four major bases, adenine, thymine, guanine and cytosine are present. About 5% of an additional base was also found. This has been tentatively identified as 5-methyl cytosine. If the latter material is taken as equivalent to cytosine, the base composition of pea embryo chromatin DNA is complementary.

<table>
<thead>
<tr>
<th>Base</th>
<th>Chromosomal DNA</th>
<th>Chromosomal RNA</th>
<th>Ribosomal RNA</th>
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<tbody>
<tr>
<td>A</td>
<td>31.8</td>
<td>23.8</td>
<td>24.3</td>
</tr>
<tr>
<td>T (or U)</td>
<td>30.5</td>
<td>30.9</td>
<td>22.0</td>
</tr>
<tr>
<td>G</td>
<td>19.2</td>
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<tr>
<td>C</td>
<td>13.4</td>
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<tr>
<td>5-MeC</td>
<td>4.8</td>
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The nucleotide composition of pea embryo chromosomal RNA is, on the contrary, not base complementary. The base composition of chromosomal RNA is not similar to that of the ribosomal RNA. It does possess, however, a base composition close, although not identical, to that expected for copying of a single strand of the chromosomal DNA.

It may well be that only a portion of the chromosomal DNA is active in RNA synthesis since a majority of such DNA is covered with histone. A study of the base ratio of the active portion of the DNA will be needed to illuminate in detail the DNA-copying mechanism.

Publications:


128. INTRANUCLEAR PROTEIN SYNTHESIS

Investigators: Max Birnstiel, Margaret I. H. Birnstiel, Beal B. Hyde, Phil Martin
Support: National Institutes of Health, U.S. Public Health Service
National Science Foundation

Optimal conditions for incorporation of labeled amino acids into isolated pea nuclei have been described in a preceding report. It is now shown: a) that amino acids are incorporated into peptide linkages; b) that this incorporation is intranuclear due to contaminating cytoplasmic ribosomes; c) what kinds of nuclear proteins are synthesized; and d) where in the nucleus this protein synthesis takes place.

a) The fact that amino acid incorporation is into base and acid stable products and that incorporation of a single amino acid requires the presence of all of the other amino acids, as well as an energy regenerating system, is taken to indicate the formation of peptide linkages. By the DNP method of Saenger it has been shown that 92% of incorporated L-valine-Cl4 is recovered as free valine-Cl4 upon hydrolysis of the DNP protein, and that this compound must therefore have not been bound terminally but internally in peptide chains. A trypsin digest of the labeled nuclear protein shows only partial hydrolysis, the bulk of the material being resistant to trypsin. In the hydrolysate labeled peptides have been demonstrated by electrophoresis and Sephadex G-25 separation. From these results it is concluded that the amino acid incorporation represents actual protein synthesis.

b) Electron microscopic examination of the nuclear preparation has revealed only little contamination by ribosomes. By the addition of P32-tagged ribosomes to a whole tissue homogenate, the contamination by ribosomes was determined for different nuclear preparations. Crude nuclear preparations contain less than 6% contamination, ficoll purified nuclei less than 4% contamination and sucrose purified nuclei are practically free from ribosomal contamination, the ribosomes amounting only to 1.5% of the preparation. After amino acid incubation of a crude nuclear preparation under conditions optimal for nuclear incorporation into protein, the ribosomes of the incubate were recovered by differential centrifugation and were found to have a specific activity one-fourth that of the nuclei as a whole. Therefore the contribution by ribosomal protein synthesis is negligible.

Further proof that the protein synthesis studied is non-ribosomal comes from its incorporation characteristics. Thus, no pH 5 enzymes or total supernatant fraction are required for maximal protein synthesis, nor is it sensitive to RNase, to Ca-ions or streptomycin; furthermore it is enhanced by sodium ions.

c) It has been shown by salt extraction that nuclei synthesize all three major protein fractions in vitro. In nuclei obtained from very young tissue synthesis of the residual proteins predominates. With increasing age of the tissue histone and soluble protein synthesis becomes more distinct.

d) By using the citrate fractionation procedures already described further evidence has been found that the nucleolus is the major site of total nuclear protein synthesis and that newly synthesized proteins are transferred to the chromatin. In short-term incubations the nucleolar histones obtain 2 to 3 times higher specific activities than chromosomal histones while the specific activity of the weak saline and residual proteins of the chromatin are of the same order of magnitude. This strongly suggests that in interphase nuclei the nucleolus is also the major source for histone synthesis and might therefore participate in the mechanism of gene activity regulation (see preceding report No. 124).
129. PROTEIN SYNTHESIS BY ISOLATED PEA NUCLEOLI

Investigators: Max Birnstiel, Margaret I. H. Birnstiel, Beal B. Hyde, Phil Martin
Support: National Science Foundation
National Institutes of Health, U.S. Public Health Service

Nucleoli were liberated from intact pea nuclei by mechanical disintegration. They were exhaustively purified, principally from contamination by chromatin, by repeated centrifugation in concentrated ficoll solutions and by a short DNase treatment. Such nucleolar preparations have been shown to be electron microscopically pure and to contain 11% RNA, 4% DNA and 85% protein. Preliminary results show that the SDS extracted RNA sediments at 4 to 12S and has a melting temperature identical with that of ribosomal RNA. Approximately 6% of the protein is 0.1 N HCl extractable and is therefore histone; 5% is extractable in low ionic strength neutral solution, leaving 89% as residual protein. Amino acid analysis of this residual protein has disclosed that it is similar to ribosomal protein in composition and contains characteristically about 10% each of arginine, lysine, glutamic acid and aspartic acid.

Isolated nucleoli incorporate 20 to 30 µmoles of labeled amino acids into acid and base stable compounds. Such incorporation lasts for 20 minutes at 37°C, and requires an energy source and a complete amino acid mixture. The nucleolar incorporation is independent of added nuclear pH 5 fraction or supernatant and is sensitive to RNase. It is not impaired by preincubation with DNase and therefore differs distinctly from chromosomal incorporation of amino acids. From chemical analyses and incorporation studies it is considered that an important part of the nucleolus is ribosomal in nature. Further investigations, combined with electron microscopy, are necessary to clarify the relationship of this ribonucleoprotein to the cytoplasmic ribosomes.

130. PROTEIN SYNTHESIS BY ISOLATED PEA CHROMATIN THREADS

Investigators: Max Birnstiel, Margaret I. H. Birnstiel, Phil Martin
Support: National Institutes of Health, U.S. Public Health Service
National Science Foundation

Isolated pea nuclei were used for the isolation of chromatin threads. The nuclei were subjected to glass bead grinding and the chromatin threads liberated from contaminating material by repeated centrifugation in concentrated ficoll solutions and separated from the contaminating nucleoli in a ficoll density gradient. Such a preparation is nucleus-free and nucleoli are very sparse.

The chemical composition of this preparation is found to be 11% RNA, 29% DNA and 60% protein. Of the protein 45% is histone and the remainder is predominantly residual protein.

Incorporation of labeled amino acid into base and acid stable compounds is enhanced by the addition of a complete amino acid mixture and an energy regenerating system. By studies of mixtures of nucleoli and chromatin it could be shown that chromosomal protein synthesis cannot be attributed to contaminating nucleoli. Amino acid incorporation by chromatin is RNase sensitive but differs from nucleolar incorporation in that it is inhibited by DNase.
131. THE SYNTHESIS OF TRANSFER RNA BY ISOLATED PEA NUCLEI

Investigators: Margaret I. H. Birnstiel, Max Birnstiel, Phil Martin
Support: National Institutes of Health, U.S. Public Health Service

An active transfer RNA was extracted by the phenol method from isolated pea nuclei. This RNA forms amino acyl-RNA with C\textsuperscript{14}-amino acids in the presence of either nuclear or cytoplasmic pH 5 fractions. This transfer RNA is synthesized in the nucleus. This is shown by an experiment in which isolated nuclei are incubated with uridine-H\textsuperscript{3}, the RNA extracted by phenol and the extracted RNA centrifuged in a sucrose density gradient. It was found that the major peak of labeled RNA radioactivity coincides with the peak of amino acid incorporating ability. In a chase experiment, in which cold uridine compounds are added after eight minutes of incubation with tritiated uridine, it was found that the labeled transfer RNA peak does not lose activity. This indicates that it is not a precursor of some larger RNA molecule but is, rather, a terminal product. Thus, nuclei--predominantly the chromatin fraction--possess the ability to synthesize RNA of the same physical characteristics as transfer RNA.

To show that the newly synthesized RNA possesses the ability to incorporate amino acids, the fractionation procedures of Stephenson and Zamecnik were used. In this procedure the transfer RNA is converted to the amino-acyl form and a dye molecule is selectively attached to the non-amino-acyl RNA. Fractionation of thus-treated labeled nuclear RNA by selective precipitation shows that uridine activity is recovered in the non-dye-bound or amino-acyl-RNA fraction. This demonstrates that transfer RNA is indeed synthesized in the nucleus.

132. A SURVEY OF THE FINE STRUCTURE OF PEA NUCLEI

Investigators: Beal B. Hyde, Max Birnstiel
Support: National Science Foundation

Nuclei isolated by several methods from the embryos of peas have been prepared for electron microscopy in a variety of ways. In addition, fractions of nuclei such as chromatin, nucleoli and a ribosome-like preparation have been surveyed for ultrastructure.

Nucleoli at first seem granular in thin sections of osmium-fixed, Epon embedded material; the granules being 100 to 200 Å in diameter. In addition, a much finer granular background material is also evident although it stains less densely. Occasionally the larger granules appear to be segments of tubes or coils of the same diameter. Some sections show attached to the nucleolus an elliptical structure intermediate in size and appearance between the nucleolus proper and chromatin. Since in addition to RNA and residual protein, these nucleoli contain DNA and histone this elliptical structure is thought to be the nucleolar organizer. Moreover, in the nucleolus proper some of the coils or tubes may represent penetrating chromosomal material. The larger granules are never uniformly distributed throughout the nucleolus but tend to be grouped in different ways in different nucleoli.

Thin sections of chromatin show a loose network usually. The smallest threads are about 40 Å across. Sometimes many such strands may be seen running parallel to each other. This latter appearance is quite common in chromatin which has not been embedded and sectioned but simply dried on a grid and stained with a heavy metal salt such as uranyl acetate. In this case the 40 Å strands tend to appear as regularly spaced pairs. The pairs do not seem to be profiles of a coil or helical arrangement.
When the fine structure of active nucleoli and chromatin is as clear as these methods allow, comparative observations will be made on these structures after enzymatic treatment and chemical modification.

133. PHYTOFERRITIN: A NEW PLANT PROTEIN

Investigators: Beal B. Hyde, Alan J. Hodge, Max Birnstiel
Support: National Science Foundation
National Institutes of Health, U.S. Public Health Service

This particle was first noticed while surveying by electron microscopy a series of fractions taken from the isolated nuclei of pea embryos. The ribosome fraction, when suspended in a medium containing 4% phosphotungstic acid showed two classes of particles. One, about 105 Å in diameter was outlined as a "doughnut" with a core some 55 Å in diameter. When this material is dried down without staining, dense square profiles are seen with a size and spacing which indicate they represent the contents of the "hole" in the doughnut. This suggestion was confirmed by a sequential combination of these two methods of preparing this material for electron microscopy. The appearance of these particles is essentially identical to the iron-containing protein of animals known as ferritin. That the phytoferritin contains iron and protein in quantities similar to that of ferritin has been confirmed by colorimetric tests as well as by emission spectroscopy. That phytoferritin may contain other heavy metals is also suggested by the emission spectroscopy. Subsequently phytoferritin has been isolated from whole embryos as well as cotyledons. It has also been identified in a ribosome preparation from isolated maize embryos. More refined methods for purifying phytoferritin using differential centrifugation and column chromatography on DEAE cellulose have produced preparations with quite uniform sedimentation characteristics in the ultracentrifuge.

An effort is being made to characterize these particles as accurately as possible both chemically and physically. In addition, attempts are being made to locate these particles in thin sections of pea embryo tissue.

134. AN ANALYSIS OF THE CORRELATION BETWEEN CELL METABOLISM AND ACTIVITY CHANGES IN THE GOLGI APPARATUS OF RAPIDLY GROWING PLANT TISSUES

Investigators: Jack M. Widholm, D. James Morré
Support: National Institutes of Health, U.S. Public Health Service

The Golgi apparatus of plant cells are similar to those found in many types of animal cells but appear more discrete and possibly of less complex structure. The Golgi apparatus has been interpreted structurally as a stack of flattened cisternae. During periods of high cellular metabolic activity, vesicles appear at the edges of the cisternae and eventually become free in the cytoplasm. The number of such vesicles in the vicinity of the Golgi apparatus as observed by electron microscopy is taken as a measure of Golgi activity. The electron micrographs were prepared from ultrathin sections of maize roots previously fixed in 2% aqueous KMnO₄ for 2 hours at 25°.

Golgi activity appears to be correlated directly with the metabolic level of the cell. Meristematic cells show more pronounced Golgi activity than the cortical cells of the elongating region. Cortical cells are more active than the corresponding epidermal cells of the same region. Only the outer root cap cells form an exception to the generality that Golgi activity parallels growth rate. Experimental modification of growth rate has been particularly helpful in establishing this correlation.
135. **THE EFFECT OF INDOLEACETIC ACID ON THE INCORPORATION OF LABELED METABOLITES INTO VARIOUS SUBCELLULAR FRACTIONS OF AVENA COLEOPTILES**

**Investigators:** Alfred C. Olson, James Bonner, D. James Morré, Judith D. Elliott

**Support:** U.S. Department of Agriculture, National Institutes of Health, U.S. Public Health Service

This study is part of a continuing investigation of cell wall biosynthesis and the possible relationship between the plant growth hormone, indoleacetic acid (IAA) and a specific biochemical pathway involved in the synthesis of new cell wall material. Avena coleoptile sections were incubated with uniformly labeled C\textsubscript{14}-glucose under a variety of conditions and in the presence or absence of IAA. The addition of IAA resulted in an increased incorporation of label into hot water and hot versene extractable fractions of the cell wall. The IAA-induced increase in these fractions, which ranged from 30 to 70\text%, is largely due to increased labeling of pectic and hemicellulosic materials.

The incorporation of radioactivity from C\textsubscript{14}-glucose into the fraction soluble in cold 0.15 M acetate buffer, pH 4.5, reflects labeling changes in both neutral carbohydrate and an acidic fraction. Of the neutral carbohydrates, the labeling of galactose was most influenced by IAA whereas the amount of label found in fructose and pentose was largely unchanged by IAA treatment. The acidic fraction was shown to contain malic acid as a major component. This organic acid was identified by its chromatographic and electrophoretic mobilities under different conditions and by preparation of its p-toluidine derivative. The IAA-induced increase in incorporation of label from glucose into this acid varies between experiments but normally lies in the range of a 1.5- to 2.0-fold increase. Incorporation of C\textsubscript{14}-malic acid and of P\textsuperscript{32}-inorganic phosphate is also increased in the presence of IAA. These results are thought to be related to increased respiration normally associated with IAA-induced growth.

The same IAA-induced changes in the labeling patterns of the hot water soluble and hot versene soluble fractions were observed when uniformly labeled C\textsubscript{14}-fructose was substituted for C\textsubscript{14}-glucose. However, in the case of similar experiments with I-C\textsubscript{14}-galactose the total radioactivity of both fractions was greater from sections treated with IAA, although this increase was not reflected in the galacturonic acid isolated from the pectic substances. A comparison of the relative incorporation levels of glucose, fructose and galactose has revealed that of these three sugars galactose is most readily incorporated into the hot water soluble fraction of the cell wall, providing additional evidence for an increase in hemicellulosic.

The incorporation of C\textsubscript{14}-labeled acetate into lipid and steroid fractions of Avena coleoptiles was not influenced by the addition of IAA.

On the basis of these results it would appear that the effect of IAA on incorporation of C\textsubscript{14}-hexoses into cell wall is reflected in a number of readily extractable components. It has therefore not been possible to conclude that IAA directs the synthesis of any individual and unique cell wall material.

136. **RESOLUTION OF A CARBOHYDRATE-PROTEIN COMPLEX FROM PLANT TISSUES. I.**

**Investigators:** D. James Morré, Alfred C. Olson, Jean Poyntz

**Support:** U.S. Department of Agriculture, National Institutes of Health, U.S. Public Health Service

Earlier work revealed that the elongation zone of maize primary roots, under the influence of concentrations of indoleacetic acid that cause increased cell
wall plasticity and increased growth rate, responds by rapid formation (2- to 10-fold increase) of a carbohydrate-like substance soluble in 0.15 M acetate buffer, pH 4.4. A portion of the contaminating pH 4.4 soluble protein may be removed by heat denaturation. The fraction is then partially purified by repeated precipitations from 70% ethanol. Substantial amounts of heat stable protein are retained throughout the purification procedure. The carbohydrate portion of the complex yields a unique absorption spectrum with carbazole in sulfuric acid (a reaction commonly employed for assay of pectic substances and uronic acids), and this has served to identify the complex. The carbazole adduct of the material prepared from roots absorbs a 543 mµ with a secondary peak at 387 mµ and can be readily distinguished from uronic acids, pectin, hexoses, pentoses and mixtures of these sugars and protein. Since the elongating region of roots is restricted to a zone of only a few millimeters in length, a more abundant source of material was sought before further characterization was attempted.

The pH 4.4 cold buffer soluble, 70% ethanol soluble (CBSEI) fraction from Avena coleoptiles contains carbohydrates in various forms, a small quantity of heat stable protein, inorganic ions (predominantly phosphate) and trace amounts of several organic acids and acidic amino acids (identified from chromatographic and electrophoretic properties). The carbohydrate when reacted with carbazole yields a characteristic hexose spectrum. The component sugars were tentatively identified as fructose, galactose, glucose and small quantities of xylose. All assays for hexosamine, sialic acid or pectin-bound uronic acid were negative.

A comparison of indoleacetic-acid-treated and untreated sections revealed that although all components of the CBSEI fraction examined increased by the IAA treatment in long-term (8 hour) experiments, these changes can be accounted for on the basis of increased tissue as a result of IAA-induced growth with the single exception of heat stable protein (30 to 100% increase due to IAA).

A fraction with similar carbohydrate and protein content can be prepared from cold buffer extracts by ammonium sulfate fractionation. This material was then precipitable by cold 5% TCA or by 70% ethanol. A comparison of ammonium sulfate fractionation patterns from IAA treated and untreated Avena coleoptiles showed no overall increase in either the protein or carbazole-carbohydrate levels. A definite alteration of the fractionation pattern of the carbohydrate does however result from IAA treatment. Although the complex is non-dialyzable and is repeatedly precipitable by both ammonium sulfate and 70% ethanol, the bulk of the protein may be removed by various procedures including prolonged treatment with 7.5% TCA and the Sevag procedure. The major portion of the carbohydrate released after removal of protein is no longer 70% ethanol insoluble and it is assumed that the association with protein was largely responsible for the ethanol insolubility of the carbohydrate. Further examination of the CBSEI fraction of Avena coleoptiles by column chromatography and several electrophoretic techniques provide no indication of a major subfraction of a homogenous nature. A cesium chloride density gradient separation (4 M cesium chloride, density 1.42) also achieved essentially complete separation of protein and carbohydrate with only a small band of intermediate density. The intermediate banding material was of insufficient quantity to permit chemical characterization.

It is therefore concluded that the cold buffer soluble, 70% ethanol insoluble fraction of Avena coleoptiles consists largely of a complex between protein and a variety of carbohydrates as well as some negatively charged low molecular weight substances. All efforts to isolate and conclusively identify the spectrally distinct carbazole adduct from this fraction of Avena by any of the above fractional precipitation techniques have been unsuccessful.
137. RESOLUTION OF A CARBOHYDRATE-PROTEIN COMPLEX FROM PLANT TISSUES. II.

Investigators: D. James Morré, James Bonner
Support: National Institutes of Health, U.S. Public Health Service General Funds

A carbohydrate-containing water soluble material has been found in Avena coleoptiles and in pea stems which is of special interest because it increases in abundance in such tissue two- to fivefold as the result of treatment of the tissue with indoleacetic acid (5 x 10^{-6}M). This material is complexed, in plant homogenates, to protein. The purified protein-free water insoluble salt of such material has been obtained by procedures which involve solubilization of the protein in alkaline copper sulfate. The carbazole adduct of the purified material possesses spectral properties identical to those of a similar material present in maize roots. The substance contains not only carbazole-positive carbohydrate, but also hexose (as determined by anthrone) and a complex lipid residue. Both moieties are increased in amount as a result of indoleacetic acid treatment of the tissue, a treatment which also increases heat stability of the associated protein as well as tissue acid phosphatase activity.

138. WALL BOUND PROTEIN IN AVENA COLEOPTILES

Investigators: Alfred C. Olson, James Bonner, D. James Morré
Support: U.S. Department of Agriculture
National Institutes of Health, U.S. Public Health Service

Carboxyl-C14 labeled proline is incorporated into protein associated with cell walls of rapidly growing Avena coleoptiles. Such cell wall protein, which constitutes about 10% of the total tissue protein, is resistant to extraction from the wall by cold 0.1 M neutral phosphate buffer and by 0.1 N boiling hydrochloric acid. It is hydrolyzed to component amino acids (including labeled proline) by 6 N hydrochloric acid at 105°C. The amount of labeled proline incorporated into cell wall protein is not influenced by the presence of the plant growth regulator indole-3-acetic acid over the incubation periods studied. This incorporated label is also not subject to rapid turnover. The major portion of the bound protein is liberated by boiling in 0.1 N sodium hydroxide and is recoverable by TCA precipitation. The cell wall residue remaining after sodium hydroxide extraction still contains additional firmly bound material which may be hydrolyzed to its component amino acids by the boiling 6 N hydrochloric acid treatment. The cell wall protein of Avena coleoptiles evidently is firmly associated with the cell wall structure.

In order to find out whether this wall bound protein is structurally important to the Avena cell wall a method has been devised for measuring the elastic and plastic properties of coleoptiles. By clamping the wet coleoptile in a small pin vise it has been possible to measure stress strain relationships on a suitable device, the Instron. From such measurements it has been found that loss of turgor (e.g. by washing the coleoptile with ethanol) causes no loss in wet strength of coleoptiles. Preliminary observations suggest that trypsin treatment of ethanol washed coleoptiles causes them to become more elastic. This in turn suggests that cell wall protein may play a role in determining cell wall properties.
139. BIOSYNTHESIS OF CELL WALL PROTEIN IN NEUROSPORA

Investigators: Alfred C. Olson, P. C. Huang, Judith D. Elliott
Support: U.S. Department of Agriculture
National Institutes of Health, U.S. Public Health Service

Wild-type Neurospora, which has a well-defined cell wall, and the mutant slime, which is known to be plasmodioid and deficient in cell wall, have been studied to see if they may be useful in a comparative study of cell wall biosynthesis, particularly cell wall protein biosynthesis. Proteins which are presumed to be cell wall related were extracted with hot 0.1 N NaOH from cell wall residues from which cytoplasmic proteins had been removed. Such presumptive cell wall protein corresponds to 2 to 5% of the total cell proteins and is found in both the wild-type and the slime mutant of Neurospora. Relative incorporation of $^{14}$C proline into cytoplasm and wall is similar to the two strains. The wall proteins of Neurospora are different from those reported for certain other plants since the former contain less than 1% hydroxyproline, an amino acid reported to comprise as much as 20% of the cell wall protein of a variety of higher plants. Chromatographic examination of wall protein hydrolysates of the wild strain reveals a significant amount of an unknown, labeled material, not hydroxyproline, in addition to labeled proline. This material is not observed in the mutant. Thus, even though synthesis of cell wall protein occurs in the slime mutant, the nature of this protein differs from the normal.

140. THE EFFECT OF IAA ON THE ACID PHOSPHATASE LEVELS IN PLANT TISSUES

Investigators: D. James Morré, Vernon Bliss
Support: National Institutes of Health, U.S. Public Health Service
National Science Foundation

The increase in acid phosphatase activity associated with indoleacetic acid (IAA)-induced growth of various plant tissues has been reinvestigated. The activity of the enzyme was assayed in crude extracts under standard assay conditions with p-nitrophenylphosphate or β-glycerolphosphate as the substrate. Activity ratios for the two substrates remain constant although specific activity is higher in the case of p-nitrophenylphosphate.

The acid phosphatase activity of pea stems, Avena coleoptiles, maize roots and mung bean hypocotyls was examined after an 8-hour pretreatment with and without IAA. In all cases, the IAA-induced increases in activity are restricted to a readily soluble enzyme fraction. Both the total activity and the residual activity reflect only small changes under the influence of IAA.

141. THE ACTION OF 2,6-DICHLOROPHENOXY-ALPHA-PROPIONIC ACID (2,6-DP)

Investigators: Jack M. Widholm, James Bonner
Support: National Institutes of Health, U.S. Public Health Service

Studies on Avena coleoptiles with 2,6-DP, a compound which at low concentrations behaves as an antiauxin, indicate that at higher concentrations this compound acts as a weak auxin. Treated Avena sections exhibit an initial rapid growth for 3 hours after which auxin-induced growth ceases. The addition of 2,4-D after 3 hours of incubation of sections in 2,6-DP partially reverses the action of the latter. Addition of 2,4-D or replacement of 2,6-DP by 2,4-D after 6 hours results in essentially no growth response.
It appears that the 2,6-DP binds to the auxin receptor site in a partially reversible manner to cause the initial burst of growth, but that the material then binds in such a way as to permanently block the site from further action.

142. THE INHIBITION OF AUXIN-INDUCED CELL WALL PLASTICIZATION BY DINITROPHENOL

Investigator: D. James Morré
Support: National Institutes of Health, U.S. Public Health Service

The auxin-induced growth of Avena coleoptile sections can be largely suppressed by addition of dinitrophenol to the incubation medium. These observations have been extended to cell wall plasticization in the following experiments.

Avena coleoptile sections were incubated two hours in varying concentrations of the auxin, indoleacetic acid in the presence and absence of dinitrophenol. The resulting cell wall deformability was measured by the bending method of Tagawa and Bonner. The dinitrophenol effect was proportional to dinitrophenol concentration and at a concentration of $10^{-4}$ M completely negated the cell wall plasticizing effect of the indoleacetic acid.

143. INCORPORATION OF $^{14}C$ GLUCOSE INTO INSOLUBLE CELL WALL MATERIAL USING SINGLE CELL DISPERSIONS OF HIGHER PLANT TISSUES

Investigators: D. James Morré, Mary O'Leary
Support: National Science Foundation

Cells of single cell dispersions prepared from immature cucumber fruits retained their protoplasts intact and demonstrated a capacity for steady state incorporation of $^{14}C$ glucose into insoluble cell wall material for a minimum period of four hours after isolation. The cells were obtained by treating blocks of intact tissue with a mixture of buffered versene and the enzyme pectinase. In contrast to many tissues studied, the cell walls of this tissue remain intact under these conditions whereas the cementing materials between the walls are solubilized.

144. ISOLATION OF AN ALCOHOL SOLUBLE GLUCOSE DERIVATIVE FROM AVENA COLEOPTILES

Investigators: Alfred C. Olson, Judith D. Elliott
Support: U.S. Department of Agriculture

An investigation of the soluble components of Avena coleoptiles has led to the isolation of a derivative of glucose which may be of some significance as a precursor of cell wall material. The compound (I) is isolated in cold buffer extracts of Avena and is purified by chromatographing 80% ethanol dilutions of the aqueous fractions. Chromatographically the material is fast moving in the usual carbohydrate solvents, which suggests it may have a lipid component. The glucose moiety, which is liberated from (I) by boiling 10 minutes in 2 N HCl, was identified by chromatography and measured quantitatively by the glucose oxidase procedure. The glucose in (I) corresponds to 1 to 2% of the dry weight of the coleoptiles. There is no indication of ninhydrin positive material, phosphate or reducing sugar in (I) and only a positive test for reducing sugar has been obtained on chromatograms of acid hydrolysates. (I) is stable to hydrolysis in boiling ammonium or sodium hydroxide.
Glucose-U-C\textsuperscript{14} is incorporated solely into the glucose portion of (I) without any detectable turnover of label. C\textsuperscript{14}-labeled-(I) is recovered unchanged on incubating it with Avena sections. Fructose-C\textsuperscript{14} and galactose-C\textsuperscript{14} are also incorporated into materials which are chromatographically similar to (I). No effect of indoleacetic acid has been observed on the incorporation of these labeled carbohydrates into (I).

The large amount of (I) present in Avena coleoptiles and the ease of incorporation of label into the compound suggest an important biological role for the material. Failure to reincorporate (I) into coleoptiles may indicate that it is an end product of metabolism or that it has been altered in some way to biologically inactive material by the method of isolation. The overall properties of (I) suggest it may be related to the elusive cellulose precursor of Kahn and Colvin (J. Polymer Sci. 51:1, 1961).

145. STUDIES ON THE INCORPORATION OF A KININ, BENZYLADENINE, INTO RNA OF MAIZE ROOT TIPS

Investigator: D. James Morrè
Support: National Institutes of Health, U.S. Public Health Service

Previous studies with benzyladenine (6-benzylaminopurine) labeled in the purine moiety have shown that a small fraction of the C\textsuperscript{14} is recovered as RNA. Since these tissues were also shown to convert benzyladenine to adenine by an enzymatic debenzylation reaction, the problem was reinvestigated with C\textsuperscript{14} benzyladenine specifically labeled in the benzyl ring. Under these conditions, debenzylated purines derived from benzyladenine would contain no C\textsuperscript{14} and any radioactivity recovered in RNA should indicate incorporation of the intact benzyladenine molecule. In preliminary experiments maize root tips were found to have a high capacity to incorporate benzyladenine.

RNA was isolated by the method of Ts'o and Sato following a 20-hour incubation of roots with 1 and 10 ppm benzyl-labeled C\textsuperscript{14}-benzyladenine. The isolated RNA was hydrolyzed with 0.2 N potassium hydroxide and the specific activity of the ribonucleotides determined. At this point the radioactivity measurements indicated an apparent incorporation of 3.4 molecules of benzyladenine per 1 molecule of RNA of molecular weight 1 million. The ribonucleotides were, however, subjected to further purification by paper electrophoresis. All of the radioactivity was recovered in a zone corresponding to free benzyladenine rather than to that of the corresponding ribonucleotides. It is therefore concluded that the kinetin analog benzyladenine is not incorporated into the interiors of RNA chains.

146. CARBOHYDRATE METABOLISM OF PLANTS

Investigators: Clifford J. Pollard, Alfred C. Olson, A. J. Haagen-Smit
Support: U.S. Atomic Energy Commission
U.S. Department of Agriculture

The incubation of acetone powders of germinating Mung beans with C\textsuperscript{14}-glucose, adenosine triphosphate, and magnesium chloride results in the incorporation of an appreciable amount of radioactivity into an 80% hot ethanol insoluble, water soluble fraction. The incorporation is not affected by the presence of unlabeled glucose-1-phosphate, fructose, fructose-6-phosphate, xylose, gluconic acid or glucuronic acid. On the other hand, unlabeled glucose-6-phosphate or mannose effectively decreases the incorporation of glucose into the fraction. Glucose-1-C\textsuperscript{14}, glucose-6-C\textsuperscript{14} and uniformly labeled glucose appear to be incorporated to the same extent. No other cofactors appear to be necessary. The incubation of glucose-1-C\textsuperscript{14} with the preparation and TPN results in a marked decrease in activity in the fraction.
This information suggests that glucose is converted to higher molecular weight compound(s) without scission of the carbon chain. Furthermore, the inability of unlabeled glucose-1-phosphate or fructose-6-phosphate to diminish the incorporation of glucose into the product(s) and other facts noted above eliminate a number of well-known pathways such as the hexose monophosphate shunt or those involving fructose-6-phosphate or glucose-1-phosphate.

The bulk of the material migrates to one spot in both paper chromatographic and paper electrophoretic systems. It is barely separable from fructose-1-6-diphosphate. Large-scale preparation of the product with labeled and unlabeled glucose, purification by paper chromatography and analysis reveal that the molar ratio of anthrone-reacting material (based on glucose) to phosphate is 1 to 2. This suggests a diphosphate ester of a monosaccharide.

Periodate oxidation of the product, prepared from uniformly labeled glucose, results in a loss of 66% of its radioactivity as volatile material. No radioactivity, as volatile material, is lost from the material prepared from glucose-6-C14.

Further work has suggested that, in fact, two diphosphate esters of sugars are formed by the system. Thus, hydrolysis with calf intestinal alkaline phosphatase results in the formation of two monosaccharides. One product migrates with fructose in a variety of chromatographic systems. However, neither appears to be glucose or fructose. For example, acid hydrolysis of the original product(s) produces one major product which migrates with glucose-6-phosphate and is separable from fructose-6-phosphate. On the other hand, the original material is not oxidizable with bromine water, denoting the absence of a free carbonyl group on carbon 1. Although glucose oxidase oxidizes the glucose-like product resulting from the alkaline phosphatase hydrolysis, the product is not gluconic acid.

The ratio of glucose-like to fructose-like product varies from preparation to preparation. This latter fact tends to rule out the possibility of the formation of a disaccharide of the two sugars.

These experiments suggest the existence of a previously unrecognized, but significant, pathway of glucose metabolism in higher plants.

147. SUSPENSION CULTURES OF CELLS DERIVED FROM TOBACCO PITH

Investigator: Philip Filner
Support: National Institutes of Health, U.S. Public Health Service

The recent successful cultivation of higher plant cells in liquid medium in several laboratories has made the techniques of microbiology available for use in the study of the properties of proliferating higher plant cells.

Cell lines were started from pith tissue of mature tobacco. Callus cell growth was induced on an agar medium containing White's nutrients, casein hydrolysate, 2,4-dichlorophenoxyacetic acid, and malt extract. Most of the cells produced are highly vacuolated, large, and spherical; a minority substantially smaller and less vacuolated was also produced. After several subcultures on agar, cells were transferred to a liquid form of the same medium. An initial but brief period of cell multiplication was followed by a period during which most of the cells died. Those that survived overgrew the culture and remained stable to further subculturing in liquid medium. These cells closely resemble the few small cells found on agar, rather than the dominant large spherical type. By plating out the liquid-grown cells on agar, it was demonstrated that the small cells can generate the large type.
The multiplication of the cells passes through a lag phase, an exponential phase, and a complex terminal phase. During the exponential phase, the generation time is 1.4 days, and final titers of about $2 \times 10^5$ cells/ml are obtained. The terminal phase seems to be composed of several distinct periods of progressively slower growth. It is noteworthy that cells in the terminal phase greatly increase in size, and come to resemble the cells of the original callus. This suggests that the terminal phase may correspond to the initiation of developmental processes due to modification of the environment. Lag phase may then involve a process of differentiation so that the cells may multiply in a developmental steady state, which is determined by the medium.

148. FREQUENCY DISTRIBUTION IN CELL CHAIN POPULATIONS

Investigator: Philip Filner  
Support: National Institutes of Health, U.S. Public Health Service

The population of tobacco cell suspension cultures is principally composed of linear chains containing various numbers of cells. These chains increase in cell number by division of their members so that each new wall is laid down perpendicular to the chain axis. Chains are reduced in cell number, and new chains generated, by dissociation of cells. When total cell number is increasing exponentially, total chain number is found to be increasing exponentially also, and at the same rate. A simple relationship can be formulated between $k_d$, the division rate constant, and $k_s$, the dissociation rate constant:

$$k_d = k_s (\bar{n} - 1)$$  
where $\bar{n}$ is the average number of cells per chain. The frequency distribution of chain sizes is also observed to be constant during exponential growth. This means that each class of chains increases at a rate which is proportional to its frequency in the population.

An equation for the rate of change of any class of chains has been constructed on the assumption that chains of any given class are created or destroyed by division and dissociation. This has been equated to the observed rate law, and solved for the frequency factor, $A_n / A_T$, where $A_n$ is the number of chains in the class $n$, and $A_T$ is the total number of chains:

$$\frac{A_n}{A_T} = \left( \frac{k_s}{k_s + k_d} \right) \left( \frac{k_d}{k_s + k_d} \right)^{n-1}$$

Equation (2) describes a frequency distribution which falls off exponentially as $n$ increases, and depends only upon the two rate constants. If (1) is substituted in (2), the following simple form results:

$$\frac{A_n}{A_T} = \frac{1}{\bar{n}} \left( 1 - \frac{1}{\bar{n}} \right)^{n-1}$$

The theoretical distribution for tobacco cells, calculated from the observed value of $\bar{n}$, fits the observed distribution quite well.

The treatment outlined above should be applicable to any steady state cell population made up of chains, such as certain cocci and bacilli. Frequency distributions for B. cereus, B. megatherium, and E. coli were found to agree with distributions calculated from observed $\bar{n}$ values. It would seem reasonable, therefore, to propose that experimentally determined values of $\bar{n}$, and derived values of $k_s$, may be considered fundamental properties of a cell population, perhaps as fundamental as the growth constant.
Gibberellins are a group of plant growth hormones which have been discovered some 30 years ago, but the general occurrence and significance of which have been recognized only in the last 6 years or so. Gibberellins cause a variety of responses in plants, including promotion of cell enlargement and multiplication (cell division), induction of flowering, breaking of dormancy, etc. However, most information on gibberellin action stems from experiments in which gibberellin was applied to plants or plant organs, and the response was registered. Also, occurrence of gibberellins in numerous higher plants has been demonstrated, although mostly in at best a semi-quantitative manner. The objective of the present research is to obtain more precise and specific information on the role of gibberellins, as endogenous growth hormones, in the regulation of plant development.

This goal is pursued by means of various approaches. These include comparative studies of the effect of applied, different gibberellins; quantitative determinations of the gibberellin content of plants in different states of development, which can be controlled both by specific environmental conditions and by application of gibberellins; organ culture, which can supply information about the site of gibberellin action in the plant; the use of substances which seem to act, at least in certain systems, as gibberellin antagonists and therefore may permit indirect conclusions on the need of gibberellin for a given growth response. While the work is in progress, it has already been possible to obtain a much more precise picture of the over-all action of gibberellin in certain developmental processes.

References:


I. EFFECT OF GIBBERELLINS A₁ THROUGH A₉ ON FLOWER FORMATION IN COLD-REQUIRING, LONG-DAY AND LONG-SHORT-DAY PLANTS

Investigators: Marian Michniewicz, Anton Lang
Support: Rockefeller Foundation
            National Science Foundation

Treatment with gibberellin causes flower formation in numerous cold-requiring and long-day plants grown under non-inductive (i.e. flower-inhibiting or flower-delaying) conditions; it substitutes for the long-day but not for the short-day part of induction in long-short-day plants. However, not all representatives of these plant types show this response; some respond only with stem elongation, with no flower formation; others do not respond at all.

Almost all previous work was done with gibberellin A₃ (gibberellic acid). However, at present no less than nine different gibberellins (GA₁ through GA₉) are known, and it is also known that their effectiveness in different plants and different responses is not the same. It was therefore decided to compare the effect of the nine gibberellins on flower formation in selected plant species, in order to determine whether the response can be improved by use of the "right" gibberellin. The "new" gibberellins were obtained through the courtesy of the Imperial Chemical Industries, Ltd., and of Professor Y. Sumiki, University of Tokyo.
The following plants were studied: a) A cold-requiring strain of Centaurium minus; b) *Myosotis alpestris* (forget-me-not), another cold-requiring plant; c) *Crepis parviflora*; and d) *Silene armeria*, two long-day plants; and e) *Bryophyllum creanatum*, a long-short-day plant. The first two species were grown under non-inductive temperature conditions, the latter three on short days. *Centaurium*, *Crepis* and *Bryophyllum* respond to GA₃ readily, *Silene* and *Myosotis* slowly or not at all.

The results are summarized in the following table:

<table>
<thead>
<tr>
<th>Flowering Effect</th>
<th>Myosotis</th>
<th>Centaurium</th>
<th>Silene</th>
<th>Crepis</th>
<th>Bryophyllum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongest</td>
<td>GA₇</td>
<td>GA₃</td>
<td>GA₇</td>
<td>GA₄,GA₇</td>
<td>GA₃,GA₄,GA₇</td>
</tr>
<tr>
<td>Medium</td>
<td>GA₁</td>
<td>GA₁,GA₄,GA₅</td>
<td>--</td>
<td>GA₁,GA₃</td>
<td>GA₁,GA₂</td>
</tr>
<tr>
<td></td>
<td>GA₇,GA₉</td>
<td></td>
<td></td>
<td></td>
<td>GA₅,GA₉</td>
</tr>
<tr>
<td>Weak</td>
<td>GA₆</td>
<td>--</td>
<td>GA₂,GA₅,GA₉</td>
<td>GA₆</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>GA₂,GA₆,GA₈</td>
<td>GA₁,GA₆,GA₆,GA₈</td>
<td>GA₈</td>
<td>GA₈,GA₉</td>
<td></td>
</tr>
</tbody>
</table>

It can be seen (1) that the different gibberellins differ greatly in their flower-inducing ability; (2) that different plants exhibit certain specific differences in their response to the different gibberellins; and (3) that GA₇ is more active in several species than GA₃. It is very probable that some of the earlier negative results were caused by the use of a relatively non-effective gibberellin. However, different plants still exhibit considerable difference in their sensitivity to gibberellins; for example, *Silene* is considerably less sensitive than *Crepis*, even if the responses to the most active gibberellins are compared.

Except in *Crepis*, flower formation was always preceded by substantial stem or internode elongation but the correlation between the effect of the different gibberellins on stem elongation and flower formation was not complete.

No correlation between the flower-inducing activity of the gibberellins and their chemical structure could be recognized.

Reference:


Publications:


II. ANALYSIS OF NATIVE GIBBERELLINS IN RELATION TO FLOWER INDUCTION

Investigators: Manfred Ruddat, Elliot N. Light, Norman McCann, Willard G. Spiegelman
Support: National Science Foundation

Previous investigations indicated quantitative and qualitative changes in the content of endogenous gibberellin-like substances in black henbane (Hyoscyamus niger) as a result of treatment with flower-inducing environmental conditions (see Biology 1961, p. 111). In repeating and expanding these experiments some difficulties were however encountered in obtaining reproducible results. It had also been observed that some fractions were not active on dwarf peas, our routine assay for gibberellins, but were active on dwarf mutants of maize. Since the available material of induced and non-induced plants is restricted in quantity, the extraction and assay procedures were re-examined, using other plant materials. The work included tests of the efficiency of different extractants, different purification procedures, the suitability of different charcoal preparations for chromatographic separation of active materials, and varying responses in different bioassays. Among other findings, it was observed that immature seeds, seedlings and vegetative shoots of Japanese morning glory (Pharbitis nil) contain appreciable gibberellin activities which, however, could not be detected with the dwarf pea assay but could be measured with dwarf maize mutants. These results indicate that it is necessary to use several bioassay systems in attempts at extraction of unknown gibberellin-like growth substances.

III. EXTRACTION OF GIBBERELLIN-LIKE MATERIALS FROM INDUCED AND NON-INDUCED PERILLA, A SHORT-DAY PLANT

Investigator: Marian Michniewicz
Support: Rockefeller Foundation, National Science Foundation

Tips of Perilla plants which had been maintained on long days or had been exposed to 10 or 20 short days were extracted and the extracts, after partial purification, adsorption to activated charcoal and fractionation into 20 fractions by means of elution with increasing concentrations of acetone, tested for the occurrence of gibberellin-like growth substances. The long-day plants were vegetative, the plants which had received 10 short days were partially induced to flower formation, and the plants which had received 20 short days possessed small visible flower buds. No evidence for qualitative changes in the gibberellin composition of the plants was found. The quantity of gibberellin-like materials seemed to decrease with increasing length of inductive treatment. This finding is in contrast to the results of similar experiments with long-day plants where both an increase of the total content in gibberellin-like substances, and appearance of active substances which were absent in the vegetative plants, had been found after photoinductive treatment (i.e. exposure to long days). This is in agreement with the fact that gibberellins do not seem to be limiting factors in flower formation of short-day plants.

IV. RELATIONSHIP BETWEEN GIBBERELLIN AND FLORAL STIMULUS IN THE LONG-SHORT-DAY PLANT, BRYOPHYLLUM DAIGREMONTEANUM

Investigators: J. A. D. Zeevaart, Anton Lang
Support: National Science Foundation, Frasch Foundation

Bryophyllum daigremontianum will form flower buds only if a minimum number of long days is followed by a minimum number of short days. Hence, it is
called a long-short-day plant. Flower formation will also occur when gibberellin is applied in continuous short day, but not in long day. Thus, gibberellin can replace the long day, but not the short day part of photoperiodic induction.

It was established that this plant has a relatively low night temperature optimum for flower formation. Both upon the change from long to short day and after gibberellin application in short day, flower buds are formed only at night temperatures of 11° and 15°C, but not at 19°C.

In grafting experiments the transmission of a floral stimulus can easily be demonstrated from a flowering stock to a vegetative receptor scion in both long and short day. Donor stocks induced by photoperiodic induction or by gibberellin treatment in short day are equally active in this function, although direct gibberellin treatment under long-day conditions would not result in flowering.

Flower formation induced by the change from long to short day as well as by gibberellin treatment in short day is always associated with an increased length of newly formed internodes.

It has been concluded that gibberellin and the floral stimulation are not identical, but that gibberellin is a factor which normally limits production of the floral stimulus in Bryophyllum under short-day conditions, and that the transfer long day – short day results in gibberellin formation in the plant.

Publication:

V. EFFECT OF PLANT RETARDANTS AND OF GIBBERELLIN ON PHOTOINDUCTION IN A LONG-DAY PLANT

Investigators: Bilandrai Baldev, Anton Lang
Support: American Cancer Society
National Science Foundation

Previous investigations have shown that gibberellin application causes flowering in many long-day rosette plants kept under short-day conditions, an effect which is usually preceded by stem elongation; and that on the other hand application of certain plant retardants reduces stem elongation in caulescent plants, an effect which can be overcome by gibberellin application. (Rosette plants are plants which lack an elongate stem in part of their life, caulescent plants have such a stem throughout their life.) The effect of two of these plant retardants (Amo-1618 and CCC, see next report) on photoinduction in a long-day rosette plant, Samolus parviflorus, was studied. It was found that the retardants, when used in sufficiently high concentrations, suppressed both the stem-elongating and the flower-inducing effect of long days without suppressing growth of the plants (formation of new leaves). The inhibitory effect of the retardants could be overcome by small quantities of GA. The results are indicative of participation of endogenous GA-like materials in stem elongation and flowering.

Reference:
VI. CHEMICAL AND ENVIRONMENTAL CONTROL OF MORPHOGENESIS OF SEX ORGANS IN CUCUMBER

Investigators: Esra Galun, Hossein-Ali Sadri, Yvonne Jung, Anton Lang
Support: National Science Foundation

Monoecious cucumber plants bear unisexual flowers which, however, develop from bisexual floral buds. During the development of a bud into a flower the initials of either the stamens or the pistil are inhibited; this results in the production of pistillate (female) and staminate (male) flowers, respectively (see Biology 1961, p. 111). The factors which control this alternate mode of morphogenesis in the individual floral buds are responsible for the flowering pattern (sex expression) of the plant. The purpose of the following projects was to study some of these factors.

(a) Effect of environmental and chemical factors on sex expression in cucumber (Galun, Sadri, Lang). The effect of chemical factors, namely gibberellic acid and of certain "plant retardants," and of environmental factors, namely day length and temperature, was investigated by using intact plants. Use was made of pure genetic monoecious cucumber lines in which any change in sex expression is readily detected. It was shown that the effectiveness of gibberellic acid (GA) to cause the development of male flowers at sites of potential female flowers is very much dependent on the temperature and day length regimes during its application. GA treatment causes a very strong shift towards maleness when plants are kept at low temperature and short (8 hours) photoperiods, but is almost not effective under conditions of high temperature and long photoperiods (16 hours).

The shift in sex expression caused by GA was accompanied by a promotion of elongation in the GA-treated plants. The possibility of a causal relationship between sex determination and elongation led to the use of substances which are known to cause retardation of elongation in several plants. The substances were: (1) Amo 1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride); (2) GCC (2-chloroethyl trimethyl ammonium chloride); (3) Phosfon D (tributyl-2,4-dichlorobenzylphosphonium chloride).

It was found that all three substances shifted sex expression towards female-ness and at the same time reduced elongation of the plants, thus having in either respect the opposite effect of GA. Moreover their effect could be counteracted by GA application. Quantitative experiments with two of the retardants showed that both the change of sex expression and the reduction of growth can gradually be reversed by increasing concentrations of applied GA.

(b) Studies with floral buds cultured in vitro (Galun, Jung, Lang). As previously reported (Biology 1961), a technique was developed to culture excised (isolated) floral buds on agar nutrient media. Such buds were dissected out of intact plants at early stages of their development—the bisexual (BS) stage or earlier. They were then planted on the agar medium. The basal medium was supplemented with various concentrations of GA (0.0, 0.3, 1.0 and 3.0 mg/l), or of indoleacetic acid (0.0, 0.1, 0.3 and 1.0 mg/l), or with combination of the two growth substances. Results evaluated so far are from potential male floral buds, taken from the lower nodes of monoecious plants. Such buds react differently according to the stage at which they were excised and cultured. When excised very early—before BS—they tend to develop in the direction of female flowers and an almost normal ovary is produced. When cultured at a late stage, after BS, they develop in the direction of male flowers. In both cases the development is very little changed by the addition of growth substances. But at an intermediate stage they do react to the additional indoleacetic acid (IAA) which causes the potential male buds to develop in the direction of female flowers. The simultaneous addition of GA counteracts the IAA. The buds thus are passing through a stage in which their sex expression can be determined by chemical factors applied to the bud itself; it furthermore seems that GA may not be acting directly but only by modifying the action of auxin.
(c) Extraction of gibberellin from cucumber (Galun, Sadri, Lang). With use of methods for gibberellin extraction and determination developed in this laboratory, plants of monoecious cucumber were assayed for presence of GA-like substances. Presence of GA activity could be detected in plants which had been grown under conditions favoring male sex expression but not under conditions favoring female sex expression. The results, while preliminary in nature, indicate that male sex expression in cucumber is associated with a higher endogenous content. They are thus in agreement with the findings that application of GA to whole plants causes increased maleness and that GA, as explained above (section b), maintains the maleness of excised floral buds grown in the presence of auxin.

Publication:


VII. INFLORESCENCE GROWTH IN HEMP AND GIBBERELLIN

Investigators: Diethard P. Köhler, Christel Huber
Support: German Federal Research Council (Deutsche Forschungsgemeinschaft)
National Science Foundation

It was shown by Heslop-Harrison that treatment of female hemp plants with gibberellic acid makes their inflorescence grow like that of the males but does not alter their sex. In present experiments it was found that sex expression in monoecious strains of hemp is not altered by treatment with gibberellic acid, but that both monoecious plants and the short males developed the long inflorescence characteristic of typical males. It was concluded that in hemp unlike cucumbers sex is not dependent on gibberellin, but that the inflorescence type may be determined by the endogenous gibberellin content. In accord with this assumption it was found that extracts of long males showed some gibberellin activity, whereas no measurable activity was found in extracts from female plants.

Reference:


VIII. LIGHT-INHIBITION OF PLANT GROWTH AND GIBBERELLIN

Investigators: Diethard P. Köhler, Christel Huber, Hossein-Ali Sadri
Support: German Federal Research Council (Deutsche Forschungsgemeinschaft)
National Science Foundation

Plants grow usually faster in darkness than in light. The light inhibition in some dwarf varieties is particularly strong, with very small quantities of light sufficient to depress growth to a fraction of growth in darkness. With tall varieties much more light is needed for a comparable growth inhibition (Gorter, 1961). Application of gibberellin brings the growth of light-grown peas (both tall and dwarf varieties) to the dark value but not above. It was therefore concluded that light lowers the effective endogenous gibberellin level in the plants, this effect being greater in dwarfs than tall (Lockhart, 1961). In order to determine whether the growth differences in light and in dark are associated with differences in the gibberellin content of the plants, light- and dark-grown peas were extracted with methanol. The extracts, after
purification by means of petrol ether and ethyl acetate, were fractionated by elution from a charcoal column with increasing concentrations of acetone, and the fractions were tested on dwarf peas (see Biology 1961, p. 112). The work is still in progress. So far the test did not reveal differences in the gibberellin content of light- and dark-grown plants.

References:


IX. STUDIES ON THE INTERACTION OF DIFFERENT GIBBERELLINS

Investigators: Marian Michniewicz, Anton Lang
Support: Rockefeller Foundation
National Science Foundation

At present, nine different gibberellins (GA1 through GA9) are known chemically. It is also known that they differ considerably in their activity in different plants and different growth responses. It was decided to investigate possible interactions between the different gibberellins, particularly in view of the fact that in the case of synthetic auxins it has been shown that auxins with low activity can act as antagonists (competitors) for auxins with strong activity. The following responses were studied: (a) Growth of the first leaf of dark-grown oat seedlings; (b) growth of the first two leaf sheaths of the d-1 dwarf mutant of maize; (c) growth of cucumber seedlings; (d, e) flowering in the long-short-day plant Bryophyllum calycinum, and the long-day plant Crepis parviflora, both grown on short days. In each case, a gibberellin which showed high activity in the particular response was given simultaneously with another gibberellin which was known to be little active, or not active at all, in the same response; the latter was given 3 to 100 times in excess of the former. Some of the experiments are still under way, but so far the results indicate the following: 1) If the weak gibberellin was given at a level where it did not produce an effect of its own the response equalled that to the strong gibberellin given alone; 2) if both the weak and the strong gibberellins, when used separately, gave significant responses, the response to the combination was additive or sub-additive. No evidence for synergistic or antagonistic interaction was so far found.

X. GIBBERELLIN ACTIVITY OF A PLANT DITERPENOIDS

Investigators: Manfred Ruddat, Elliot N. Light
Support: National Science Foundation

Most gibberellin responses are highly specific, i.e. they have not been produced by any other chemicals. It is therefore of interest that a plant diterpenoid, steviol (sodium salt), gave a distinct response in a system, the d-5 dwarf mutant of maize, which so far has responded only to gibberellins. Steviol is the aglycon of stevioside, a glycoside found in Stevia rebaudiana, a member of the composites, and has been prepared and supplied by the late Dr. Erich Mosettig, National Institute of Arthritis and Metabolic Diseases.
The structures of steviol and of a gibberellin (gibberellin A₃ or gibberellic acid) are shown below; the other chemically identified gibberellins differ from A₃ by lacking one or both of the double bonds and/or having different numbers of hydroxyl groups.

The activity of steviol on d-5 maize was highly significant, although between 10 and 100 times lower than that of gibberellin A₃. In other gibberellin-sensitive maize mutants (d-1, d-2, d-3 and an-1) and other gibberellin-sensitive systems (dwarf peas and cucumber seedlings) no consistent responses with steviol were obtained. Isosteviol, in which the C/D ring has a spatial arrangement different from that in the gibberellins, was inactive in all tests, including the d-5 mutant.

A particularly interesting aspect of the gibberellin-like activity of steviol is the fact that this compound lacks the lactone ring which is present in all chemically known gibberellins and which had been thought to be essential for their biological activity. The activity of steviol on d-5 may therefore become useful in determining the point of the biosynthetic sequence leading to gibberellin formation at which the lactone ring is made.

150. ENVIRONMENTAL CONTROL OF PLANT GROWTH AND DEVELOPMENT

I. CYCLE LENGTH AND PLANT GROWTH

Investigators: Hendrik Ketellapper, Dick M. Chang
Support: The Rockefeller Foundation

Plants are normally exposed to alternating periods of light and darkness. The combined length of a light period and the following dark period is called cycle length. Cycle length strongly influences plant growth (Biology 1961, p. 118). Maximal growth occurs when the cycle length is approximately 24 hours. It has also been shown (Biology 1961) that plant growth responds to the length of the light period within a 24-hour cycle. Generally growth is maximal when the photoperiod is 20 hours. This leaves a 4-hour dark period. In all such experiments, temperature is kept constant.

Plants possess an endogenous rhythmicity with a period of 24 hours and it has been suggested that the environmental light-dark periodicity must be properly synchronized with the endogenous periodicity of the plant for maximal growth to occur. According to this view, growth is reduced in cycle lengths other than 24 hours because such synchronization is lacking. Some new facts indicate that this hypothesis may be correct. At least a thorough testing of the hypothesis is now possible.

The following information has been obtained:

a) The effect of a cycle length depends on the length of the photoperiod. The longer the photoperiod, the smaller the growth reduction caused by a given cycle
length as compared with growth on a 24-hour cycle having the same ratio between light and darkness. When the light period constitutes 5/6 of the total length of an unfavorable cycle, growth under such a cycle is equal to growth under a 24-hour cycle with a light period equal to 5/6 of 24 hours.

b) The relation between growth and photoperiod for a 24-hour cycle is linear between 8 hours and light saturation. Generally light saturation is found with a photoperiod of 20 hours, or 5/6 of the cycle length.

c) The curve showing the relation between growth and photoperiod for cycles other than 24 hours is bimodal. When the photoperiod becomes longer than half the cycle length the growth reducing effect decreases progressively. Light saturation occurs when the photoperiod is 5/6 of the cycle length.

d) Such results have been obtained with tomato, soybean, red kidney bean and pinto bean using 36-, 48-, and 72-hour cycles.

e) A preliminary study of the course of the endogenous periodicity in kidney beans under a 48-hour cycle has shown that the rhythm is affected by the length of the photoperiod. When the light period is 5/6 of the cycle (40 hours light, 8 hours dark) the rhythm proceeds in exactly the same manner as in a 24-hour cycle. When the 48-hour cycle consists of equal periods of light and dark, abnormalities occur. These need further clarification.

It should now be possible to study the effect of atypical cycles on growth and on the endogenous periodicity in the bean plant. The question can then be asked whether the length of the photoperiod in an atypical cycle affects growth and rhythm in a matching manner. If it does, one may conclude that cycle length influences growth through interference with the endogenous periodicity. The evidence obtained so far seems to indicate that this is indeed the case.

II. TEMPERATURE FLUCTUATION AND GROWTH OF TOMATO PLANTS IN CONTINUOUS LIGHT

**Investigators:** John Berry, Hendrik Ketellapper

**Support:** The Rockefeller Foundation

Tomato plants become chlorotic when grown in continuous light and constant temperature. Such chlorosis can be prevented by a daily dark period or a daily period at a lower temperature. A 4-hour dark period is adequate to insure normal development. It has been found that for the prevention of chlorosis lowering the temperature by 20°C for 4 hours is equivalent to a 4-hour dark period. When the temperature is lowered by 16°C, 6 to 8 hours at the lower temperature are required. Lowering by 12°C for 8 hours is not adequate.

Darkness and lower temperature are to some extent additive. However, darkness followed by lower temperature is much more effective than the reverse. This may be useful for a further approach to the problem of the mechanism of action of continuous light on tomato plants.

III. THE EFFECT OF ROOT TEMPERATURE ON THE GROWTH OF TOMATO PLANTS

**Investigators:** Hans Kende, Hendrik Ketellapper

**Support:**
- National Science Foundation
- Rockefeller Foundation

Effects of varied root temperature on the growth of tomato plants have been investigated. Particular attention was given to the action of continuous light since it
has been reported that cooling of the roots is able to prevent chlorosis of the leaves caused by continuous light. Tomato seedlings were planted in glass tubes and pre-grown under optimal conditions. After the roots reached a length of approximately 20 cm the tubes were transferred to water tanks the temperature of which had been adjusted to 10°, 20°, 30°, and 40°C. The plants were exposed to continuous illumination at an air temperature of 27°C. Optimal growth has been observed at a root temperature of 30°C, which is considerably higher than the temperature optimum for growth when tops and roots are kept at the same temperature (23° day, 17° night: Went). At the extreme soil temperatures of 10° and 40°C growth was greatly retarded. Cooling of the root system did not prevent the development of chlorotic lesions in the leaves.

IV. PHOTOPERIOD AND THE GROWTH AND FLOWERING OF PEAS

Investigator: Hendrik Ketellapper
Support: The Rockefeller Foundation

It has been reported that peas do not require a diurnal periodicity for normal development (Biology 1961, p. 118), in particular that there is no optimal photoperiod for dry matter accumulation. Further experiments have shown, however, that there is an optimal photoperiod for stem elongation in the pea variety Progress #9. Moreover, the exact length of the optimal photoperiod depends on light intensity: The lower the light intensity, the longer the photoperiod that is optimal for elongation. This is true during the logarithmic phase of elongation.

The dry weight of the plant tops increases with increasing photoperiod up to 16 or 20 hours. Increase of light intensity above 1,300 foot candles at the top of the plants does not cause any further increase in dry weight accumulation under photoperiods longer than 16 hours.

When plants are harvested after fruit development has started, but before complete maturity has been reached, the fruit weight increases with increasing photoperiod up to and including continuous light. Stem weight behaves as before.

These results indicate that the effect of photoperiod, including continuous light, on the growth of peas in constant temperature is a direct light effect and does not involve the endogenous periodicity.

In the variety Progress #9 the number of the nodes to first flower is not affected by light intensity nor by the length of the light period. In the variety Unica the number of the nodes to first flower decreases by 6 when the photoperiod is increased from 12 hours to continuous light.

V. TEMPERATURE EFFECT UPON SHOOT AND NEEDLE ELONGATION OF PONDEROSA PINE

Investigator: Henry Hellmers
Support: U.S. Forest Service
General Funds

This study was undertaken to determine the effect of temperature during the current and previous summer upon the growth of shoots and needles of Ponderosa pine. Pines are an interesting subject for this study because cell division in the needles occurs at a different time of the year than it does in the shoot.
The needles have a meristematic region at their base and both cell division and cell elongation occur during the current season. Therefore the needles were not affected by the previous season's temperature. The needles grew more slowly under current cool conditions and after 180 days they were only half as long as needles grown under warm conditions, but had not terminated their growth. The needles under warm temperature ceased growth after approximately 110 days.

The shoots, which have the cells laid down in the bud during the previous growing season, were affected by both the temperature of the previous season and that of the current season. Buds formed under warm conditions and then transferred to cool conditions for elongation grew the most. Buds formed in the cool conditions and allowed to elongate in the cool conditions grew more than did buds grown in either cool or warm conditions and then placed under warm conditions. The shoots in the warm conditions grew faster but the shoots in the cool conditions grew more before setting new buds.

VI. SEED GERMINATION IN BITTERWEED

Investigator: Donald Caplenor
Support: National Science Foundation

This investigation deals with one of a series of problems involving the life cycle of the common pasture pest of the southeastern United States, bitterweed or sneezeweed (Helenium amarum [Raf.] H. Rock). Earlier studies have indicated that, though seeds may mature in the most southern states in May or early June, they do not germinate until autumn, and that germination was not affected by light but was inhibited by relatively high temperatures. The purpose of the present study was to determine the details of the control of germination by temperature in Helenium. This has involved four areas of investigation: The effect of various constant temperatures and, in close connection, of the length of storage of the seeds; the effect of various alternating temperatures; and the effect of short periods of stratification.

A. Effect of constant temperatures upon germination; dependence upon length of storage. Freshly collected seeds germinated in high percentages, without statistically significant differences, at 11°C, 15°C, and 19°C, but not at 3°C; only a small number germinated at 27°C (5 to 7% of optimal germination) and at 31°C (1%). For seeds stored at room temperature for 1 or 2 years, optimum germination temperature was 19°C. The most marked effects of storage were (1) general loss of viability, and (2) relatively better germination at higher temperatures. The latter is well illustrated by the following data:

<table>
<thead>
<tr>
<th>Collection year</th>
<th>1961</th>
<th>1960</th>
<th>1959</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination per cent at 27°C</td>
<td>5.3</td>
<td>47.3</td>
<td>20.0</td>
</tr>
<tr>
<td>Maximum germination per cent</td>
<td>86.7</td>
<td>65.3</td>
<td>26.0</td>
</tr>
</tbody>
</table>

B. Alternating temperatures which greatly enhance germination in many plants did not generally increase the percentage of germination in Helenium. Only 2 experiments out of 15 showed significantly, although slightly higher per cent of germination of the seeds subjected to alternating temperatures; in all others there was either a significant decrease in germination or there was no significant difference.

C. Effects of stratification. Contrary to their activity when untreated, soaked seeds held at approximately 4°C for 6 to 22 days germinated almost equally well at all temperatures between 7°C and 31°C. Alternating temperatures of 4°C and 31°C were also effective in increasing germination but alternation between 15°C and 31°C was not.
Seeds were not permanently affected by being held at 31°C after inhibition. In all experiments in which seeds were removed from 31°C to a lower temperature, they germinated promptly in percentages characteristic of the "new" temperature.

VII. THE EFFECT OF 5-FLUORODEOXYURIDINE ON GERMINATION OF SEEDS OF BITTERWEED

Investigator: Donald Caplenor
Support: National Science Foundation

Seeds of Helenium amarum (Raf.) H. Rock are very sensitive to temperature. About 85% of mature, freshly harvested seeds germinate at 19°C, but very few germinate at either 27°C or 31°C.

It has been considered possible that the inhibition of germination at certain temperatures depends upon inactivation, or lack of activation of enzymes controlling DNA synthesis. Zeevaart has shown that 5-fluorodeoxyuridine (5-FDU) is effective in inhibiting photoperiodic induction in Pharbitis and that this inhibition is reversed by simultaneous application of thymidine (see Biology 1961, p. 108, 109). This inhibition has been interpreted as interference with DNA synthesis. It was believed that, if the temperature sensitivity of Helenium seeds were due to a defect in the synthesis of DNA, 5-FDU might suppress germination at optimum temperatures.

Freshly collected seeds were soaked in 10^{-6} M 5-FDU and incubated at 19°C. Per cent germination, as determined by the appearance of a hypocotyl longer than 1 mm, was identical to that of controls soaked in tap water.

It appears that germination of Helenium is not dependent upon DNA synthesis, or that 5-FDU at the level used does not inhibit DNA synthesis in these seeds.

VIII. THE EFFECT OF GERMINATION TEMPERATURE ON THE GROWTH OF PLANTS

Investigators: Hans Kende, Yvonne Jung
Support: National Science Foundation

Earlier experiments at the Earhart Laboratory gave evidence that plants raised from seeds which had been germinated at different temperatures grow at different rates, even if transferred into the same conditions. Seeds of corn cockle (Agrostemma githago), false flax (Camelina dentata), French weed (Galinsoga parviflora), groundsel (Senecio vulgaris), Tridax triplubata, and mustard (Brassica juncea) have been germinated at temperatures ranging from 3°C to 31°C. After germination the plants were grown under identical conditions. In all species tested growth was markedly promoted when the seeds had been germinated in low temperature (7°C or 11°C) or in high temperature (31°C). The response was most pronounced in the early growth phases and seemed to be modified by the conditions under which the plants were grown. The aim of further experiments will be to specify the effect of the growing conditions and to investigate the biochemical processes which are affected by specific germination temperatures and cause increased growth of the young plants.

Reference:

IX. ENVIRONMENTAL CONDITIONING IN PEAS

Investigators: Harry R. Highkin, Susan R. Sherbet
Support: National Science Foundation

A. Significance of light and temperature in conditioning effects. It has been shown that subjecting genetically pure strains of peas to a constant temperature-artificial light environment for a number of generations results in a decrease in vigor of the line. This decline in vigor can be reversed by subjecting the "degenerated" lines to natural light and daily fluctuating temperatures; however this "recovery" requires a number of generations. These earlier experiments, however, did not permit a determination whether temperature or light, or both, were responsible for these conditioning effects. The experiments are being repeated by using both natural and artificial light and combining them with both constant and fluctuating temperatures. Since installation of new lights in some of the Earhart artificial-light chambers, it has been possible to begin a comparison of growth in natural light as well as in two different artificial-light sources differing mainly in intensity. The plants are presently in the third generation.

B. Carry-over effects of different light-dark conditions. Preliminary results of experiments were reported (Biology 1961, p. 119) in which a carry-over effect of photoperiod and of light-dark cycles of different length on growth and flowering of pea plants (Pisum sativum) was shown. Plants from seeds produced in a continuous-light regime were compared to those produced in regimes of 16 hours light and 8 hours dark, 24 hours light and 24 hours dark, and 12 hours light and 12 hours dark. It was shown that if all plants were grown in a common test environment there was a marked difference in growth behavior as well as time of flowering among these "lines."

These studies are being continued with special light boxes designed for this purpose. Temperature and light conditions are common to all treatments, the only differences being in photoperiod and cycle length. A large population of the variety L5 was divided into four smaller populations and grown in four conditions: 1) Continuous light; 2) 24 hours light and 24 hours dark; 3) 12 hours light and 12 hours dark; 4) 16 hours light and 8 hours dark. In each of these treatments the population has been replanted for three generations. In the fourth they will be grown side by side in a number of different environments in order to determine the extent of the carry-over effect as well as the time (number of generations) needed for disappearance of the latter "recovery."

C. Effect of cotyledons in environmental conditioning of peas. Pea seeds contain large amounts of stored food materials in their cotyledons. An experiment has been designed to test whether growth differences in the progeny which are caused by environment can be, at least in part, ascribed to the presence of different "food" materials in the cotyledons as a result of maturation under different environmental conditions.

Seeds from a single environmental source have been used. The seeds have been cotylectomized and the "embryo" grown on a minimal sterile agar nutrient containing mineral salts and sugar. When approximately five nodes have been formed the seedlings have been transplanted to standard growing medium (vermiculite) and watered with nutrient. Seeds produced on these plants have again been cotylectomized and grown on the agar-nutrient. This has been done for three generations and will be done for one more generation before the seeds obtained in the single constant environment will be distributed in a spectrum of temperature and light environments in order to determine the response of plants.
Reference:


X. INDUCED HERITABLE CHANGE IN FLAX

Investigators: Alan Durrant, Margaret K. Genthe
Support: National Science Foundation

Heritable changes are induced in flax plants of the variety Stormont Cirrus when supplied with different fertilizer combinations and grown in a warm greenhouse for the first five weeks from sowing. For example, a NPK combination induces a large plant and NK a small plant when applied to soil compost used at Aberystwyth, but it is suspected that the balance of N and P is particularly important for inducing heritable change. The large and small types so induced are stable in inheritance and behave as ordinary genetic types when they are crossed or grafted.

The effects of a number of day/night temperature (°C) regimes, and of the addition of N and P separately to Hoagland's nutrient solution, in inducing heritable change are being studied at the Earhart Laboratory. The first-generation plants are too young at present for a reliable assessment but there is good evidence that N has induced large plants and it is probable that P has induced small plants, these changes being in the expected directions. Temperatures of 27/19 and 27/15 appear to be effective and 19/11 and 15/7 not effective, the other temperatures used giving no evidence either way at present.

Further tests on the large and small types previously induced suggest that their weight difference, but not other character differences, diminishes, or disappears, when the plants are grown for more than one generation in Hoagland's solution and a temperature regime of 27/7, that is, an inherited change maintained only in these conditions. These observations require to be checked on the mature plants and in later generations, and by crossings.

Reference:


XI. INHIBITION OF ELONGATION OF PEA STEM SECTIONS BY LIGHT

Investigator: Joseph E. Scheibe
Support: National Institutes of Health, U.S. Public Health Service

Elongation of the excised apical segments of the stems of dark-grown peas has long been used as a parameter in assays for growth-mediating substances such as auxins and gibberellins. For technical reasons, the excision of these segments has always been accomplished under conditions of more or less dim light, usually green or blue. In order to study effects of light on growth in material which had not been previously exposed to light, a method was devised for obtaining pea sections in total darkness.

Exploratory experiments with these sections revealed the following interesting facts: (1) The optimal (for elongation) concentration of the auxin 3-indoleacetic acid (IAA) was 10 to 100 times lower than that previously reported in the literature; (2) exposure to dim green "safelight" resulted in a somewhat higher IAA optimum;
(3) brief exposures to red light reduced the total elongation by at least one half, and this effect was partially (but not completely) reversed by the addition of either IAA or gibberellic acid; (4) the time course of elongation was linear up to 14 hours, in contrast with a reported sharp decline in rate of growth after 5 hours.

The assay has been developed to test volumes of solutions as small as 0.8 ml. For this purpose, antibiotics may be used to minimize possible interference by microbial activity. The combination of penicillin and streptomycin (100 parts per million each) is satisfactory; it was found to cause no significant change in growth over controls.

Extracts of briefly illuminated dark-grown peas are now being examined for the presence of an inhibitor of elongation induced by light and antagonized by gibberellic acid and/or IAA.

151. PHYSIOLOGY OF FLOWER FORMATION

I. INHIBITORS OF FLOWER FORMATION IN JAPANESE MORNING GLORY

Investigator: J. A. D. Zeevaart
Support: Frasch Foundation
National Science Foundation

Young seedlings (four days old) of the Japanese morning glory, Pharbitis nil, variety Violet, with both cotyledons developed, respond to exposure to one dark period at about 28°C by initiation of flower primordia. The floral stimulus starts to move out of the cotyledons after 15 hours in darkness. The translocation rate of the hormone is calculated to be 0.3 mm/hour. The rate is the same in light and darkness, but is strongly temperature dependent.

The inhibitor of cholesterol biosynthesis SK&F 7997, tris (2-diethylaminoethyl) phosphate, is the only substance thus found to inhibit inductive processes in cotyledons during the dark period. No compounds have been found that can reverse this effect.

Application of either 5-fluorouracil and 5-fluorodeoxyuridine fully suppresses flower formation. These compounds exert their effects only in the bud. Such precursors of DNA as thymidine overcome this inhibition. The application of 5-fluorodeoxyuridine to the plumule in a concentration which fully inhibits flower formation essentially stops cell division in the apex for one to two days. Aminopterin also inhibits flower formation, but colchicine does not. It has been concluded from these results that the floral stimulus in Japanese morning glory can express itself in the initiation of floral primordia only in an apex which contains multiplying DNA.

Further experiments with growth retardants CCC and Amo-1618 have shown that these compounds cause almost complete inhibition of flowering if applied before the inductive dark period, but not if applied one day after the beginning of induction. In both cases the compounds cause a dwarf growing-habit. The flower-inhibiting effect is completely overcome by application of gibberellin to the apex before arrival of the hormone. The dwarfing effect can, however, be reversed much later. The results suggest that the growth retardants block cell division and DNA multiplication in the apex and thus block expression of the hormone in flower primordia.
Publications:


II. A NEW INHIBITOR OF PHOTOPERIODIC INDUCTION IN COCKLEBUR AND ITS EFFECT ON STEROL BIOSYNTHESIS

Investigators: J. A. D. Zeevaart, Erich Heftmann, James Bonner
Support: National Institutes of Health, U.S. Public Health Service
Frasch Foundation
National Science Foundation

Inhibitors of cholesterol biosynthesis developed by Smith, Kline and French Laboratories, Philadelphia, have been tested for their ability to inhibit photoperiodic induction of cocklebur.

Two compounds which block cholesterol biosynthesis between mevalonic acid and squalene cause yellowing of cocklebur leaves and have a slight flower-inhibiting effect.

The most interesting results have been obtained with two compounds which block cholesterol biosynthesis after lanosterol. The effect on photoperiodic induction of one of these compounds, SK&F 7997, tris (2-diethylaminoethyl) phosphate, has been studied in detail. This compound fully suppresses flowering when applied to the leaves shortly before the inductive dark period, but application to the leaves after the inductive night, or to the buds, has no effect. No substances so far have been found which can reverse the flower-inhibiting effect of SK&F 7997.

The hypothesis, based on these results, that the flower hormone may be a steroid has been tested with labeled precursors. Cl4-acetate and Cl4-mevalonate were fed to cocklebur leaves before a long night. Rapid incorporation into the nonsaponifiable fraction was obtained, but no differences between induced and non-induced plants could be detected. Application of SK&F 7997 suppresses sterol biosynthesis (the leaves contain β-sitosterol and stigmasterol). At the same time precursors believed to be farnesol and squalene accumulate. It is possible that SK&F 7997 inhibits another unknown metabolic pathway which leads to flower hormone, but it is also possible that the steroidal differences between induced and non-induced plants are in minor components and have not yet been detected.

Reference:

III. THE JUVENILE PHASE IN BRYOPHYLLUM DAIGREMONTIANUM

Investigator: J. A. D. Zeevaart
Support: National Science Foundation

The initial period in the life cycle during which photoperiodically, plants do not respond with flower formation after exposure to an inductive day length is called juvenile phase. Bryophyllum daigremontianum, a long-short-day plant, has a very long juvenile phase and was therefore chosen for the present investigation.

Young plants of Bryophyllum, which do not form flower buds in response to the change from long day to short day, can easily be induced to flower by grafting them onto flowering old plants. This indicates that whereas the growing points of young plants are fully capable of responding to floral stimulus supplied by the flowering donors, the leaves of juvenile plants are not capable of producing the floral stimulus.

Publication:

IV. THE EFFECT OF METABOLIC INHIBITORS ON VERNALIZATION IN CEREALS

Investigators: Agnieszka Barbaro, Hendrik Ketellapper
Support: Rockefeller Foundation

Many plants require a period of low temperature in order to flower. How such vernalization induces flowering is still not known. Furthermore, the effect of vernalization can be completely reversed during the first few days following the termination of the cold treatment, provided the cold treatment has not been overly long. Such reversal is effected by high temperature. For this reason it seems likely that during or immediately after vernalization biochemical events essential to the process take place. Experiments were undertaken to determine whether nucleic acids or steroids are involved in such events. To this end rye and barley seeds were vernalized at 3 to 5°C at 50% moisture content of the seed. Before, during and after vernalization inhibitors of RNA, DNA or steroid synthesis were applied. The following substances were used: 5-fluorouracil, 5-fluorodeoxyuridine, 6-azauracil, 2-thiouracil, 4-ethylthiamin, 2,6-dimethyl pyrimidine, 5-hydroxyuridine, 8-azaguanine, 2,6-diaminopurine and three different steroid inhibitors, SKF 3301 A, 7732 and 525 A. Results based on the evaluation of dissected growing points and on the number of leaves formed before flowering did not show significant specific inhibition of floral induction by any of the inhibitors. The delays in flowering obtained can be explained on the basis of inhibition of development.

V. COREOPSIS, A SHORT-LONG-DAY PLANT

Investigators: Agnieszka Barbaro, Hendrik Ketellapper
Support: The Rockefeller Foundation

The purpose of these experiments was to determine the environmental conditions which Coreopsis plants require for flowering. Coreopsis will not normally flower in long days (16 to 18 hours' light) nor in short days (8 hours' light). However, if plants first receive a period of short days (SD) and are then transferred to long day (LD) conditions, they flower. Thus Coreopsis belongs to the group of short-long day plants. One month to 6 weeks of SD is adequate for flowering to occur subsequently
in LD. The SD treatment can be replaced by vernalization at 3°C. The day length during vernalization is immaterial. Four to six weeks at 3°C is generally sufficient.

Very young plants do not respond to SD treatment. Seedlings are, however, fully sensitive to inductive SD treatment when they are 10 weeks old.

There are indications that the effect of SD treatment is dependent on the temperature during the dark period. The results are not conclusive due to the variability of the material. The population used is extremely variable with respect to sensitivity to SD treatment.

Application of gibberellic acid (GA) elicits striking responses from Coreopsis. The effect of GA depends on the photoperiod. Plants raised in SD and kept in SD during GA treatment respond to GA with stem elongation and the initiation of buds which develop into flowers. Plants raised in LD and kept in LD during GA treatment respond with stem elongation comparable to that which normally accompanies flowering. However, the growing points remain vegetative. Finally, plants raised in SD and transferred to LD at the beginning of GA treatment also show very striking stem elongation. GA markedly inhibits flower formation in these plants as compared with control plants which flower normally.

Day length has a very striking effect on the vegetative development of Coreopsis. In SD prostrate rosette plants are produced. Leaves are small and prostrate; the petioles short. In LD plants grow as erect rosettes. The leaves are large and erect; the petioles are long. When LD plants are transferred to SD, all the leaves with the LD character die very quickly.

VI. PHOTOPERIODIC CONTROL OF FLOWERING IN JASMINE

Investigator: Wesley O. Griese
Support: American Academy of Arts and Sciences

Various species of the genus Cestrum show different responses to photoperiod. The responses of two species, C. diurnum (day-blooming jasmine) and C. nocturnum (night-blooming jasmine) were investigated. Plants were grown at 23°C day temperature and 19°C night temperature, with either an 18-hour light period (long day = LD) or an 8-hour light period (short day = SD).

Four-month old seedlings of C. diurnum grown on LD formed floral buds at more than 90% of the potential sites when given two SD. Defoliating experiments showed that the stimulus originates in the mature leaf and is transported throughout the plant. Plants maintained on LD for 18 months bloomed sporadically three times; in each case, less than 1% of the floral sites were affected. Plants maintained on SD showed a similar response. These experiments indicate C. diurnum is a long-short-day plant with regard to its flowering response.

Four-month old seedlings of C. nocturnum grown on LD formed floral buds at more than 90% of the potential sites when given two SD, but seedlings maintained on LD formed flower buds at more than 90% of the sites in from 7 to 9 months. In previous work neither plants grown on LD, defoliated and then placed on SD, nor plants maintained on SD for 18 months had formed floral buds (Sachs, 1956). A seedling maintained on LD has bloomed continuously for one year after its floral initiation. These data suggest that C. nocturnum is not as strict a long-short-day plant as is C. diurnum but can also flower on LD.

The floral stimulus could not be transferred by graft between the two species although both bloomed on the same grafted plant when leaves of both were retained.
The stimulus was transferred through stem intergrafts of the non-donor species and caused floral initiation in the donor. These data suggest that the floral stimulus is not transferable between C. diurnum and C. nocturnum, and that at least two different hormones may be involved.

Reference:


VII. EFFECT OF LONG-DAY INTERRUPTION ON PHOTOPERIODIC INDUCTION IN A SHORT-DAY VARIETY OF HEMP

Investigator: Diethard P. Köhler
Support: German Federal Research Council (Deutsche Forschungsgemeinschaft)
National Science Foundation

In short-day plants which need more than a single night for flower induction it had been found that long days intercalated between short days inhibited flowering, one long day being often sufficient to abolish the flower-promoting effect of more than one short day (Schwabe, 1959). This effect was thought to be a common feature of this type of short-day plants. In a short-day variety of hemp (Cannabis), it could be shown, however, that long days intercalated between inductive short days have no effect on the flowering response; in this plant, then, the effect of short days is, at least within the limits of the experiments, simply additive.

Reference:


152. CONTROL OF THE ORIENTATION OF CELL DIVISION IN ROSETTE PLANTS

Investigators: Bilandrai Baldev, Aurora Agatep, Anton Lang
Support: American Cancer Society
National Science Foundation

It is well known that excessive but irregular cell division results in the formation of a mass of unorganized tissue, forming localized tumors in both plants and animals. In work with shoot apices of some rosette plants it has been shown earlier that it is not only possible to enhance cell division but also control orientation, i.e. the direction of the mitotic spindle and of wall formation (Biology 1960, p. 90; 1961, p. 113). Since then some additional information has been obtained.

Crepis parviflora (hawksbeard) is a long-day plant and remains in a rosette condition when kept under short days. In this stage there is only slight mitotic activity at the shoot tip. Application of gibberellic acid increases cell activity to a very great extent and the resulting divisions are mostly vertical, thus contributing to stem elongation. If the plant is completely defoliated immediately after the application of gibberellic acid there is only a slight decrease in cell division activity but the orientation of the spindle occurs at random. To study further the role of leaves on the orientation of mitotic figures different degrees of defoliations were tried. These results as well as previous information strongly suggest that leaves play a part in determining orientation of cell division in the stem and thus contribute to the control of the morphogenesis of the latter.
Reference:


153. BENZYL-ADENINE AND SENESENCENCE IN PEAS

Investigator: Hendrik Ketellapper
Support: The Rockefeller Foundation

Peas suffer two types of senescence: leaf and tip. Both are influenced by environmental factors. In studies of the effects of photoperiod on peas (above) it was noted that photoperiod strikingly influenced senescence: the longer the photoperiod, the earlier the plants flower and the earlier the growing point dies and elongation stops. In the case of the variety Unica, the number of nodes to first flower is also reduced. With increase in photoperiod the plants go through their life cycle more quickly.

Application of the kinetin analog benzyl-adenine delays leaf senescence. The formation of new nodes continues for as long as benzyl-adenine is supplied. However, it appears that benzyl-adenine does not prevent the dying of the apical growing point, but promotes the development of an axillary bud on the uppermost node. As a result the number of nodes and the number of flowers and fruits are increased. Since the new internodes are very short, height is little affected.

Benzyl-adenine also has bad effects. The number of flowers which aborted increases as a result of treatment with the chemical. Growth is inhibited by concentrations of 50 mg/l and higher applied as leaf sprays once a week. The inhibition depends on the photoperiod. In continuous light even 100 mg/l is not inhibitory. The degree of inhibition also depends on the physiological age at which the application is started: The older the plant, the smaller the inhibition.

It can be concluded that benzyl-adenine does delay the onset of leaf senescence, but that it has little or no effect on tip senescence.

154. A NEW SEX TYPE IN HEMP

Investigator: Diethard P. Köhler
Support: German Federal Research Council (Deutsche Forschungsgemeinschaft)
National Science Foundation

As a foundation for physiological studies on sex determination and flowering in hemp, genetical work which the author had initiated in Germany was continued. Hemp occurs in male and female plants. The males are heterozygous (XY), the females homozygous (XX). The typical males differ from the females also in the shape of the inflorescence, since after flower induction they grow faster than the latter and thus form a loose inflorescence with the individual flower clusters widely separated. An altered X chromosome was discovered (Xm); XmXm homozygotes are males but their inflorescence is like that of females (XX or XmX). This shows that sex expression in hemp is not correlated with inflorescence growth.

Publication:

VIROLOGY

PART A.--ANIMAL VIRUSES

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155. NUCLEOTIDE SEQUENCES IN THE AMINO ACID CODE

Investigators:
John D. Smith, Renato Dulbecco

Support:
National Institutes of Health, U.S. Public Health Service

In a cell-free E. coli system polyuridylic acid specifically directs the synthesis of polyphenylalanine. The composition of the nucleotide sequences directing the incorporation of the other amino acids is known. An attempt is being made to determine the order of the nucleotides in the sequences coding for individual amino acids. Polyribonucleotides consisting of a known oligonucleotide sequence at the 5'-end of polyuridylic acid have been prepared using polynucleotide phosphorylase. These polymers are tested in the E. coli system to find whether they will direct the synthesis of a polyphenylalanine chain terminated by an amino acid specified by the terminal sequence of the polyribonucleotide. Polyuridylic acids terminated by a sequence of cytidylic or adenylic acid residues will direct the synthesis of polyphenylalanine. Those terminated by one or more cytidylic acid residues also stimulate the incorporation of small amounts of serine into polyphenylalanine. However, since the location of the serine in the polypeptide chain is not yet known it is not certain that the synthesis is specified by the terminal sequence of the polyribonucleotide.

156. PURIFICATION OF POLYOMA VIRUS

Investigator:
Ernest Winocour

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

Many aspects of the investigation of the polyoma tumor virus have been hampered by the lack of a concentration and purification procedure. The main difficulty arises from the fact that only relatively low titers of the virus are routinely obtained in mouse cell cultures infected in vitro. This difficulty has now been overcome by the use of cultures made from the kidneys of baby mice that were inoculated with the virus a few days after birth. Such cultures of in vivo infected mouse kidney cells release large amounts of virus (2 x 10^9 to 4 x 10^9 plaque forming units per milliliter) after 7 to 9 days of in vitro incubation. The virus is then concentrated by two consecutive cycles of sedimentation onto a cesium chloride layer, in a manner similar to the method devised by Crawford for Rous sarcoma virus (Crawford, Biology 1961). Finally, the virus is purified by banding in a cesium chloride density gradient.
centrifugation. The density gradient centrifugation of the virus has also revealed the presence of non-infectious hemagglutinating virus which bands at a lower density than that of the infectious virus.

The over-all recovery of infectious virus in the above purification procedure is of the order of 40 to 50%, and titers up to $10^{11}$ plaque forming units per milliliter have been obtained. A preparation of $4 \times 10^{10}$ plaque forming units per milliliter yielded 40 micrograms per milliliter of polyoma virus DNA when extracted with phenol. The homogeneity of the final virus preparations has been attested to by (1) the sharpness of the infectivity peak in the density gradient centrifugation, and (2) the presence of a single sedimenting component in sedimentation velocity runs carried out in the analytical centrifuge in cooperation with Dr. J. Vinograd.

157. POLYOMA DNA-DIRECTED SYNTHESIS OF RNA

Investigator: Ernest Winocour
Support: American Cancer Society
National Institutes of Health, U.S. Public Health Service

In attempts to establish a cell-free system for the synthesis of polyoma virus related materials, the first stage that was studied concerned the synthesis of RNA on a polyoma DNA template. In the presence of the enzyme RNA polymerase (derived from E. coli bacteria), polyoma DNA directs the incorporation of the four ribonucleoside triphosphates into an acid-insoluble product. The effectiveness of polyoma DNA in priming this reaction was found to be comparable to that of calf thymus and T4-phage DNA. Currently under study are (1) the nature of the product formed in the above reaction, (2) its effect, when co-melted, on the infectivity of polyoma DNA, and (3) the ability of the product to direct incorporation of amino acids into polyoma-specific proteins.

158. THE PHYSICAL STATE OF DNA DURING REPLICATION IN ESCHERICHIA COLI

Investigator: Ronald Rolfe
Support: National Institutes of Health, U.S. Public Health Service
American Cancer Society

A study of the replication of DNA in E. coli has been undertaken using density and radioactive labeling. Preliminary experiments have revealed the existence of a DNA fraction with unusual physical properties. The kinetics of labeling of this fraction suggest that it is an intermediate in DNA synthesis and its physical properties are consistent with the possibility that it represents a template for the synthesis of new DNA. A complete characterization of this DNA fraction is in progress.

159. MUTANTS OF POLYOMA VIRUS

Investigator: Michael Fried
Support: American Cancer Society, Nutrition Foundation
National Institutes of Health, U.S. Public Health Service
The National Foundation

An attempt is being made to isolate temperature sensitive mutants of polyoma virus. It is hoped that by the use of such mutants the properties of the virus and of the virus-host cell relationship leading to neoplastic conversion can be further clarified.
Upon treatment of polyoma virus with nitrous acid first order kinetics of inactivation are produced for at least 3.5 log units. This indicates that a single deamination in the DNA of the virus is enough to destroy its plaque forming ability.

Two probable temperature sensitive mutants have been isolated from nitrous acid treated virus. These temperature sensitive mutants have the property of forming plaques at 30°C but not at 37 to 39°C. The wild-type virus is capable of plaque production at both the high and low temperature. Preliminary results with the two lines indicate that the effect of the high temperature is to interfere with the production of plaque forming virus during a step which occurs at the end of the latent period. This result suggests that a late function, possibly involved in the synthesis of the capsid protein, is temperature sensitive.

160. RADIOBIOLOGICAL STUDIES WITH POLYOMA VIRUS

Investigator: Thomas L. Benjamin
Support: National Institutes of Health, U.S. Public Health Service
American Cancer Society
The National Foundation

A start has been made on a study of polyoma virus using radiological methods. To date, the X-ray and ultraviolet sensitivities of the virus have been determined for the cytocidal interaction of the virus with mouse embryo cells. Preliminary results have been obtained on the radiosensitivity of the capacity of these cells to reproduce the virus. It is hoped that these results, along with those from a similar study of the ability of polyoma to transform hamster embryo cells, will give some information on the nature of the neoplastic conversion.

161. ORIGIN OF THE NEoplastically TRANSFORMED CELLS IN HAMSTER EMBRYO CULTURES INFECTED BY POLYOMA VIRUS

Investigators: Marguerite Vogt, Renato Dulbecco
Support: American Cancer Society
National Institutes of Health, U.S. Public Health Service
The National Foundation

It has been previously shown that hamster embryo cultures infected by polyoma virus become "transformed" because of the appearance and selection of a new cell type, which has neoplastic properties. The origin of these cells has now been studied.

Hamster embryo cultures were infected by the virus and at various times afterwards (between 2 and 20 days) they were dispersed with trypsin; the cells from one culture were used to start a group of new cultures with low cell density (sparse cultures). The sparse cultures were maintained for two weeks; some of them showed mitotically active foci of distinctive morphology, with a frequency of about $10^{-2}$ per cell seeded. The foci were picked and the properties of the cells they contained were studied over a number of serial transfers.

A striking result of these experiments is that although each focus must have originated from a single cell (from statistical considerations), they contained cells which, on subcloning, gave rise to clones of different types. In general there was one predominant cell type called early transformed cell, which produced clones of relatively low abnormality. In addition there was a variable proportion of cells giving rise to very abnormal clones of various types, probably similar to the cells studied in the previous experiments in mass cultures. The latter cell types are called "late" transformed cells.
Early transformed cells have low efficiency of cloning (similar to normal cells) and are not transplantable into adult hamster; late cells have high efficiency of cloning and are transplantable. Neither type of cultures contain infectious polyoma virus.

In mixed cultures late transformed cells become selected for.

These results suggest the hypothesis that the first product of the transformation caused by polyoma virus is the early transformed cell, which has a relatively normal morphology and enhanced growth ability; and that these cells produce the late cells by a genetic variation.

Since the variation leading to the late transformed cells does not occur with a comparable frequency in non-infected cultures, it must be itself virus-induced. Studies on the mechanism of variation are under way. The late cells are frequently found to be aneuploid, with chromosome numbers of 45 to 49 (euploid is 44); the variation may therefore be due to the production of chromosomal alterations.

Cytological studies have shown that the early transformed cultures very frequently have anaphase bridges. The formation of the bridges causes a high cell mortality in the cultures. Bridge formation may also be the cause of the chromosomal alterations leading to the production of transformed cells. This problem is under study.

162. TIME LAPSE PHOTOGRAPHS OF CULTURES TRANSFORMED BY POLYOMA VIRUS AND OF NORMAL CULTURES

Investigators: Renato Dulbecco, Marguerite Vogt
Support: American Cancer Society
National Institutes of Health, U.S. Public Health Service
The National Foundation

One of the outstanding characteristics of culture of late transformed cells present in cultures transformed by polyoma virus is their culture pattern. This is characterized by extensive multilayering and by a criss-cross arrangement of the cells. It was thought that the alteration of the culture pattern may depend on the special social behavior of the transformed cells in the culture. This was shown to be the case by studying time lapse photographs of growing cultures, which were taken at the Pasadena Foundation for Medical Research in the laboratory of Dr. C. Pomerat.

The study of these photographs showed two main differences between normal and transformed cells. The first difference concerns the movement of the cells: Normal cells, when they meet, tend to slip past each other, without crawling on top of each other; on the contrary transformed cells upon meeting continue in the original direction and thus crawl on top of each other. This difference may be due to an alteration of the property of contact inhibition in the transformed cells, or to low adhesion of the transformed cells to the substrate.

The second difference concerns the behavior of mitotic cells. In the normal cultures they remain attached and partly spread, whereas in the cultures of transformed cells they round up and partly detach from the substrate. This phenomenon suggests low adhesiveness of the transformed cells to the substrate.

Thus the alteration of the pattern of cultures of transformed cells suggests alteration of the cell surface and of the regulatory mechanisms involved in contact inhibition.
STUDIES OF THE PERMEATION SYSTEMS FOR AMINO ACIDS IN NORMAL AND TRANSFORMED CELLS

Investigator: Renato Dulbecco
Support: American Cancer Society
        National Institutes of Health, U.S. Public Health Service
        The National Foundation

The finding that neoplastically transformed cells (of the late type) are considerably altered in their surface suggested a study of some permeation system of the cells. The object was to find out whether the neoplastic cells had a more efficient permeation system.

A series of experiments were carried out by determining uptake of C\(^{14}\) threonine into acid-soluble precursor pool and into acid-insoluble protein. Cell lines of various kinds were used: secondary mouse embryo and hamster embryo cultures, lines of "early" transformed cells and a line of late transformed cells. Preliminary experiments were also carried out for the uptake of C\(^{14}\) lysine and C\(^{14}\) phenylalanine. All the results were normalized to the same weight of cell protein. The results obtained can be summarized as follows:

1. The uptake of the amino acids follows a curve of the Michaelis-Menten type, and is abolished in the cold; this shows that uptake is caused by a permease.
2. Uptake is very rapid; pool equilibration reaches 50% in about one minute. The initial rate of uptake is unchanged in the presence of puromycin.
3. The rate of uptake varies quite widely even for normal cultures, depending on their physiological state. This appears to be a sensitive criterion for characterizing the physiological state of the cultures.
4. For a given amino acid the rate of uptake and the rate of incorporation into protein vary in a parallel way; for different amino acids the same culture may show large differences in the ratio of the two rates.

These results show that efficiency of amino acid permeases are an expression of the general growth ability of a culture. Since point (4) suggests that the uptake of amino acids is not the rate limiting step in protein synthesis, the differences in permease efficiency may be a consequence rather than a cause of the degree of growth ability of the cells. The results suggest that general growth ability may depend on a mechanism which independently controls the rate of permeation and the rate of protein synthesis.

ANTIGENIC DIFFERENCES BETWEEN NORMAL AND POLYOMA VIRUS TRANSFORMED HAMSTER CELLS

Investigator: Robert E. Bases
Support: American Cancer Society
        National Institutes of Health, U.S. Public Health Service
        The National Foundation

Antigenic differences between normal hamster embryo cells and those transformed by polyoma virus were investigated in vitro. Rabbit antisera to pooled normal embryo cells and to transformed cells were prepared. These sera reacted with either cell type in vitro and gave a prompt cytolytic effect in the presence of complement. The reaction was studied quantitatively by means of an assay of surviving cells based on their ability to multiply in vitro and form macroscopic colonies. The method was applicable to both cell types. At low concentrations of antiserum (1/1000) killing was
entirely complement dependent; at high concentrations (1/50) killing was independent of complement and appeared due to another mechanism.

Studies of the kinetics of cell killing by antibody and complement revealed (1) an initial lag period, inversely proportional to antisera concentration during which cells absorbed antibody and were prepared for killing by complement; (2) a second phase during which cell survival decreased rapidly with time. A plot of log survivors versus time gave a straight line for 2 or 3 decades. The second phase slope was a function of complement concentration at constant antibody concentration and a function of antibody concentration at excess complement concentration.

Application of this method provided the following information:

(1) Normal hamster embryo cells were more sensitive to antibody and complement than transformed cells, and absorbed antibody was better than the transformed cells. Use of whole cells or cell membranes as antigen leads to similar results.

(2) Anti-transformed cell sera, after exhaustive absorption with normal embryo cells failed to react with such cells but did so with five established hamster cell lines. Two were polyoma induced malignant cells that produced progressive tumors in hamsters. But a third (malignant) line arose spontaneously. Not only did the latter line react with the absorbed sera but itself removed all residual activity against the other lines. This indicates that while the above investigations failed so far to reveal a cellular antigen associated with polyoma virus infection in hamster cells they did reveal a surface antigen common to five established hamster cell lines, the presence of which may be related to this property of the cells.

165. STUDIES IN THE QUANTITATIVE ASPECTS OF POLYOMA VIRUS MEDIATED NEOPLASTIC CONVERSION IN VITRO

Investigator: Klaus Bayreuther
Support: American Cancer Society
The National Foundation

Attempts have been undertaken to develop a quantitative assay system for polyoma virus mediated neoplastic conversion in vitro. The work has not yet led to a completely reliable routine method. However, it resulted in an increase of efficiency of conversion of up to 1% of the cells infected. The cells used are exclusively heart fibroblasts from 15-day-old Syrian hamsters. The cells are infected either in suspension or on the dish in attachment medium (0.25 M sucrose and 0.001 to 1 M CaCl₂) or on the dish for periods of 12 to 24 hours in nutrient medium reinforced with glucose and tryptose phosphate. The infected cells are plated in medium containing 20% fetal calf serum. After three days the fetal calf serum has to be replaced by 10% calf serum since fetal calf serum has been found to interfere with the expression of the neoplastic phenotype. In several instances a linear relationship between the number of neoplastic foci and virus input has been found.

166. STUDIES OF POLYOMA VIRUS INFECTION IN MOUSE CELLS

Investigator: Robert A. Weisberg
Support: National Institutes of Health, U.S. Public Health Service
The National Foundation
American Cancer Society

A line of polyoma virus (Py) transformed cells deriving from a clone of mouse embryo cells has been obtained (Weisberg, Biology 1961, Report 181). This line is
relatively resistant to Py. Subclones from this culture were studied to find Py-sensitive revertants. Two subclones out of 50 were almost as sensitive to Py infection as a control line of uninfected mouse embryo cells. The virus-sensitive culture possessed the typical morphology and culture-habit associated with cells transformed by Py. It is concluded that the maintenance of the transformed state is not causally related to immunity to reinfection with Py. Superinfection immunity probably arises as a result of secondary selection for virus resistant mutants in an infected culture.

The properties of these subclones are being further studied along lines suggested by several recent developments in the tumor virus field; namely, (1) that virus induced tumors possess a new and virus-specific antigenic determinant; and (2) cells transformed in vitro by Py and relatively resistant to Py infection can produce larger quantities of interferon (an inhibitor of virus multiplication) when infected with vaccinia virus than can untransformed cells.

The heterogeneous response (cell degeneration and cell proliferation) of clonal isolates of mouse embryo cells after Py infection has been reinvestigated and confirmed. Twenty-two out of 43 clones which were originally derived from an inbred strain of mice infected with Py and then reimplanted into the strain of origin gave rise to tumors. However, almost as high a proportion of non-infected control clones also produced tumors. These control clones were maintained in vitro for 4 to 6 months before reimplantation. Most, although not all, had undergone changes in morphology and culture-habit from freshly explanted mouse embryo tissue. The rapid occurrence of "spontaneous" transformation in uninfected cultures of mouse embryo cells renders the relation between Py infection and the acquisition of transplantability obscure. The transplantability, antigenicity and behavior in culture of these lines are being studied in an effort to clarify this relation.

167. STUDIES ON THE DNA OF POLYOMA VIRUS

Investigator: Roger Weil
Support: Damon Runyon Fund
National Institutes of Health, U.S. Public Health Service

The present studies were undertaken in order to elucidate the nature of the DNA of polyoma virus and to compare it with the DNA of other oncogenic viruses. Most of the studies were performed with a subviral infective agent (SIA) extracted from mouse embryo cells infected with polyoma virus or from purified virus preparations. The SIA contains the genetic information for the synthesis of polyoma virus. Its properties differ markedly, however, from those of the intact virus. Preliminary investigations in the analytical centrifuge on the DNA extracted from purified virus preparations suggest that the behavior of the SIA actually reflects that of the viral DNA. The DNA content per infective virus particle is about $6 \times 10^6$. The DNA of polyoma virus is base-paired and has a buoyant density in CsCl of 1.709 g cm$^{-3}$. The behavior of polyoma DNA is unusual however: the infectivity increases regularly by a factor of 2 to 3 upon heat denaturation. The infectivity is unusually heat stable. About 50% of heat denatured polyoma DNA renatures spontaneously during density gradient centrifugation. Experiments are under way in order to determine the structural basis of the unusual properties.

References:


168. INTERACTION OF CYTOCIDAL VIRUSES WITH CHROMOSOMES OF ANIMAL CELLS

Investigator: Klaus Bayreuther  
Support: American Cancer Society  
The National Foundation

Mouse and chicken embryo cultures have been infected with cytocidal viruses of four different types: 1) DNA-virus with intranuclear phase of reproduction (polyoma, herpes); 2) DNA-virus with cytoplasmatic phase of reproduction (vaccinia); 3) helical RNA-viruses (influenza); and 4) isometric RNA-viruses (EMC, Sindbis). Infection with these viruses results in an immediate and almost complete blockage of the mitotic activity. The mitotic index sinks from normally 3 to 5% to 0.003 to 0.005%. Addition of glucose and insulin to the medium increases the mitotic frequency of infected cultures about tenfold. Infected cells entering mitotic division show at metaphase heavily fragmented chromosomes. The breaks are of the nature of chromosome mutations and are located randomly. Cells incubated with interferon prior to infection with virus show the same time sequence for mitotic blockage and chromosome fragmentation as do controls, indicating that these two events are probably very early steps in the course of virus reduplication not affected by the action of interferon.

169. ALTERATIONS OF THE SYNTHESIS OF POLIOVIRUS AT SUPRANORMAL TEMPERATURE

Investigator: Herbert Subak-Sharpe  
Support: The National Foundation

It has been shown by Lwoff that there is a critical temperature above which the synthesis of infectious virus is inhibited, and that this temperature varies for different virus lines, and is genetically determined.

The alterations of the synthesis of poliovirus type 1, Brunhilde, in monkey kidney cultures at 42°C were examined. The following determinations were carried out: infectious virus; infectious RNA; incorporation of precursors into either protein or RNA which will end up in mature virus when the temperature is lowered and synthesis of virus is allowed to resume; synthesis of poliovirus antigen by serum blocking power; formation of non-infectious particles having physical properties of poliovirus particles.

The results showed that cells infected at high temperature do not synthesize any material recognizable by the tests listed above. It appears therefore that the high temperature blocks an initial step in synthesis.

170. CHARACTERISTICS OF AN INHIBITOR OF ROUS SARCOMA VIRUS

Investigator: John P. Bader  
Support: National Institutes of Health, U.S. Public Health Service  
American Cancer Society

An inhibitor of cellular transformation by Rous sarcoma virus appearing in chick embryo cell cultures infected by the virus has been identified as an interferon. The interferon acts upon the virus-cell complex and not upon virus alone, and cells must be exposed to the interferon prior to infection for effective inhibition. The
inhibitory effect of interferon is rapidly lost in dividing cultures; this loss cannot be attributed to dilution of interferon alone. The dose-response curve for the interferon follows single-hit kinetics for low multiplicities of virus per cell, and an increase in virus/cell ratio up to 10 does not alter the kinetic curve of the reaction.

Production of the interferon after exposure of cells to inactivated Rous sarcoma virus has been analyzed. Only small amounts of interferon are present after 12 hours and maximal interferon production does not occur until the 24th hour. Interferon production can be inhibited by prior exposure of cells to X-rays (5000 r) or to actinomycin (1 µg/ml). The time course of interferon synthesis after exposure to virus is under investigation.

Reference:


171. THE EFFECT OF INTERFERON IN DEVELOPMENT OF ROUS SARCOMAS

Investigator: John P. Bader
Support: National Institutes of Health, U.S. Public Health Service
American Cancer Society

Interferon which is produced by chick embryo cell cultures exposed to inactivated Rous sarcoma virus is able to prevent or delay the appearance of tumors in chicks injected with Rous sarcoma virus. The possible effect of interferon in preventing viral metastases has been examined. Chicks injected with large numbers of cells which had been transformed by Rous sarcoma virus in vitro are susceptible to an injection of virus at another site. No interferon could be demonstrated in the serum of chickens having large virus-induced Rous tumors, and although interferon is produced in large quantities in cultures of Rous tumor cells dispersed and grown in vitro, the tumors themselves contain only small amounts of interferon.

Observations on the regression of Rous tumors did not support the notion that interferon plays an important role in such regressions. These, and other observations, indicate that if interferon plays any role in Rous tumor development in the chicken, it is a subtle role.

172. A STUDY ON CHICK EMBRYO CELLS INFECTED BY HEAT INACTIVATED ROUS SARCOMA VIRUS

Investigator: Robert C. Y. Ting
Support: National Institutes of Health, U.S. Public Health Service
American Cancer Society

Chick embryo cells infected by heat inactivated Rous sarcoma virus exhibit the following characteristics:

(1) Resistance to superinfection of active Rous sarcoma virus (Temin, 1960).

(2) Production of interferon-like substance (Bader, 1962).

However, little is known whether any of these properties can be transmitted to progeny. For this purpose a set of culture of chick embryo cells was infected with heat-inactivated Rous sarcoma virus (37°C for 6 days, focus-forming units before heating 5 x 10^5/ml, after heating 0/0.4 ml), and another set treated with medium was
used as control. Two plates from each set were then infected with 400 to 600 FFU. A reduction of 80 to 90% of foci was observed on plates of the culture which had received heat-inactivated virus.

Cells of treated and untreated cultures were transferred every three days. Two plates of each were again challenged with 400 to 600 FFU. The treated culture showed resistance to challenge even after the seventh transfer. Supernatant of treated culture, however, does not induce resistance of chick embryo cells. This rules out the possibility that these cultures are able to produce interferon-like substance indefinitely. The nature of such inheritable property is being investigated.

173. VIRUS RELEASE FROM CLONES OF ROUS SARCOMA CELLS

Investigator: Robert C. Y. Ting
Support: National Institutes of Health, U.S. Public Health Service
American Cancer Society

Chicken embryo cells infected by Rous sarcoma virus cloned on X-rayed duck feeder layers often release very low amounts of virus. Of 40 clones (2,000 to 5,000 cells/clone) tested, more than half released 10 to 12 focus-forming units (FFU). When fresh chick embryo cells were added to these clones and incubated for 3 or 4 days, the amount of virus increases 30 to 50 times as compared to clones grown by themselves or clones grown with hamster feeder cells. One of the 40 clones showed no virus before enrichment, but had 20 FFU after enrichment. Thus no virus-free clone of Rous cell could be obtained. The enrichment technique is essential to disclose whether a clone of Rous cells is virus free.

174. HEREDITARY TRANSMISSION OF A VIRUS INDUCING CO₂-SENSITIVITY IN DROSOPHILA

Investigator: Robert Seecof
Support: National Institutes of Health, U.S. Public Health Service
The National Foundation

Certain strains of Drosophila melanogaster maintain a virus infection (Sigma virus) which is transmitted in hereditary fashion through the flies' eggs and sperm. Ordinarily, infected strains maintain high titers of the virus, the average fly containing about $10^4$ infectious units. However, there is a body of evidence that suggests the virus may exist within fly cells and be transmitted by gametes in a noninfectious form (L'Heritier, 1958, Advances in Virus Research, Academic Press, Inc.).

Virus-infected flies are being studied in order to determine the nature of the noninfectious form of the virus. A strain has been discovered in which all of the flies carry the infection, but the average fly contains only about 150 infectious units. The infectious virus from this strain has been shown to be capable of initiating high titer infections in genetically similar fly strains, and to be similar to ordinary infectious virus in several other respects. This "low-titer" infection is being studied further in an attempt to identify the noninfectious virus form and its relationship to the host Drosophila cells. These results will be presented more fully in the Cold Spring Harbor Symposium on Quantitative Biology, 1962.
175. LACK OF INFECTIVITY OF BACTERIOPHAGE λ DNA IN THE PROPHAGE AND VEGETATIVE STATES

Investigator: Jean J. Weigle
Support: The National Foundation

Kaiser has shown that the DNA extracted from mature λ phage particles can participate in the production of phage particles of its own genetic type in bacteria infected with helper λ phages. This property is called the infectivity of λ DNA.

The genetic material of λ phage can exist in several states: as prophage in lysogenic bacteria; as material injected into a sensitive host; as multiplying or vegetative DNA; and as the DNA of mature phages. Experiments to determine the infectivity of the DNA in these different states were performed.

To study the infectivity of the phage DNA during lytic growth, sensitive bacteria were infected with λ phage at a multiplicity of 5. After different times of incubation aliquots were removed to measure 1) the amount of DNA synthesized, 2) the infectivity of this DNA, and 3) the number of mature intracellular phages. The results show that immediately after injection the phage DNA loses its infective property and that only a very small fraction of the vegetative DNA is infective until just before the appearance of mature phages in the infected cell, at which time a large proportion becomes infective.

In the case of lysogenic bacteria similar experiments show that there is no detectable infectivity in the prophage state. During vegetative multiplication following induction of lysogenic cells, just as after infection, infective DNA appears only just before the production of mature viruses.

These results suggest that, although λ DNA in mature phage is infective, this property is lost immediately after injection into a host bacterium. Infectivity is only re-acquired immediately before the formation of mature phage particles.

This work was done in collaboration with William F. Dove, Division of Chemistry, California Institute of Technology.
176. RECOMBINATION OF BACTERIOPHAGE LAMBDA IN BACTERIA DEPRIVED OF THYMIDINE

Investigator: Jean J. Weigle
Support: The National Foundation

The following experiments were performed using a strain of E. coli K-12 which requires thymidine for growth. This strain when deprived of thymidine undergoes "thymineless death": unable to synthesize DNA it nevertheless synthesizes proteins and RNA. Such bacteria, starved for thymidine, lyse after being infected with phage \( \lambda \). The yield of phage produced is very low, on the order of one phage/bacterium with an input multiplicity of four. The mean burst size increases with the addition of thymidine to the growth medium, the maximum burst size being reached with about 10 gamma/ml. Genetic crosses have been performed in bacteria deprived of thymidine to varying degrees. It is found that, as the burst size drops, the frequency of recombination increases. In bacteria completely deprived of thymidine the frequency of recombination, for markers giving 5% in the control, increases to about 30%. Single bursts show that in most cells only one phage is produced and that is often a recombinant. Unlike the control, many of the phage produced in the absence of thymidine are heterozygous.

A three-factor cross (using the markers \( m_6, c \) and \( m_1 \)) was performed in the presence (control) and absence of thymidine in the growth medium. A large number of the progeny plaques were picked and replated to test for heterozygosity. The results show that in the absence of thymidine there is about a fivefold increase in the frequency of the various recombinant types. In addition, at least 25% of the progeny were heterozygous for at least one locus. No heterozygotes were found in the control. The structure of many of the heterozygotes was complex (e.g. some phage were heterozygous for the terminal markers, homozygous for the central marker).

It thus seems that an extreme fragmentation and reassortment of the \( \lambda \) genetic material takes place in bacteria starved for thymidine. It is tempting to relate these facts to the lack of DNA synthesis in the absence of thymidine and to draw the conclusion that for phage \( \lambda \) replication "obsures" recombination, and that recombination can occur in the total absence of DNA synthesis. It thus becomes important to know if phage DNA synthesis is completely blocked by thymidine starvation of the bacteria. Experiments are in progress to test various possibilities such as breakdown and reutilization by the phage of bacterial DNA, or the "leakiness" of the bacteria. Further experiments are also in progress to determine more precisely the genetic and structural properties of the phage produced under these conditions.

177. THE ULTRAVIOLET INACTIVATION OF 5-BROMODESOXYURIDINE SUBSTITUTED T4D BACTERIOPHAGES

Investigator: Seymour Rapaport
Support: National Science Foundation
The National Foundation

When viruses or bacteria are grown in the presence of the thymidine analog 5-bromodesoxyuridine (5-BD), this substance becomes incorporated into their DNA in place of thymidine. Organisms with such substituted DNA have proved of value in genetic studies in the past. It has been found that such organisms are also much more sensitive to ultraviolet and "visible" light than their unsubstituted counterparts.

In hopes of more accurately defining this increased sensitivity and obtaining further information regarding the ultraviolet inactivation of viral DNA in general an action spectrum of inactivation of plaque forming ability of 5-BD substituted T4 was
determined. This was done using an ultracentrifuge purified fraction of 5-BD T4 which was uniform in respect to the total amount of 5-BD substitution that had occurred in its DNA.

The ratio of sensitivity of 5-BD T4 to unsubstituted T4 was studied at various wavelengths. This ratio was 4 to 5 at wavelengths less than 2890 Å, rose very rapidly to a maximum of 475 at 3130 Å, before falling again at about 3440 Å, where it became only 200. Other studies at 3650 Å and at 4040 Å indicated no significant difference in sensitivity between substituted and unsubstituted DNA. These differences in sensitivity have potential use in the selective inactivation of portions of 5-BD substituted DNA genetically incorporated into otherwise unsubstituted DNA.

From the relative magnitudes of the increases in sensitivity it was possible to conclude that exciton travel, i.e. transfer of light energy absorbed by one nucleotide in the DNA chain to another prior to ultraviolet inactivation, must occur in the inactivation of unsubstituted DNA by ultraviolet light. The study also indicated that the increased sensitivity to ultraviolet light showed by 5-BD substituted DNA directly involved the 5-bromodesoxyuridine and that significant amounts of inactivation were not caused by a possible electronic transition absorption band that appeared to be present in the 5-bromodesoxyuridine absorption spectra at about 3200 Å.

178. TEMPERATURE SENSITIVE MUTANTS OF T4D

Investigators: R. S. Edgar, Millard Susman
Support: The National Foundation
National Institutes of Health, U.S. Public Health Service

Isolation. Wild-type T4D forms plaques at an incubation temperature of 25°C with the same efficiency of plating as at an incubation temperature of 42°C. One can isolate "temperature sensitive" mutants (ts mutants) which form plaques at 25°C but are unable to form plaques at 42°C. To date, 176 independent mutants have been isolated; 42 have been induced with nitrous acid, 33 with 2-aminopurine, 98 with 5-bromodeoxyuridine and one spontaneous mutant was also isolated. Mutant stocks can be grown at 25°C. The particles obtained are not more sensitive (in the majority of cases) to heat inactivation than the wild-type particles.

Complementation tests. One would expect that mutants affected in different functions could complement each other. That is, upon mixed infection of one bacterium with two such different mutants, the cycle of growth of each could be completed at 42°C while each single mutant could not complete its cycle of growth. Such a test permits the division of the mutants into groups affecting the same function. On the basis of a "complementation spot test," the 176 mutants fall into about 50 complementation groups, or cistrons. Although all the possible pairwise combinations were not tested, all mutants fall into discrete classes giving negative tests within the group and only positive tests between groups. However, some groups of mutants behave in an exceptional fashion, giving some positive tests between members of the group. More refined complementation tests have been performed which consist of measuring the burst size of bacteria infected with various pairs of mutants, incubating them at the high temperature. Pairwise combinations of mutants give burst sizes either close to that of wild type (complementation), or a burst size of less than 20% of wild type (no complementation). The results of these experiments in general parallel the results of the spot tests.

Amber mutants are another class of mutants, studied by Epstein (see Biology 1961), which are widely scattered throughout the genome. Complementation spot tests have been performed between amber and ts mutants. A number of these tests were negative, indicating that some amber and ts mutants affect common functions. A map of the amber mutants (from data of Epstein) is included in the figure, together with the correspondences between amber functional units and ts functional units.
Physiological functions of temperature sensitive mutants of bacteriophage T4.
Mapping. To locate the mutants on the genome of T4D, one mutant from each cistron was selected for mapping. Standard crosses between ts mutants were performed and wild-type recombinants scored by plating at 42°C. On the basis of over 600 crosses, a map of the ts mutants has been constructed. The relationship of this map to the previously known map of T4D has been determined by crosses between ts mutants and plaque morphology mutants. The results are summarized in the figure. The ts mutations are widely distributed over the linkage structure. A number of crosses have been performed between mutants within the same cistron. In most cases the mutants fall in the same region of the genome; in general, the recombination values being less than 7%.

In one portion of the map the mutants used for mapping are rather densely but uniformly distributed. Utilizing data from crosses involving the mutants in this region, a mapping function, relating recombination values to map distance, was constructed, with a metric based on intervals averaging 6%. Because of the phenomenon of high negative interference, recombination values are not directly proportional to map distance. This mapping function permits the conversion of recombination values into map values, which are probably more closely related to physical distance along the chromosome. On the basis of this function, the map of T4D is greater than 800 map units in length. The distances presented in the figure are given as map values rather than recombination frequencies.

Physiology experiments. If bacteria are infected with a ts mutant and the complex incubated at a high temperature, the infection is abortive, since the gene or its product affected by the mutation is inoperative at that temperature. Studies on the nature of such abortive infection by different mutants throw light on the specific function of the affected gene. To this end, various observations have been made on abortive complexes: 1) The amount of DNA during the course of the infection was measured. A lack of increase would suggest that phage DNA synthesis is not initiated. 2) The complexes were examined visually in the optical microscope for characteristic cytological changes after infection (nuclear breakdown, etc.). 3) The optical density of the infected culture was followed for the occurrence of lysis. 4) If cells did not lyse, artificial lysates were made. Lysates were examined serologically for the presence of tail fiber antigen. 5) Lysates were examined in the electron microscope for the presence or absence of morphological components of the phage.

The vast majority of the mutants are able to initiate DNA synthesis; and lysis of the cells occurs at the normal time with the production of phage specific material. However, only incomplete phage are produced. These mutants can be categorized in the following way. With one group of mutants (A20, N30, A4 and N41) morphologically normal looking phages are produced. These phage, however, are inactive. These mutants probably control the synthesis and assembly of tail fibers, since in this region are the previously studied host range and co-factor requirement mutations. Another group of mutants (A3, N8, and N16) synthesize tail fiber protein and a normal number of tail cores to which are attached end-plates. However no heads, either full or empty, are manufactured. These mutations probably concern the synthesis and assembly of the head. These mutants are in the same region of the genome as the osmotic shock resistant mutant which has previously been studied. A third group of cistrons (those within the segment A2 to A7) appear to affect the synthesis of tail components and their assembly since with these mutants a normal number of heads are synthesized, but the tail is either missing, unattached, or contracted.

Three mutants which affect DNA synthesis have been found. One mutant (A41) appears to be delayed in growth at the high temperature. However, the other two mutants appear to synthesize neither DNA, lysozyme, tail fiber protein, nor phage components. Nuclear breakdown does occur in these complexes.
The above preliminary results suggest that the genome of T4D shows a high degree of order, all mutations affecting similar functions being located in contiguous segments of the linkage structure.

Time course experiments. In a given ts mutant a particular function is absent when growth takes place at a high temperature, but present at a low temperature. Information about the time during growth at which the gene acts can be obtained by transferring complexes from low to high or high to low temperature during the growth cycle. Some experiments of this type have been performed with a number of the mutants. The experiments support the following picture. Genes controlling the structure or assembly of phage particles act during terminal (tail functions) or near terminal (head functions) stages of growth (these may be designated late function genes). The genes affecting DNA synthesis act early during growth ("early functions"). Late mutants complete their cycle of growth even at the high temperature although producing no infectious particles. The early mutants, on the other hand, appear to be arrested in growth at an early stage. The late functions depend upon the expression of the early functions for their initiation. If, at a time after the normal cycle of growth would have been completed, the complexes of early mutants are transferred to a lower temperature, they do resume the synthesis of phage.

179. TEMPERATURE SENSITIVE MUTATIONS OF BACTERIOPHAGE T4 AFFECTING EARLY STAGES OF GROWTH

Investigator: John B. Gurdon
Support: Gosney Fund

Temperature sensitive mutations have been obtained in bacteriophage T4D as described in the previous report (Edgar and Susman). These mutants will form plaques at 25°C but not at 39.5°C. Most of the mutants isolated by Edgar will initiate DNA synthesis at the high temperature, but 3 out of 176 of these mutants will not do so. In bacteria infected with one of these mutants (A14), phage multiplication appears to be suspended at 39.5°C but will start as soon as the infected bacteria are placed at 25°C. This mutant is of interest since it appears to control the initiation of the activity of other genes which function later in the growth cycle. In order to investigate this effect, a method of selecting more of these mutants affecting DNA synthesis was devised, and some physiology and time course experiments have been begun on the mutants obtained.

The selection method consisted of infecting bacteria with phage treated with 5-bromodeoxyuridine and growing them at 39.5°C. The phage produced by bacteria which lysed at the normal time were inactivated by antiserum. Infected bacteria which were transferred to 25°C between 50 and 150 minutes after infection yielded 62 temperature sensitive mutants which constituted 4% of the phage tested. Complementation and crossing experiments showed that these mutants are located in 14 functional groups or cistrons, and that these lie in 2 linkage groups. The first linkage group is between r48 and r67, and the second between rII and r48. The mutants in about half of the cistrons were found to initiate little or no DNA at the elevated temperature. The selection method employed therefore provided about 50 times as many mutants affecting DNA synthesis as would have been obtained without any selection.

Physiological studies have been started using representative mutants from each cistron. No DNA synthesis takes place at 39.5°C in bacteria infected with some of the mutants in the rII to r48 linkage group, while normal DNA synthesis is supported by all mutants in the r48 to r67 group. Bacteria infected with mutants in the r48 to r67 group have undergone lysis within 100 minutes at 39.5°C, while those in the rII to r48 group either fail to lyse at all, or else lyse only when returned to 25°C.
180. TEMPERATURE SENSITIVE MUTANTS OF PHAGE T5

Investigator: Michael Nesson
Support: National Institutes of Health, U.S. Public Health Service
The National Foundation

Wild-type phage T5 forms plaques on E. coli strain FCb with the same efficiency of plating at both 40°C and 30°C. It is possible to isolate mutants which behave like wild type at 30°C, but which do not form plaques at 40°C. In a phage stock grown in the presence of 0.5 mg/ml of 5-bromouracil, such temperature sensitive mutants arise with a frequency of 1 in 10^3 phage.

These mutants have made it possible to demonstrate that genetic recombination occurs in phage T5. Five purified temperature sensitive mutants have been used in nearly all pairwise combinations as parents in crosses performed by procedures similar to the standard T-even cross procedures. A preliminary, but self-consistent, genetic map can be prepared from the cross data; the map appears to extend for at least 40 recombination units. Recombination values from individual crosses range from 1.5% to 27%.

181. THE DISTRIBUTION OF BASE ANALOG INDUCED r MUTANTS IN PHAGE T4D

Investigator: Michael Nesson
Support: National Institutes of Health, U.S. Public Health Service
The National Foundation

The vast majority of spontaneous r mutants of T4D are located in 1 of 4 genes: rI, rIIA, rIIB, and rII. Preliminary tests indicate that a significant proportion of r mutants induced by 2-amino-purine are located at new loci. Of 26 induced r mutants examined, 6 do not fall into any of the 4 known r genes. At least 4 of these 6 are at loci distant from one another.
VISITING LECTURERS

A number of visiting scientists gave general biology seminars during the year. The lecturers and their subjects were:

Alfred Tissieres, Harvard University Biological Laboratories: "Some Aspects of Protein Synthesis."

David Bonner, University of California at La Jolla: "Genetics of Tryptophan Synthetase Formation in Neurospora."

Harry Grundfest, College of Physicians and Surgeons, Columbia University: "The Physiology of Electric Fishes."

Harry Rubin, Virus Laboratory, University of California at Berkeley: "Biology of an Avian Leukemia Virus: Congenital Transmission, Tolerance and Immunity."

Rowland H. Davis, University of Michigan: "Genetic Interactions in Pyrimidine, Proline and Arginine Synthesis in Neurospora."

Daniel Mazia, University of California at Berkeley: "Recent Studies on the Biochemistry of Mitosis."

Curt Stern, University of California at Berkeley: "Developmental Genetics of the Sex Comb of Drosophila."

J. Y. Lettvin, Massachusetts Institute of Technology: "The Coding of Visual Environmental Changes in the Optic Nerve of Animals."

Dan Lewis, University of London and University of California at Berkeley: "Suppressor Genes and the Regulation of Methionine Synthesis in Coprinus."

Paul Berg, Stanford University Medical School: "Attempts to Correlate Nucleotide-Sequence with Amino Acid Specificity of Amino Acid-Acceptor RNA."

L. Provasoli, Haskins Laboratories, New York City: "Nutritional Requirements of Marine Algae."

Jerard Hurwitz, New York University College of Medicine: "Role of DNA in RNA Synthesis."

Theodore J. Voneida, California Institute of Technology: "An Approach to the Problem of Central Pathways in Learning."

Kenneth C. Cole, National Institute of Neurological Diseases and Blindness, Bethesda: "Membranes in Voltage Clamps."

G. Wittman, Max-Planck-Institut für Biologie, Tübingen: "Genetic Coding in Tobacco Mosaic Virus."

Eric Shooter, Stanford University Medical Center: "Specificity in the Assembly of the Hemoglobin Molecule."

Leslie Brent, University College London: "A New Look at Immunological Tolerance."

Drew Schwartz, Biology Division, Oak Ridge National Laboratory: "Genetic Studies on Mutant and Hybrid Enzymes in Maize."

James V. McConnell, University of Michigan: "Memory Transfer through Cannibalism in Planaria."


Donald Kennedy, Department of Biological Sciences, Stanford University: "Organization of the Input to Central Neurons."

Charles C. Congdon, Oak Ridge National Laboratory: "Structural Changes in Lymphatic Tissues During an Immune Response."

GRADUATES

Sixteen students were awarded the B.S. or the Ph.D. degree in Biology in June, 1962. The names, degrees conferred, the titles of doctoral theses, and the plans of the recipients for future work are as follows:

John W. Berry, B.S. Military service.

Gary C. Chamness, B.S. To University of California at Berkeley for graduate work in biochemistry.

Thomas Creighton, B.S. To Stanford University Medical School for graduate work in biochemistry.

Kerry L. Donovan, B.S. To the University of Washington for graduate work in embryology.

David Drummond, B.S. To Jet Propulsion Laboratory.

David K. Edwards, B.S. To University of California at Los Angeles for graduate work in English.

Randall Griepp, B.S. To Stanford University Medical School.

Vincent C. Hascall, Jr., B.S. To the Rockefeller Institute for graduate work.

Stephen F. Heineman, B.S. To Harvard University for graduate work in molecular biology.

Garland R. Marshall, B.S. To Rockefeller Institute for graduate work.

Carl M. Rovainen, B.S. To Harvard University for graduate work in physiology.

George Guthrie, Ph.D. Thesis: "Studies on the Interaction of Subviral Particles of the Bacteriophage øX174 with Protoplasts of Escherichia coli." He is now doing postdoctoral research in the Department of Biology at the Massachusetts Institute of Technology.
Martin Mendelson, Ph. D. Thesis: "Studies on the Activation of Crustacean Mechanoreceptors. The Movement Receptors of the Crab Pachygrapsus crassipes Randall and the Movement and Stretch Receptors of the Crayfish Procambarus clarkii (Girard)." He is now doing postdoctoral research in the Department of Psychology of the Columbia University College of Physicians and Surgeons.

Donald C. Shreffler, Ph. D. Thesis: "I. Genetic Studies of Mouse Serum Protein Types. II. Molecular Hybridization of Sheep Hemoglobins." He is now a Research Associate in the Department of Human Genetics at the University of Michigan.


Colwyn Boyd Trevarthen, Ph. D. Thesis: "Studies on Visual Learning in Split-Brain Monkeys." He is now doing postdoctoral research at the California Institute of Technology.

HONORS, AWARDS, APPOINTMENTS

James Bonner was appointed a member of the Biochemistry Training Committee of the National Institutes of Health and of the NAS-NRC Agricultural Board Executive Committee; he is serving as Vice-President, Section G, American Association for the Advancement of Science; he will be George Eastman Visiting Professor, Oxford University, 1963-1964.

Henry Hellmers has been appointed to the California State Forester's Advisory Committee on Reforestation.

Alan J. Hodge was elected a member of the New York Academy of Sciences; is chairman of the Nominating Committee of the Biophysical Society.

N. H. Horowitz is Consultant to the Bioscience Subcommittee on Exobiology Aeronautics and Space Administration; was appointed to the Committee on Exobiology of the Space Science Board; was chairman of the Program Committee, Genetics Society of America.

E. B. Lewis was elected Secretary of the Genetics Society of America.

Ray D. Owen was elected President of the Genetics Society of America; appointed to Board of Scientific Counselors of the National Institute of Allergy and Infectious Diseases.

R. L. Sinsheimer was Chairman of the Program Committee of the Biophysical Society.

Albert Tyler is President of the Society of General Physiologists and was appointed as a Consultant to the Ford Foundation.
**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 from or contracts with individuals, companies, foundations and U.S. governmental agencies for specific projects; from unrestricted annual gifts; and from contributions to general funds provided by Industrial Associates and Institute Associates, as follows:

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